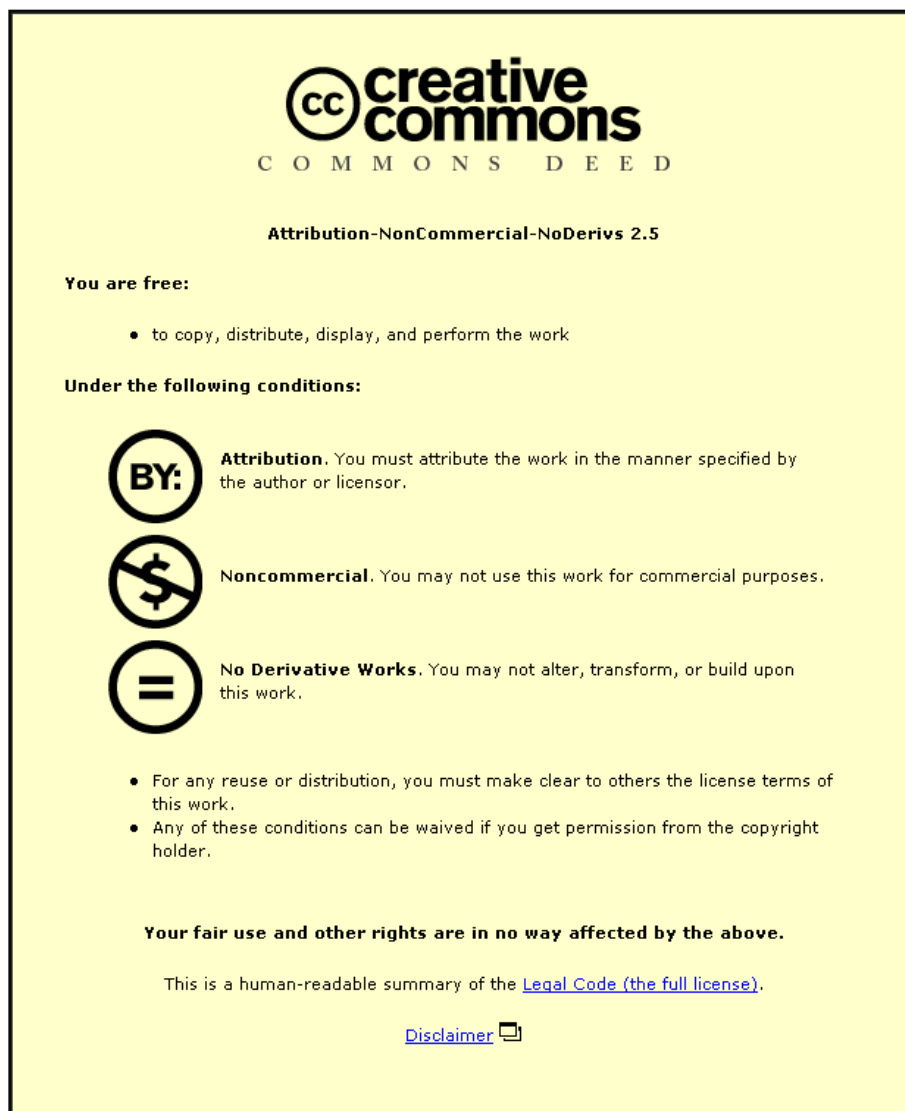


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DETERMINATION OF PROPYLENETHIOUREA AND RELATED

COMPOUNDS BY HPLC

by

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A Master's Thesis submitted in partial fulfilment
of the requirements for the award of
Master of Philosophy of
Loughborough University of Technology

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Supervisor: Dr. R. M. Smith

Department of Chemistry

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*In loving memory of my mother,
my grand-mother and my brother,
who no longer witness this
thesis.*

*To my beloved husband ALMIR
and my lovely son CONRADO.*

*To my friends BJA and ANTONJO CARLOS,
without their support this work would
not have been possible.*

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Lastly to my brothers for their cooperation and patience.

ABSTRACT

Propylenethiourea (4-methylimidazolidine-2-thione) is the major breakdown product of Propineb, a fungicide of the propylenebisdithiocarbamate group.

The degradation pattern of Propineb is analogous to those of ethylenebisdithiocarbamates which give ethylenethiourea as the principal metabolite. This compound has been reported to possess a carcinogenic activity.

The primary purpose of this investigation was to evaluate, by qualitative and quantitative determinations, the breakdown pathway of propylenethiourea (PTU) on ultraviolet irradiation. The analysis of PTU was performed by HPLC on an ODS-Hypersil column with either UV or electrochemical detection. To increase selectivity and sensitivity a derivatisation with *p*-nitrophenacyl bromide was carried out and the structure of derivative was characterized by spectroscopic methods. The derivative was monitored by HPLC on a PS-DVB column with UV detection at 263 nm.

Results of the different methods were compared and the direct method (UV detection); which appeared to be the most suitable procedure, was employed to determine propylenethiourea in sprayed lettuces and also in Propineb commercial formulations.

Propylenediamine (1,2-diaminopropane) was identified as degradation product and analysed by HPLC with fluorometric detection of its fluorescamine derivative. This label reacts with primary amines in alkaline medium to form highly fluorescent derivatives detectable at very low concentrations.

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CHAPTER I

1. INTRODUCTION

CHAPTER I

1. INTRODUCTION

1.1. - GENERAL

Population growth affects almost every aspect of everyday life and forces us to change the way the land is used.

An expanding population means we need to grow more food. Thanks to the impressive success of the agricultural revolution (1), man's food-growing capacity is now a hundred times larger than it was at the turn of the century and we are now feeding more people than at any time in history. However, there appear to be many problems still to be solved. One of them is to grow enough food while avoiding the side-effects of further pollution of the soil, water and air.

Much effort must be put into assuring that enough food is available to feed an increasing population and this can only be achieved by increasing productivity. Attempts are being made to increase the yield per hectare (2) by improving, developing and selecting plants, fertilisers, pesticides and by using modern agricultural methods and modern management techniques (3). Although the benefits of pesticides can be demonstrated experimentally (4, 5) on affected crops, it is less easy to prove their overall value or to quantify the various risks they present.

During the last twenty years of intensive technical and industrial development a wide range volume of synthetic compounds has been used in large volumes.

This ever increasing use of pesticides has already resulted in a great deal of controversy about the effects of these compounds on the environment. Questions have been raised about the possible hazards to man and to wildlife and therefore strategies must be adopted to minimise the impact on the ecosystem (6).

Nowadays pesticides can be present in many foodstuffs, in man, in animals and in the environment. Although they may be found in low concentrations, their variety, toxicity and persistence are affecting the ecosystem and they may eventually affect human health (7). Precisely because pesticides are designed to kill or upset the metabolism of a living target organism, they are potentially dangerous to other living organisms. Most of them are highly toxic in concentrated amounts and in some instances they have caused illness and the death of people and wildlife.

There is a real need to understand more deeply the properties of these compounds and control the long term impact on the ecosystem. The pesticides constitute only one facet of the general problem of pollution but efforts need to be combined to devise alternative methods for pest control with minimal risks and maximum gains. In this work, concern regarding the potential carcinogenicity, mutagenicity and teratogenicity of propylenethiourea (PTU) (8), a breakdown product of Propineb, zinc propylene-1,2-bisdithiocarbamate, has led to the development of suitable methodologies for its determination and a study of its degradation.

1.1.2. - Definition and systemicity of pesticides

Pesticides are chemical formulations largely employed to control the attack of pests on agricultural crops. They can be classified into three major groups:

- 1) Insecticides: used mainly against insects.
- 2) Fungicides: capable of killing or inhibiting fungi.
- 3) Herbicides: designed to kill weeds.

There are also minor groups including rodenticides, nematicides, acaricides and molluscicides.

The systemicity of a pesticide is its capacity to penetrate the plant tissue and to be translocated throughout the plant. Contact or non-systemic pesticides do not appreciably penetrate plant tissues and are consequently not translocated within the plant vascular system (9, 10). As a result they are very susceptible to the effects of weathering (wind, rain and sunlight) over long periods (11, 12, 13, 14). New plant growth will be left unprotected and therefore open to attack by insect and fungal pests.

Contact pesticides are uncommon and most of them are transferred at least weakly into the plant tissues. They usually remain on the surface

of the plants (leaves) and are then decomposed, lost by volatilisation, or washed off by rain.

Many pesticides are partially systemic, only a proportion is translocated to the plant vascular system. Consequently the degradation product may be observed as photodecomposition and oxidation products on the surface of the plant and metabolites from the material which was incorporated into the plant tissues.

The highly systemic pesticides penetrate into the plant vascular system (10) and are translocated throughout the plant. The metabolic pathways depend upon tissue absorption and translocation of the plant. It may be generalised that contamination problems with pesticides are closely related to their persistent nature. The most important factors determining the degree of persistence are the chemical characteristics of the pesticide compound (15).

1.2. - FUNGICIDES

Fungicides are chemical compounds capable of killing or inhibiting the development of fungi.

A desired selective toxicity is more difficult to achieve with chemicals for fungal diseases than for insects because the fungus is also a plant living in close quarters with its host (16). This explains why it is difficult to find chemicals which kill the fungus without harming the plant and none of the protectant fungicides currently commercially available is completely non-phytotoxic.

Fungicides should be applied to protect plants during stages in their growth when they are susceptible to inoculation by pathogens, before there is any evidence of disease (17).

Systemic fungicides can help to control certain diseases after the fungal spores reach the plant (18). They are absorbed by the plant via roots, leaves or seeds and are translocated within the plant. Also protectant fungicides are widely used even after symptoms of disease have appeared. Eradicant fungicides are commonly applied directly to the fungus during its "over-wintering" stage, a long time before disease has begun (19).

Fungicides are applied not only to the foliage but also to the seeds, soil and harvested plants for controlling fungal spores. They are usually applied as a water suspension but are sometimes used as dusts, granules or fumigants (9, 19), which are particularly beneficial when the

chemical is applied in a waxy leaf surface. The fungicide as a dried deposit on the leaf must generally be stable towards photochemical oxidation, hydrolysis and carbonation (16). Sometimes repeated applications of protective fungicides are necessary due to plant growth dilution and removal by rain and other weathering (18).

There are about 150 fungicidal products available in the market (17), most of which are recently discovered organic compounds. The majority of these fungicides act as protectants, preventing spore germination and subsequent fungal penetration to plant tissues.

The earliest fungicides were inorganic materials such as sulphur, lime-sulphur, copper, silver and mercury. The relative toxicity of various metal cations to fungi is as follows $Ag > Hg > Cu > Cd > Cr > Ni > P > Co > Zn > Fe > Ca$ (16). Although silver is the most toxic metal cation to fungi, only the compounds of copper and mercury have been used as fungicides but the latter is not used any more because of its hazardous build up in man, soil and animals (20). The toxicity of the metals is related to their position in the periodic table and generally increases with the atomic mass. The toxicity is also related to relative chelating powers of the metals, the stability of the metal and electronegativity of the cations.

Copper sulphate (21) has been used since the eighteenth century for seed treatment against cereal bunt. Copper ions in solution are toxic to all plant life; selective fungicidal action can therefore only be achieved by application of water insoluble copper compound to the foliage such as copper oxychloride, copper carbonate or bordeaux mixture (17, 20, 22). Copper compounds are not easily washed off leaves by rain because of their low solubility in water, thus giving longer protection against disease than do most of the organic materials.

Bordeaux mixture (16, 17, 21, 22, 23) is the most important of the copper fungicides and it was accidentally discovered when sprayed on grapes to scare off "freeloaders". It was soon observed that downy mildew, a disease of grapes disappeared from treated plants. Bordeaux mixture is a chemically undefined mixture of copper sulfate and hydrate lime. The precise mode of fungicidal action is complex. The proportion of ingredients used and the method of preparation have considerable influence on the fungicidal effectiveness of the product.

Sulphur (19) in several forms is probably the oldest effective fungicide known and still today a very useful home-garden fungicide. There is a considerable evidence that sulphur can penetrate fungal spores and the improved activity observed in the presence of urea, hydrocarbons or lime is due to enhanced penetration (18). In its fungicidal action sulphur is eventually converted to hydrogen sulphide (H_2S) which is toxic to the cellular proteins of the fungus (17). A host of synthetic sulphur and other organic fungicides have been developed over the past 30 years to replace the more harsh, less selective inorganic materials.

The newer organic fungicides possess several outstanding qualities. They are extremely efficient, therefore smaller quantities are required than of those used in the past; they usually last longer, and they are safer for crops, animals and the environment. Most of these fungicides show very low phytotoxicity and are readily degraded by soil microorganisms, thus preventing their accumulation in soils.

1) Dithiocarbamates: The accidental discovery of the fungicidal activity of the dithiocarbamates launched a new era for controlling a wide range of fungus diseases (18, 24). They are some of the most widely used organic protectant fungicides and are usually applied against a wide range of phytopathogenic fungi such as downy mildews, potato and tomato blight. In these group are included fungicides such as thiram, maneb, ferbam, ziram, zineb, mancozeb and propineb (25). (See section 1.3.).

2) Substituted aromatics: this is a somewhat arbitrary classification assigned to benzene derivatives that possess long-recognised fungicidal activity. Quintozene which was introduced in the 1930's but has only recently been widely used as soil fungicide against pathogenic fungi, such as damping-off diseases (26). Chlorothalonil (27) was first developed in 1964 and was proved to be an effective foliage protectant fungicide for beans, sweet corn, carrot, cucumber, watermelon and onions.

3) Triazines: although the triazines are usually thought of as herbicides, dyrene (28) was introduced as a fungicide in 1955 and since then has been widely employed on vegetables, for potato and tomato leaf spots along with fungus diseases of lawn and turf grass.

4) Thiazoles: the group of thiazoles includes the over popular terrazole (29), which is used as soil fungicide. It is highly recommended as turf fungicide for controlling of phytium diseases and also very effective against the seedling disease complex of several garden and field crops.

5) Dicarboximides: in 1951 Captan (30), an extremely useful foliage protectant fungicide was discovered. It is very effective especially for control of apple and pear scab, black spot on roses and seed-borne diseases. Analogues which have been subsequently developed as foliar fungicides include folpet and difolatan (31), which are more effective against potato blight.

The dicarboximides are regarded as the safest pesticides available (17) and are recommended for lawn and garden use, for seed treatment and as protectants against mildews, late blight and other diseases.

1.2.1. - Systemic fungicides

A systemic fungicide is a compound that is taken up by a plant and is then translocated within the plant, protecting it from attack by pathogenic fungus or limiting the spread of an established infection (32). They have become available only in recent years. Most systemic fungicides have eradicant properties which stop the progress of existing infections. They are therapeutic since they can be employed to cure plant diseases.

Systemic fungicides offer a much better control of diseases than is possible with a protectant fungicide that requires uniform application and remains essentially where it has been sprayed onto the plant surfaces (33). There is, however, some redistribution of protective fungicides residues on the surface of sprayed or dusted plants, giving them longer residual activity than would be expected.

Benzimidazoles are systemic fungicides and the most important members of this group are benomyl and thiabendazole (33). Both have a wide acceptance against a broad spectrum of diseases including powdery mildews and soil-borne pathogens. Benomyl, introduced in 1968 has the widest spectrum of fungitoxic activity of the all newer systemics, including control of powdery mildews and apple scab. Thiabendazole is good against post-harvest diseases of apples and pears and also against common bunt of wheat. It is better than Benomyl for eradication of dutch elm disease, although neither chemical was sufficiently active to be a practical means of large-scale control (34).

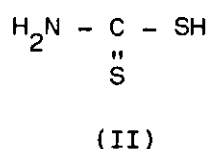
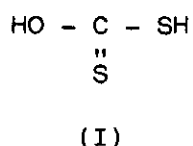
Thiophanates are a new group of systemic fungicides based on thiourea (35). Initially, they do not possess fungicidal properties, but they are metabolically converted by the host plant to an active benzimidazole derivative.

The overall fungicidal spectrum of the thiophanates is analogous to that of the benzimidazoles; in particular benomyl and thiophanate methyl whose antifungal properties are closely similar.

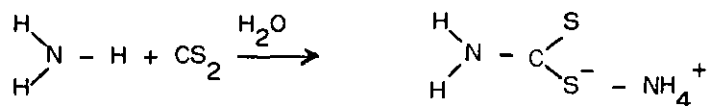
1.3. - DITHIOCARBAMATES

The development of purely organic fungicides really began with the discovery of the fungicidal activity of the dithiocarbamates, which had been originally developed as vulcanisation agents for the rubber industry. The dithiocarbamates and their derivatives are one of the most important groups of organic fungicides for controlling plant diseases (33).

The fungicidal dithiocarbamates are derived from dithiocarbonic acid (I) (36). The parent compound dithiocarbamic acid (II), is the amide of dithiocarbonic acid:



The basic synthesis of these compounds is given by the following equations:



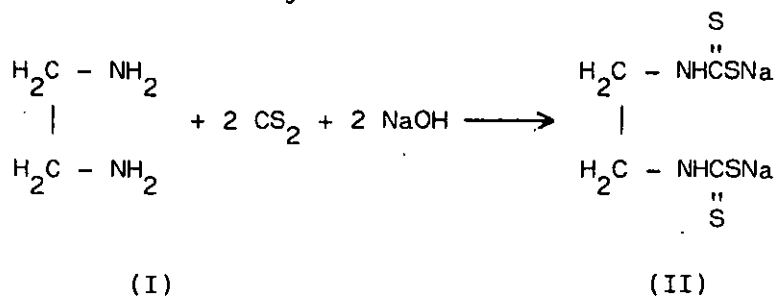
The ammonium salt of
dithiocarbamic acid

Although the first dithiocarbamates were patented by Tisdale and Williams (37) in 1934, they were not developed as fungicides until the early 1940's. The insecticidal activity of dithiocarbamates was firstly reported by Guy (38) in 1936. He demonstrated that some thiuram disulphides strongly repelled a variety of leaf-feeding insects. In more recent investigations MacMullen (39) reported the zinc and manganese salts, of ethylenebisdithiocarbamic acid to be almost as effective as one of the better insecticides for controlling the greenhouse whitefly.

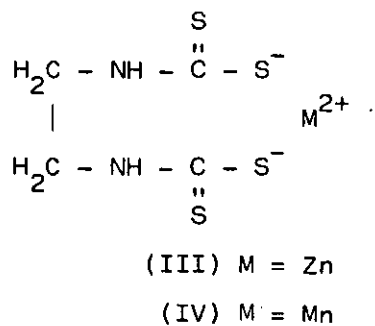
Dithiocarbamates were reported to have strong metal binding properties (probably due to the insolubility of metal salts) with the exception of these sodium and other alkali and the alkaline earth metals, and to the capacity to form chelate complexes (36).

The ethylenebisdithiocarbamates are the most widely used organic protectant fungicides and are applied for the control of a broad spectrum of phytopathogenic fungi such as downy mildews, potato and tomato blight. Table 1.3.1. (26) illustrates the structure of some derivatives of dithiocarbamates and their applications.

They are obtained by reaction of diamines with carbon disulphide in the presence of sodium hydroxide:

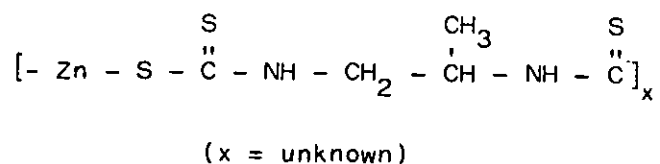


ethylene diamine



Nabam, (II) which is water soluble is converted into either zineb (III; M = Zn) or maneb (IV; M = Mn), which are insoluble, by reaction with an aqueous solution of zinc or manganous sulphate.

Propineb (zinc 1,2 propylenebisdithiocarbamate) (V) is a typical propylenebisdithiocarbamate and it was marketed in 1963 under the trade name Antracol (40). Propineb is obtained by the precipitation of water - soluble propylenebisdithiocarbamate with a solution of zinc salts.



(V) Propineb

TABLE 1.3.1. - Name, structure and application of some dithiocarbamates

COMMON OR TRADE NAME	CHEMICAL NAME	STRUCTURE	APPLICATIONS
Zineb	zinc ethylene-1,2-bisdithiocarbamate	$\begin{array}{ccc} \text{S} & & \text{S} \\ & & \\ \text{[-S.C.NH.CH}_2\text{.CH}_2\text{.NH.C.Zn-] }_x \end{array}$	early blight, blue mould, stem rust, septoria, mildew
Propineb	zinc propylene-1,2-bisdithiocarbamate	$\begin{array}{ccc} \text{S} & \text{Me} & \text{S} \\ & & \\ \text{[-S.C.NH.CH}_2\text{.CH.NH.C.S.Zn-] }_x \end{array}$	downy mildew, blight scab, blue mould and red spider mites
Maneb	Manganese ethylene-1,2-bisdithiocarbamate	$\begin{array}{ccc} \text{S} & & \text{S} \\ & & \\ \text{[-S.C.NH.CH}_2\text{.CH}_2\text{.NH.C.S.Mn-] }_x \end{array}$	early and late blight mildew
Nabam	disodium ethylene-1,2-bisdithiocarbamate	$\begin{array}{ccc} \text{S} & & \text{S} \\ & & \\ \text{Na}^+ \text{S.C.NH.CH}_2\text{.CH}_2\text{.NH.C.S}^- \text{Na}^+ \end{array}$	Late blight, root rots
Mancozeb	Manganese ethylene-1,2-bisdithiocarbamate mixed with zinc ethylene-1,2-bisdithiocarbamate	$\begin{array}{ccc} \text{S} & & \text{S} \\ & & \\ \text{[-S.C.NH.CH}_2\text{.CH}_2\text{.NH.CS.Mn-] }_x \text{ (Zn) }_y \end{array}$	early and late blight
Ferbam	dimethyl dithiocarbamate	$\begin{array}{ccc} \text{S} & & \text{Fe}^{3+} \\ & & \\ \text{[Me}_2\text{NC.S}^- \text{] }_3 \end{array}$	apple rust diseases
Ziram	zinc dimethyl dithiocarbamate	$\begin{array}{ccc} \text{S} & & \text{Zn}^{2+} \\ & & \\ \text{[Me}_2\text{N.C.S}^- \text{] }_2 \end{array}$	anthracnose diseases

It is a white to yellowish powder decomposing at approximately 160°C and at 300°C only a slight residue remains. It is practically insoluble in all conventional solvents. It is very stable in a cool dry storage, although it can be decomposed strongly in acid or alkaline solution. Propineb has a high foliar fungicidal activity and it is very effective against downy mildew on hops and wine, blight on potatoes and tomatoes, apple scab, blue mould in tobacco and sigatoka diseases of banana (40). It can be also used on gooseberries, blackcurrants, celery and cereals as an acaricide for controlling red spider mites (40).

1.4. - DETERMINATION OF DITHIOCARBAMATES

Many different methods for determining the residues of dithiocarbamate fungicides have been described in the literature. Photometric and polarographic methods have been frequently applied for residual determination of undecomposed dithiocarbamates (41, 42, 43). Paper and thin-layer chromatography were claimed to offer rapid determinations by visual spot comparisons.

A paper chromatographic method was published by McKinley and Magarvey (44), however, distinction was possible only between dimethyl dithiocarbamates and ethylenebisdithiocarbamates.

Hylin (45) described a thin-layer chromatographic methods for separating dithiocarbamate fungicides. Samples were extracted with chloroform and even maneb and zineb could be dissolved and appeared as defined spots on the silica gel plates.

The most widely used approach for the determination of total dithiocarbamates has been the release of carbon disulphide. This methodology is suitable either for the microdetermination of pesticide residues on crops or to macrodeterminations to determine the purity of ingredients in pesticide formulations. The method was introduced by Callan and Strafford (46) originally for the analysis of vulcanisation accelerators. Later, Clarke *et al.* (47) proposed a modified technique for determining residues of dithiocarbamates in plant material. The samples are firstly decomposed by hot mineral acid (usually sulphuric acid) generating the amine and carbon disulphide. The amount of hydrolysis product may be determined by colorimetry or by polarography. The carbon disulphide developed in the reaction is absorbed in an ethanolic solution of copper acetate containing diethylamine. The yellow complex formed is evaluated

photometrically in the visible region around 425 μm with a sensitivity of 0.1 mg/kg.

Although according to the procedure the method of acid decomposition gives considerably different results, the recovery and margin of error are acceptable for residue analysis. The determination of carbon disulphide released by acid is rapid and suitable for the simultaneous analysis of different dithiocarbamates, but it lacks the specificity afforded by the determination of the amine. Since each dithiocarbamate has a different chemical nature and potential to degrade to toxic metabolites, it is considered important to distinguish between them.

Newsome (48) described a method to analyse amines by electron capture gas-chromatography with prior derivatisation with trifluoroacetate. Similarly, a method has been developed by Uno *et al.* (49) for determination of Propineb in agricultural crops. Hydrolysis with acid and stannous chloride gave propylenediamine which was derivatised with dansyl chloride. The derivative was separated by TLC after 3 hours and quantitated by dual wavelength densitometry with a limit of detection of 0.1 mg/kg.

Finally a headspace gas chromatography method was performed by McLeod and McCully (50). The dithiocarbamates are quantitatively hydrolysed in a closed vessel where an equilibrium of the CS_2 is achieved between the gaseous and the liquid phases. An aliquot of the headspace gas is injected directly into a gas chromatograph fitted with an electron capture detector (ECD). This method gives reproducible results for the analysis of dithiocarbamates residues over the concentration range of 0.04 - 200 mg/kg CS_2 and has proven suitable for monitoring and controlling purposes.

1.5. - TOXICOLOGY OF DITHIOCARBAMATES AND RELATED COMPOUNDS

Until recently, the EBDC's have been regarded as relatively harmless given their general low mammalian toxicology and biodegradability, furthermore, these compounds have been continuously used for more than 40 years. However, an urgent safety review has been ordered by EPA (51) on the world most widely used fungicides sprayed on cereals, fruits and vegetables. A study carried by EPA (1989) suggested that continued use of fungicides might cause 125,000 additional cancer cases among the US population.

Zineb was found to have less than one tenth the goitrogenic potency of nabam, in a short-term feeding of male albino rats in a period up to 2 years. Death occurred in some cases after administration of nabam

preceded by diarrhea, weakness and prostration (52).

Antonovich *et al.* (53) investigated the toxicological assessment of a number of dithiocarbamates and related fungicides. Based on tests with experimental animals (oral administration and tissue culture). Ziram and maneb were more toxic than zineb. Ziram showed greater cumulative teratogenic and blastogenic effects on rats, mice and rabbits than did other pesticides. Maneb presented the strongest embryotoxic activity. Table 1.5.1. illustrates LD₅₀ for some dithiocarbamates.

An oncogenic investigation on mice with Propineb (54) detected increased hepato-cellular adenomas in male mice and increased pulmonary adenomas in female mice at 800 ppm in diet, the highest dosage level tested. Thyroid tumours were not induced in treated mice in this particular study. A NOEL (non effective level) for non-neoplastic effects could not be determined owing to insufficient data. An increased incidence of thyroid benign tumours was observed at 1000 ppm dosage level in a long term rat studies with Propineb.

The principal impurity and metabolic product of EBDC's is ETU. Ulland *et al.* (55) performed some investigations in rats which were fed with technical grade ETU in their 18 month diet. The test group was formed by 26 males and 26 females. Thyroid carcinoma was found in 17 males and 8 females, pulmonary metastasis occurring in 2 males. Thyroid enlargement was observed in 17 males and 13 females. No liver tumours were reported. In conclusion, ETU has an action similar to other thiocompounds that cause thyroid carcinoma and indirectly affect the liver.

In a long term study of PTU administration to mice (54), a great incidence of hepatocellular adenomas was observed in male mice at 1000 ppm diet, the highest dosage level tested. Increased incidence of hepatocellular carcinomas were reported in male mice at 10 ppm dosage, Thyroid tumours attributable to PTU were not observed. Although thyroid hypercellularity was found in male mice at 1000 ppm and some thyroid tumours were reported. However, at a dosage level as low as 1 ppm goitrogenic effects were observed.

Some mutagenic investigations were performed on Propineb and PTU. The results were negative or inconclusive. Increased DNA synthesis in mouse spleen cells was noted when a special study was carried.

Based on this data, a FAO meeting recommended that the temporary ADI* for Propineb be withdrawn (54). Concerned about the established carcinogenic potential of this compound, the meeting also recommended that

* ADI: Acceptable Daily Intake.

Propineb should not be used when residues in food can arise.

TABLE 1.5.1. - Acute Oral Toxicities for some dithiocarbamic acid derivatives

Common or trade name	Chemical name	Rat LD ₅₀ (mg/kg)
Zineb	Zinc ethylene bisdithiocarbamate	5,200
Propineb	Zinc propylene bisdithiocarbamate	8,500
Ferbam	Feric dimethyl dithiocarbamate	5,700
Maneb	Manganese ethylene bisdithiocarbamate	5,000
Ziram	Zinc dimethyl dithiocarbamate	500
Nabam	Disodium ethylene bisdithiocarbamate	395
Thiram	Tetramethylthiuram disulphide	450

The 1984 JMPR (Joint Meeting on Pesticide Residues, FAO-WHO) proposed the following guideline levels for residue limits of propylenethiourea:

(metabolites are not included)

apples	0.1 mg/kg
pears	0.1 "
cherries, sour	0.1 "
peaches	0.05**"
plums	0.1 "
grapes	0.1 "
celeriac roots	0.05**"
potatoes	0.02**"
tomatoes	0.1 "

**at or about limit of detection.

1.6. - DEGRADATION PATTERN OF ETHYLENEBISDITHIOCARBAMATES

EBDC's fungicides are relatively unstable compounds which are transformed to a variety of products mainly by chemical process but with relevant biological involvement. Most previous work has been done on degradation of EBDC's and little published data is available on propylenebisdithiocarbamate fungicides but the degradation pattern of both groups of fungicides is expected to be very similar.

The decomposition of these fungicides begins during production and storage of the preparations so that large proportions of degradation products are frequent in the commercial materials.

EBDC's are not systemic fungicides and therefore are mainly present as surface deposits. Consequently, significant residues of undecomposed pesticides are not present within the fruit (42). The surface deposits of dithiocarbamates decompose according to reactions known from in-vitro degradation (41).

The decomposition of dithiocarbamates is mainly influenced by oxygen and humidity. By splitting off carbon and hydrogen sulphide as well as by oxidative degradation reactions, a great number of secondary products are produced (41).

Among the first breakdown products of EBDC's in plants are 5,6-dihydro-3H-imidazo-[2,1-C]-1,2,4-dithiazol-3-thione, ethylenethiourea (ETU) and sulphur. Residues of these degradation products were found in tomatoes treated with zineb and maneb (56).

Vonk and Sijpesteijn (57) identified the following compounds from nabam ethylenethiourea (ETU), ethylene-bis-isothiocyanate sulphide (EBIS), polymeric ethylenethiuram, elemental sulphur, ethyleneurea (EU) and 2-imidazoline.

Mancozeb labelled with radioisotopes (^3H , ^{14}C or ^{35}S) gave the following metabolites detected in plants DIDT, ETU, EU, 2-imidazoline, ethylene diamine and 3-(2-imidazolin-2-yl)-2-imidazolidinethione (Jaffe's base) (58). Zineb and maneb yield similar decomposition products in aqueous solution as nabam, but at a slower rate. Fig. 1.6.1. illustrates the degradation pathway of ethylenebisdithiocarbamates.

The principal degradation product of EBDC's in terms of environmental and toxicological significance is ethylenethiourea (2-imidazolidinethione) (8,41,42,48,51,55,57). ETU occurs as an impurity in fungicide formulations. It has been claimed to be mutagenic, carcinogenic

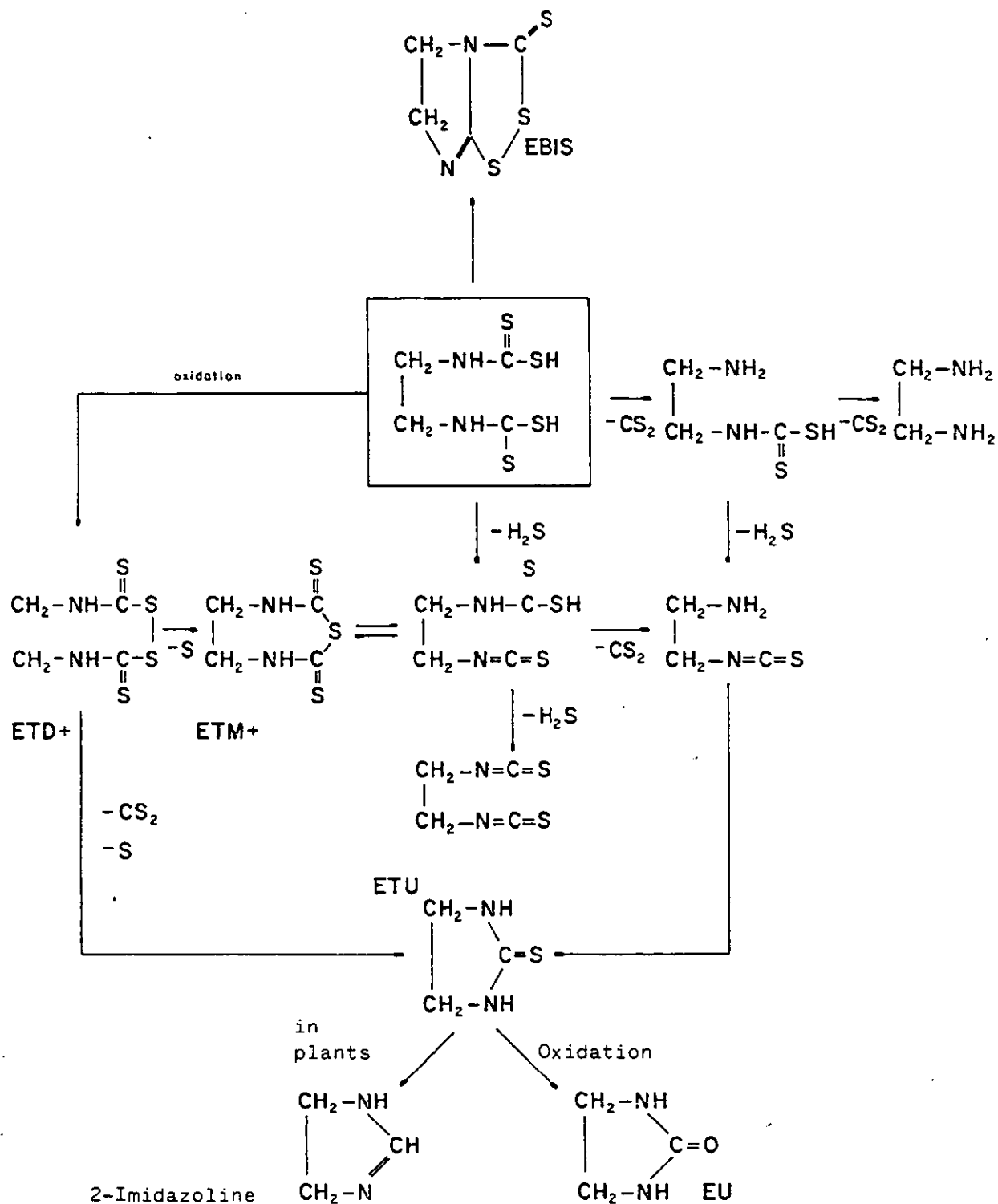


Fig. 1.6.1 - Degradation pathway of ethylenebis(dithiocarbamate)s.

and goitrogenic (8, 41, 51, 55). ETU may be present as a metabolite or degradation product on dithiocarbamate treated plants and may also be produced in large amounts as the result of cooking dithiocarbamate treated vegetables (59).

The mechanisms leading to ETU formation are not completely understood. The amount of ETU formed by degradation of EBDC's depends on the stability of the parent compound, substrate and reaction conditions. Under neutral and alkaline conditions, increased temperature promotes the formation of ETU.

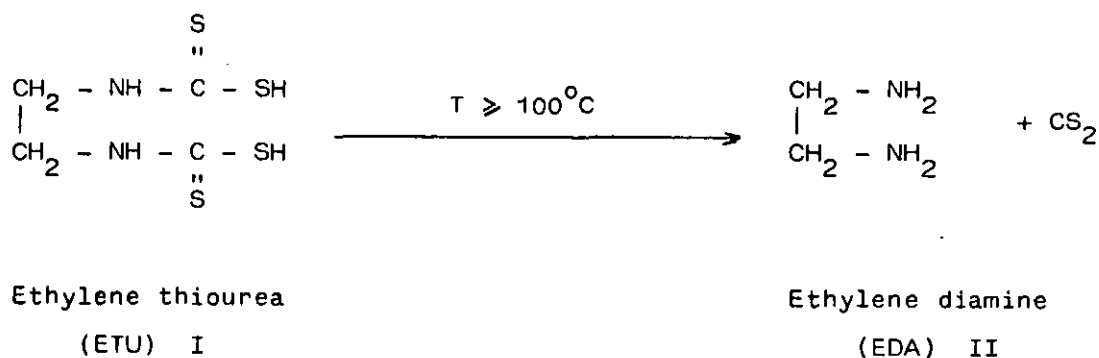
Ludwig *et al.* (60) have demonstrated ETU formation in aerated nabam solutions. When the pH was as high as 10.5, yields up to 65% of the theoretical amount of ETU have been achieved. Possibly ETU obtained in such conditions is formed from intermediate ethylenethiuram monosulphide by the loss of a molecule of CS₂.

Boiling residue amounts of EBDC's or 5,6-dihydro-3H-imidazo [2,1-c]-1,2 dithiazole-3-thione (DIDT) yielded considerable quantities of ETU (59).

Nitz and co-workers (61) investigated the fate of EBDC's and their metabolites in hops during the brewing process. They concluded that EBDC's are basically transformed to ETU and the thiourea did not undergo any chemical breakdown during brew technology.

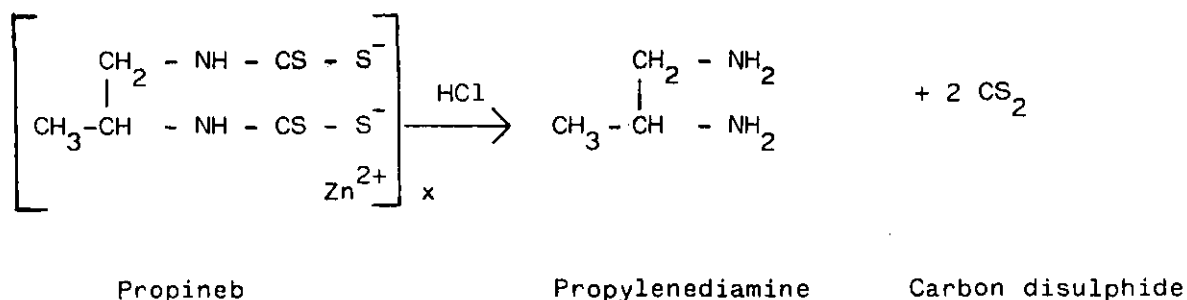
ETU is not a final product but is a relatively stable intermediate in the ultimate degradation of EBDC's fungicides. It is stable towards hydrolytic reaction but is easily oxidised to EU in biological systems and by photolytic reactions with acetone, riboflavin and other compounds (62, 63).

Under acid conditions and high temperatures, EBDC's decomposed to carbon disulphide and ethylene diamine (EDA) reversing the synthesis



After ultraviolet irradiation of ETU on silica gel 9 secondary products were identified. EU was claimed as the main breakdown product along with smaller amounts of 3-(2-imidazolin-2-yl)-2-imidazolidinethione (Jaffé's base) (62). Other secondary products of photooxidation of ETU are 2-imidazoline and glycine (63) via the intermediate product 2,4-imidazolidinedione (hydantoin).

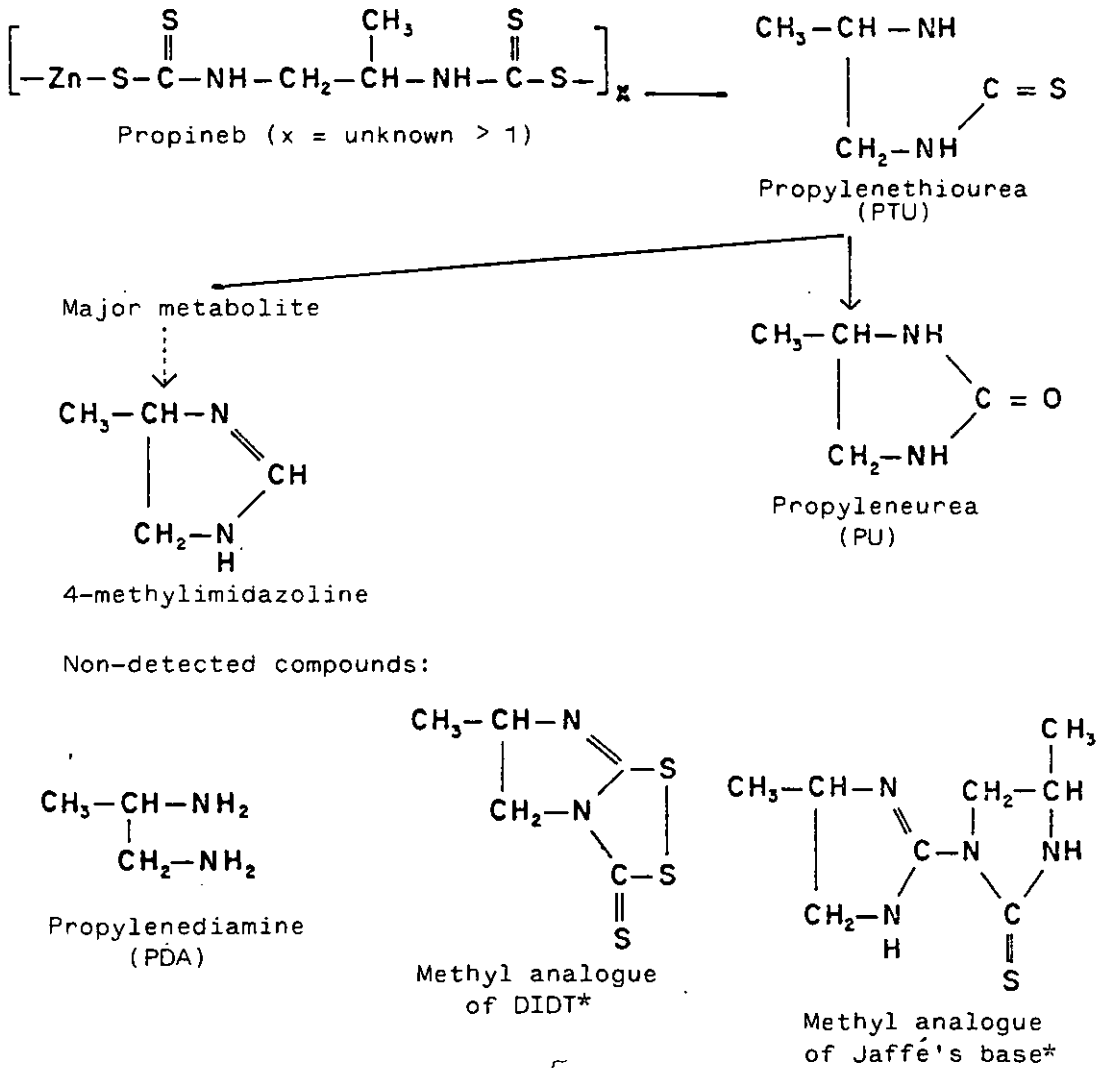
Propineb, a propylenebisdithiocarbamate fungicide, which is the methyl analogue of zineb would be expected, to follow the same degradation pattern as the EBDC's (9). Propineb decomposes to carbon disulphide and propylenediamine under acid conditions and high temperatures reversing its synthesis (49):



Similarly PTU is the major degradation product of propylenebisdithiocarbamates. Propineb undergoes thermal decomposition when hops containing residues of dithiocarbamates were boiled during a brew process. The high temperature promoted the formation of propylenethiourea (61).

Vogeler *et al.* (64) investigated the metabolism of Propineb in [^{14}C] Antracol treated apples and grapes. Two other experiments were also carried out with [^{14}C] PTU and [^{14}C] ETU on apples in order to make a comparative study. The investigations were restricted to the surface radioactivity since some analytical difficulties were raised. Consequently, it was not possible to determine the quantity of PTU in the whole fruit. The breakdown products were characterised and identified on TLC. To a large extent, Propineb and PTU degrade to the same metabolites.

Figure 1.6.2. illustrates the metabolic pathways of Propineb and PTU. Figure 1.6.3. shows the degradation of ETU. According to Vogeler (64) PTU and ETU underwent very fast degradation only 0.7 and 0.12% respectively were still present three days after application. The major



* Position of methyl groups unknown.

Fig. 1.6.2. - Propineb and degradation products.

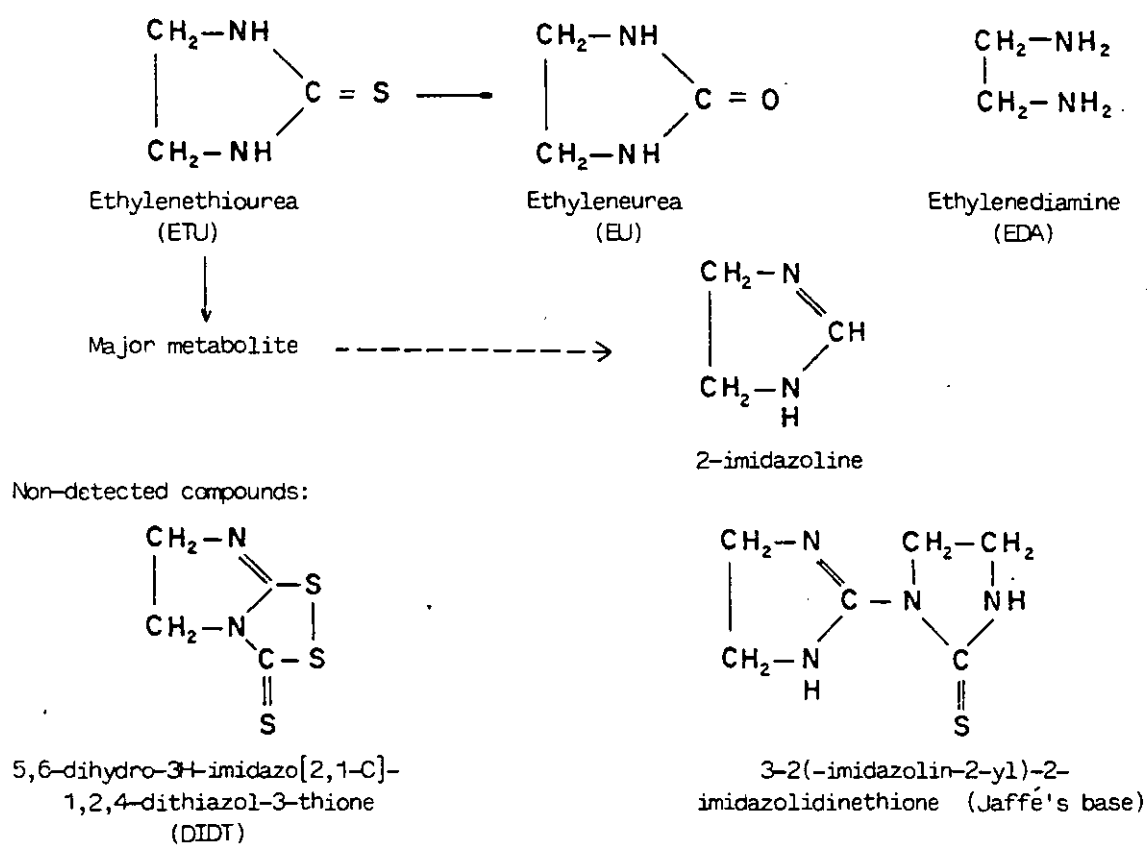


Fig. 1.6.3. - Ethylenethiourea and degradation products.

metabolite of PTU, was an unidentified basic compound which reacted with fruit acids to form a salt and penetrated into the fruit. As a free base, the main metabolite is unstable and is readily degraded to 4-methylimidazoline. Ethyleneurea and propyleneurea were formed by direct oxidation of ETU and PTU respectively. The methyl analogues of DIDT and Jaffé's base and propylenediamine were not detected as breakdown products of Propineb and PTU.

In a more recent study Vogeler (1980) (65) identified 4-methylimidazoline as a breakdown product of PTU, by comparing its mass spectrum with that of the authentic compound. The structure was also confirmed by its proton resonance spectrum in CD_3COOD .

In light of these new studies, the proposed structures of some metabolites have been corrected. The revised structures and most probable metabolic pathway of Propineb on and in plant is shown in the figure 1.6.4. (65).

1.7. - ANALYSIS OF ETU AND PTU RESIDUES

Concern regarding the important toxic role of the degradation products of dithiocarbamates ETU and PTU, has led many investigators to develop analytical methods capable of detecting nanogram amounts of these compounds.

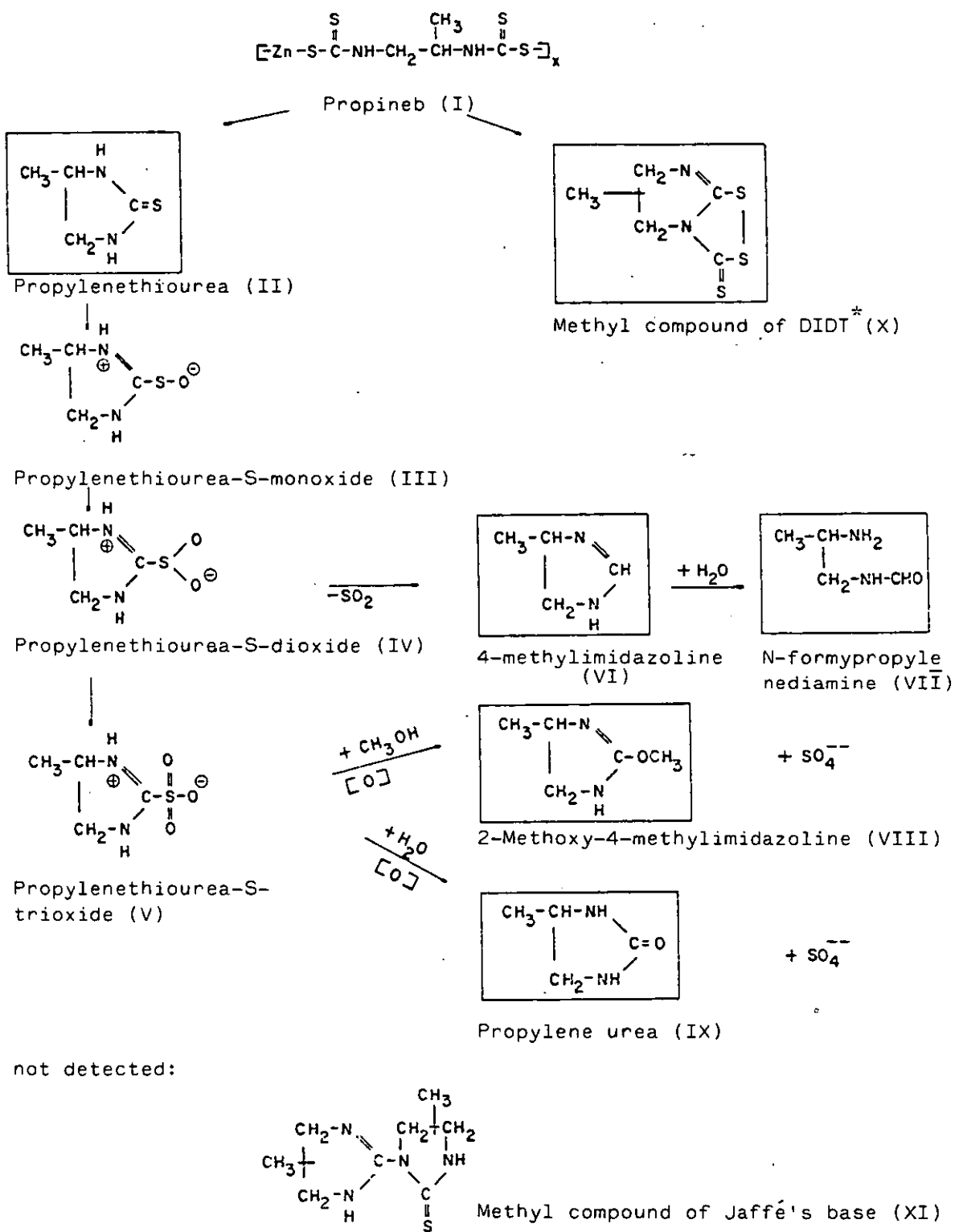
Published methods for the determination of ETU and PTU in formulations and at residue levels in foodstuffs and other substrates have mainly involved the use of chromatography techniques (66).

I - Thin layer chromatography

A number of approaches have been employed to analyse ETU and other breakdown products of EBDC's in aqueous solution or in the presence of crops or fruits. Although TLC determinations can be used to analyse ETU in foodstuffs, they are unsuitable for routine work. It was claimed to be difficult to get reproducible results since the detection limits for ETU were often very poor.

Early work on the analysis of degradation products of EBDC's was carried out by Fishbein and Fawkes (67). They separated Maneb and Zineb from the metabolites ETU, EBIS and elemental sulphur using silica gel plates impregnated with formamide and elution with chloroform.

Another procedure for the determination of ETU and other metabolites was performed by Czegledi-Janko (68). The compounds were identified on silica gel plates developed in chloroform: butan-1-ol. Blasquez (69) detected residues as low as 1 mg/kg in tomato foliage, soil and



water. The breakdown of EBDC's during hot extraction was then reported.

Vogeler measured [^{14}C] PTU in apples treated with Propineb in two dimensional TLC (64). The apples were extracted with methanol for 8 hours, and applied on polyamid sheets pre-coated with silica gel. The following mobile-phases were employed: 1st direction: benzene: methanol 2nd benzene, methanol: ammonia 25%.

II - Gas-liquid chromatography

Gas-liquid chromatography has been the most widely used technique for the determination of ETU and PTU. This is presumably because of the sensitivity combined with a wide range of different detectors.

Many methods have been reported using GLC without prior derivatisation (70, 71). However, many difficulties have been arisen with direct analysis of ETU at residue levels. Results obtained using GLC must be regarded with caution since there is possibility of breakdown of EBDC's or their intermediate degradation products. Comparison of results achieved on analysis of formulations using both GLC and HPLC techniques has shown that GLC may produce anomalous results (73, 74, 75).

However, the majority of methods using quantification include a derivatisation step to enhance sensitivity and improve its GLC properties. In all cases derivatisation involves alkylation of the thiocarbonyl group and in some instances a double derivatisation (75), involving the NH group is required.

The main disadvantages of employing derivatisation in GLC are the increased analysis time together with the possibility that the reaction steps may not proceed quantitatively in the residue range. Consequently this procedure can lead to an incomplete formation of derivatives and inconsistent yield. Some of the derivatives are unstable and must, therefore, be analysed without delay. Since the majority of derivatives are volatile, caution must be taken during the handling and concentration steps to prevent losses by evaporation. Furthermore, such methods may also require extensive clean-up procedures to remove interferences particularly those from samples of foodstuffs.

A widely used approach to determine ETU in food was made by Onley and Yip (76) who devised an elaborate extraction clean-up procedure and derivatised ETU with 1-bromobutane before quantifying the amount of S-butyl-ETU in a thermionic detector.

Newsome (77) improved the sensitivity of Onley-Yip's method by

forming the S-benzyl derivative followed by trifluoroacetylation with trifluoroacetic anhydride to produce 2-(benzylthio)-1-(trifluoroacetyl)-2-imidazoline for quantification with an electron capture detector.

A new variation for ETU analysis was proposed by Nash (75, 78) using pentafluorobenzoyl chloride as the acylating agent for the derivatisation instead of trifluoroacetic anhydride. The final product 1[(pentafluorobenzoyl)-2-imidazoline] was claimed to give more consistent and complete recoveries on account of an improved electron capture response.

King (79) performed a new approach by derivatising ETU with m-trifluoromethylbenzyl chloride which could be determined at low levels (0.01 mg/kg) without the need for a second derivatisation step. Both specific flame-photometric detector in sulphur mode or electron capture detector can be used for quantification. In addition, it was possible to confirm the structure of the compound if necessary, by further derivatisation for instance, with trifluoroacetic anhydride. This second derivatisation was reported to increase the response to the electron capture detector six times. So far, King's method was the simplest, requiring no column clean-up step and therefore no evaporation stage to remove the eluent mixture after column chromatography. Lembo *et al.* (80) applied King's method for evaluation of PTU in rat tissues and fluids with good results using electron capture detector. A limit of determination of 0.1 mg/kg and the recovery of PTU from rat urine fortified at 5 mg/kg was approximately 93%.

Based on the same derivatisation step with m-trifluoromethyl benzyl chloride, Uno *et al.* developed a method to evaluate PTU in agricultural products using flame photometric detection. Recoveries of PTU added to apples, tomatoes, cucumbers and beans ranged from 78 to 107%.

In a recent paper, Hirvi *et al.* (71) described a capillary column method for determining ETU without derivatisation using electron capture detector or nitrogen-phosphorus selective detector. The lowest detectable amounts were respectively 0.01 ng and 0.02 ng.

A simple capillary column GLC technique was developed by Nitz *et al.* (81) to assay PTU and ETU residues in beer hops and grapes without derivatisation. Later Nitz *et al.* (61) used the same technique to investigate the fate of ETU and PTU in EBDC treated hops during the brewing process.

Autio (82) devised another simple derivatisation procedure where

ETU is reacted with trimethylanilinium hydroxide in methanol. The method has been used for ETU determination in cigarette smoke condensate and also in blackcurrants. Matisova *et al.* (83) reported that direct analysis of ETU at low levels was very difficult to achieve even using capillary column. They basically used the same methods described by Onley (76) and Newsome (77) with some minor modifications. No pre-cleaning steps were required. The use of capillary columns is advantageous because of their high separation efficiency, however, practical samples generate many peaks in the chromatograms which means that the position of the derivative has always to be confirmed.

III - High-performance liquid chromatography

In earlier work, Onley *et al.* (84) devised a modified procedure of their previous method for the direct determination of ETU without the need for a derivatisation step. A limit of determination of 0.05 mg/kg was reported but was only achieved by using a 40 g sample and concentrating the final extract to 0.2 ml.

A method for determination of ETU in wine by HPLC was described by Lazzarini *et al.* (85) using UV detection. Recoveries of 60 to 75% were reported with a detection limit of approximately 2 ng.

Kobayashi *et al.* (86) demonstrated the potential of HPLC in the simultaneous quantification of thioureas in rat plasma. The following thioureas were separated: ethylenethiourea, 1,3-diethylthiourea (1,3 DETU), 1,3-dimethyl-2-thiourea, thiourea, methylthiourea and ethylthiourea.

Van Damme *et al.* (87) and Bystricky and Natora (88) reported the use of HPLC for the determination of ETU in commercial EBDC fungicide formulations. A sample of the fungicide was shaken with methanol and then filtered. An aliquot of the resulting mixture was injected onto the HPLC column without any further clean-up. Van Damme *et al.* (87) used a C18 bonded silica column, Bystricky and Natora declared they were unable to use reversed phase C18-bonded silica columns. Therefore, they employed silica gel columns.

Later, a procedure to determine ETU in beer employing an elaborate HPLC column switching system was described by Masey *et al.* (89). The method required a relatively simple clean-up. Mayer (90) developed a HPLC methodology for simultaneous determination of PTU and ETU. The samples had to be treated with potassium fluoride and ammonium chloride prior to

injection, which seems to play an important role in the elution of PTU and ETU, otherwise poor recoveries will be achieved. Apparently both salts help in separating ETU and PTU from the biological matrix. The limits of detection in beer, wine and fruit juices were below 4 ppb.

Lehotay *et al.* (91) carried out a study for determination of ETU in ethylenebisdithiocarbamate formulations Perozin and Dithane M-45. The work investigated the influence of the extraction solvent on the amount of ETU which could be determined and whether during extraction EBDC is converted to ETU. The fungicide was extracted with methanol or water. The suspension was centrifuged and the clear solution was injected into a liquid chromatograph with UV detection.

The more recent approach to PTU and ETU analysis was accomplished by Andersen (92). The techniques are quite similar to that one developed by Mayer (90). The samples were also treated with potassium fluoride and ammonium chloride. The clean-up was done in Extrelut columns eluting with dichloromethane.

In order to monitor total exposure of workers and absorption of ethylenebisdithiocarbamates by all routes, Prince (93) analysed ETU levels in the urine of people exposed to the effects of metabolites of EBDC's by inhalation or direct absorption through the skin. The methodology was a simplified version of the Onley-Yip (84) method eliminating the S-butyl derivatisation step. The detection was accomplished in this particular case by an electrochemical detector. The application of an electrochemical detector can be advantageous since they can achieve higher sensitivity and higher selectivity than UV detectors.

Finally the most recent approach to determine ETU was carried out by Madahar (94). A derivatisation step was performed with phenacyl halides to give enhanced sensitivity and improved HPLC characteristics. The derivative method was claimed to be very sensitive (LOD = 0.04 ppm) and showed less interferences from co-extractives.

1.8. - FLUOROGENIC DERIVATISATION OF DIAMINES

In order to investigate the degradation of propylenethiourea, the major metabolite of Propineb to propylenediamine (PDA) under UV irradiation, it was necessary to develop an assay method for monitoring the diamine.

Erratic or irreproducible results are often obtained when quantitative determination of aliphatic amines by GLC is attempted. Considerable difficulty is found because their reactivity and adsorption on the solid surfaces.

Aliphatic amines, are not easily detected by HPLC, since they not fluoresce, nor do they possess chromophores absorbing in the UV region. Therefore, it was planned to employ fluorogenic derivatisation to give stable products with increased sensitivity.

1.8.1. - Fluorogenic labelling

Fluorogenic derivatisation of a wide variety of structurally diverse compounds containing amino groups has dramatically advanced the development of high performance liquid chromatography. For the sensitive detection of solutes in the nanogram range, fluorometric analysis has often been adopted.

Many fluorogenic labels for amines (95), thiols, phenols and alcohols and carboxylated compounds have been developed and some of these are now being used for pre and post-column derivatisation. Under certain conditions chemical modification of the analyte can facilitate and improve its analysis.

The pre-column derivatisation offers some advantages over post-column derivatisation; greater freedom and flexibility in the selection of reaction conditions (fewer restrictions imposed on reaction kinetics) and usually less need and difficulty in removing excess reagent prior to HPLC. The major disadvantage of the pre-column technique is the possibility of forming multiple compounds from one analyte or the formation of artifacts which may interfere.

The main derivatisation agents for amines can be compared and the difference in their properties examined.

I-NBD-chloride

NBD-chloride (4-chloro-7-nitrobenzo-2-oxa-1,3-diazole) reacts with primary and secondary aliphatic amines to form highly fluorescent derivatives. It was introduced by Gosh and Whitehouse (96). The method was very selective towards aliphatic amines since other compounds such as aniline, phenols and thiols yield weakly or non-fluorescent derivatives. It

is necessary to point out that NBD-chloride reacts well with secondary amines (97) and less well with primary amines. The derivatisation is usually carried out with a five to ten fold molar excess of NBD-chloride in aqueous methyl isobutyl ketone (Fig. 1.8.1.).

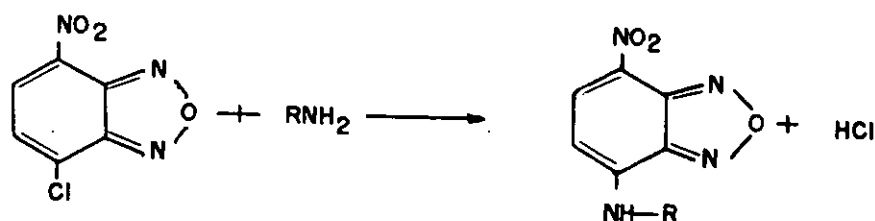


Fig. 1.8.1. - Fluorogenic derivatisation of an amine with NBD-chloride.

The strong fluorescence of the NBD amines is not observed in solvents of low polarity and is excited by visible light (464 nm) (98). Therefore quartz cells and a special ultraviolet fluorimeter are not required.

Traces of low molecular weight amines in air were derivatised with NBD-chloride in the investigation of Nishikawa and Kuwata (99). The reaction occurred in alkaline methanol and the resulting amine adducts were determined by reversed-phase HPLC with fluorescence detection at 530 nm. A wide range of amines such as methylamine, ethylamine, dimethylamine, isobutylamine were determined at picomole levels.

II - NBD-fluoride

NBD-fluoride (4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole) reacts with primary and secondary amines to yield fluorescent adducts (100) (Fig. 1.8.2.).

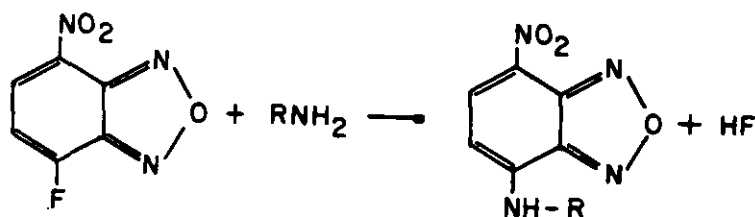


Fig. 1.8.2. - Fluorogenic derivatisation of an amine with NBD-F.

The reaction is reported to take place via a Meisenheimer complex (101). The reaction of amines in 50% ethanol - 0.1M borate buffer is usually complete within one minute by heating NBD-fluoride at 60°C with addition of hydrochloric acid to terminate the reaction and to suppress the blank fluorescence. The reactivity of NBD-fluoride with secondary amines has been thoroughly investigated (102) with proline. As the pH and temperature increased the reaction speed became faster to yield higher net fluorescence intensities.

Various organic solvents and buffers affected the reaction rate. Ethanol and acetonitrile are better solvents for NBD-F and a study of the reaction in buffers indicated that borate buffer pH 8.0 was the optimum for derivatisation of amines. The resultant adducts are quite stable in the dark under refrigeration after one week (103). The fluorescence of hydrolysis product of NBD-F namely 4-hydroxy-7-nitrobenzo-2-oxa-1,3-diazole (NBD-OH) is quenched by decreasing the pH of reaction medium to less than 1, which results in a shift of its absorption maximum. The fluorescence intensities of NBD-F adducts remained constant with the variation in pH (104).

NBD-F is a suitable label for analysis in biological matrices (105) since its adducts fluoresce at longer wavelengths (excitation at 470 nm and emission at 530 nm) then avoiding interferences of co-extracted materials.

Imai *et al.* (106) studied the fluorogenic reaction of proline with NBD-fluoride and they concluded that this label is superior in terms of reactivity and fluorescence yield to the reactions with the analogous 7-chloro and 7-bromoderivatives. Many secondary amino acids can be determined by reaction with NBD-fluoride at pH 7.5 at 70°C for 5 min and subsequent acidification to pH 1. The detection limits for proline,

hydroxyproline and sarcosine are 0.08, 0.04 and 0.17 nmol ml⁻¹ respectively. Under the same conditions, the primary amino acids, alanine, arginine and aspartic acid, are detected at 1.7, 1.7 and 3.4 nmol ml⁻¹, respectively.

Toyo'oka *et al.* (107) investigated the reactions of amines, amino acids and imino acids with NBD-fluoride with high performance liquid chromatography with fluorometric detection. All the amino compounds tested, except tryptophan, gave fluorescent derivatives at 524-541 nm with excitation at 467-472 nm. The reaction rates for secondary amines, imino acid, primary amine and amino acid decreased in this order.

III - Dansyl chloride

Dansyl chloride (5-dimethylaminonaphthalene 1-sulphonyl chloride) reacts with phenols and primary or secondary amines to form sulphonate esters or sulfonamides (108). The derivatives are highly fluorescent, whereas the reagent itself is not fluorescent. However, the hydrolysis product of DNS-chloride, DNS-OH (dansyl sulphonic acid) formed during the reaction shows strong fluorescence in the pH range from acidic to alkaline and interferes in the fluorescence analysis of water-soluble compounds (109). However, DNS-OH can be separated during the chromatographic process. For non-polar derivatives such as those from most phenols, the hydrolysed reagent can be removed by partition.

The derivatives are formed using a five to ten fold molar excess (110) of dansyl chloride in a mixture of acetone and aqueous sodium bicarbonate with either heating at high temperatures (30-50°C) or standing for several hours at room temperature (Fig. 1.8.3.).

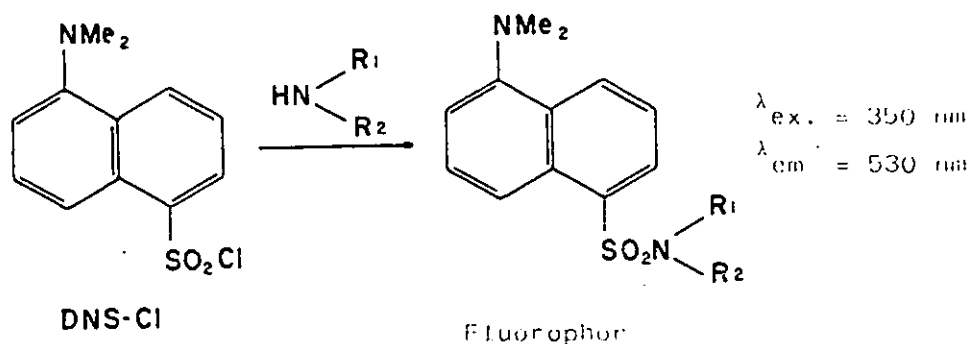


Fig. 1.8.3. - Fluorogenic derivatisation of an amine with DNS-chloride.

Dansylation has been applied to a number of drugs in pharmaceutical compounds. Frei and co-workers (111) described the dansylation of cephaeline, emetine, ephedrine and morphine in capsules and syrups. However, codeine and narcotine (lacking phenolic groups) failed to react with dansyl chloride. The sensitivity was similar either with fluorometric detection or with ultraviolet detection. Few interfering peaks were observed in the fluorescence determination therefore selectivity could be improved.

Biological amines can also be dansylated using pre-column HPLC. In this case the amine must be separated from the biological matrix prior to derivatisation. This approach has been employed for the analysis of a wide variety of polyamines of interest in cancer research (112). In their work Abdel-Monen and Ohno (113) described HPLC as a potential alternative to TLC in the separations of DNS derivatives of the diamines, polyamines and monoacetyldiamines. The separation of derivatives were generally good except for monoacetyl cadaverine and 1,3 diamino-propane.

In more recent work, Hayman (114) reported the applicability of dansyl derivatisation to the separation of biogenic amines by HPLC. The system was optimised for simultaneous determination of amines which were fairly well separated. DNS derivatives that run close together on TLC plates, like those of benzylamine and phenylethylamine or ammonia and 1,2 diamino propane were well separated by this system.

Finally, Henricks-Eckerman *et al.* (115) developed an ion-pair liquid chromatographic methodology for dansylated aliphatic polyamines which are commonly used in epoxy resins. The derivatisation was carried out by reacting the polyamines with a solution of dansyl-chloride in acetone. The mixture was heated at 55°C for 2 hours in water bath. After cooling at room temperature, the dansylated amines were extracted into ethyl acetate and analysed by HPLC with both fluorometric and UV detection.

The reaction of DNS-chloride with amines is very easily accomplished but is not free from side reactions. The typical products formed include DNS-NH and the aldehyde or ketone molecules (116) Fig. 1.8.4. The large excess of DNS-chloride as well as increased pH values in the reaction mixture accelerates the formation of DNS-NH.

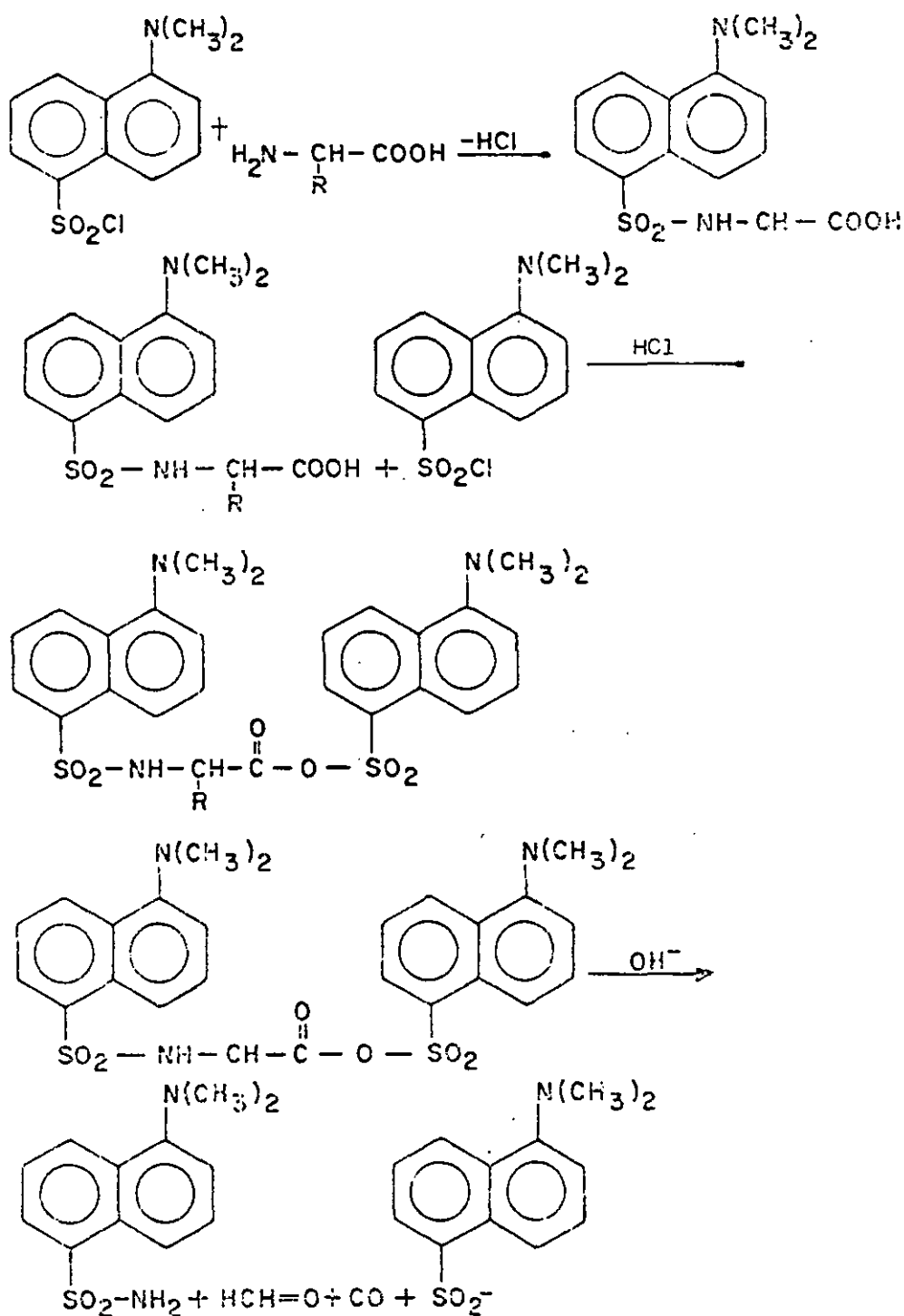


Fig. 1.8.4. - Proposed side reactions or effect of increased amount of DNS-chloride and increased pH values.

IV - o-Phthalaldehyde

Roth (117) first reported formation of fluorescent derivatives with OPA and amino acids in the presence of 2-mercaptoethanol (2-merc) in aqueous alkaline medium (pH 8.0).

o-Phthalaldehyde reacts only with primary amines in alkaline medium in the presence of certain thiols (2-mercaptoethanol or ethanethiol) to give fluorescent isoindole adducts and these can be detected with excitation at 340 nm and emission at 455 nm (117, 118, 119, 120, 121, 122).

In 1976 Simons and Johnson (121) reported the first study elucidating the structure of the derivative formed in the preparative reaction of a primary amine, OPA and 2-mercaptoethanol. Based upon the proton NMR spectra of derivatised n-butylamine they came to the conclusion that the structure of the compound was 1-alkylthio-2-alkyl substituted isoindole. In order to confirm the presence of the isoindole ring system, Simons and Johnson (120) in more recent investigation, performed UV and Mass Spectra (MS) analysis for many derivatised samples.

The mechanism of the reaction of OPA and thiol with primary amines involves the protonation of amine intermediate followed by a "partially S_N1 -like intramolecular reaction" to give a protonated isoindole (Fig. 1.8.5.) (120, 121).

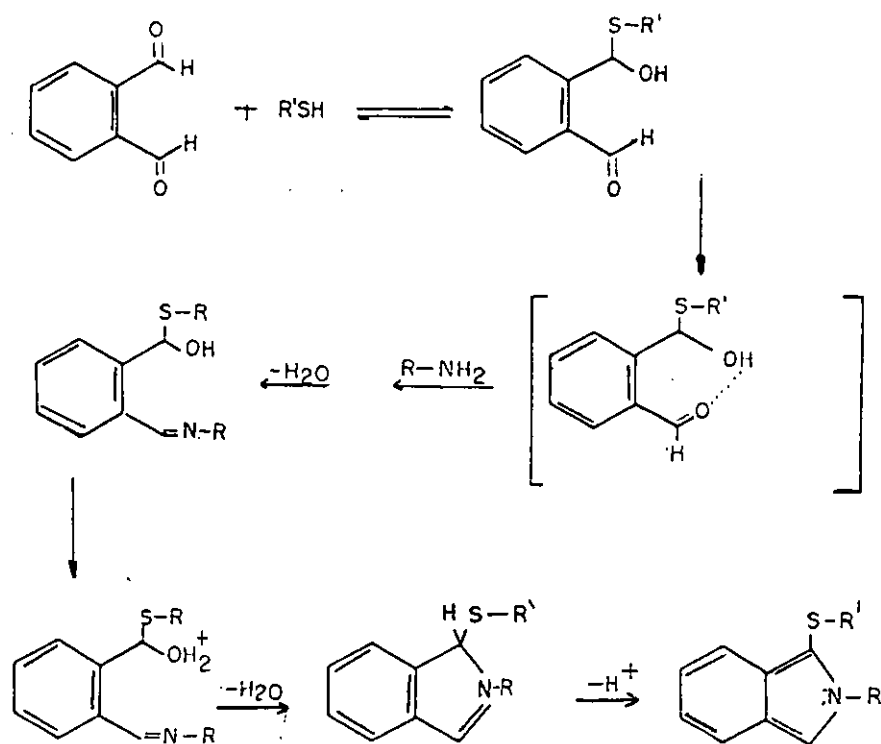


Fig. 1.8.5. - Mechanism of fluorogenic reaction of an amine with OPA.

Although OPA adducts are relatively stable at pH 9-11, they do degrade to non-fluorescent species, decomposition occurring more rapidly as pH is lowered. This reaction explains the lack of fluorescence in the reaction with secondary amines at low pH solutions. The isoindole formed with 2-mercaptoethanol has been observed to decompose to give an ethylene sulphide polymer and a non-fluorescent 2,3-dihydro-1H-isoindole-1-one (Fig. 1.8.6.).

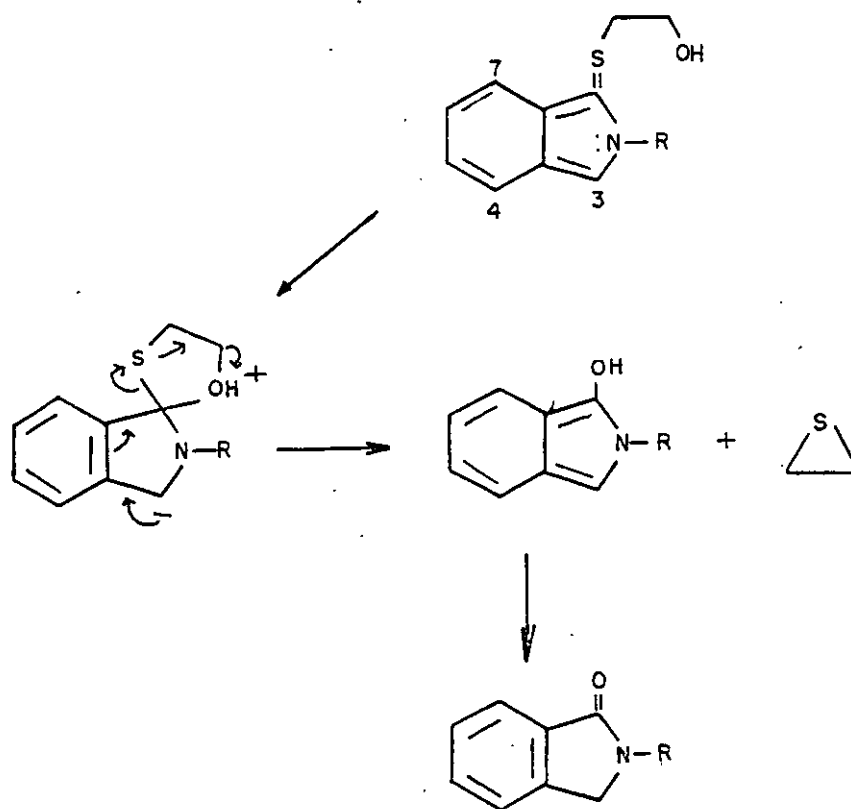


Fig. 1.8.6. - Proposed degradation mechanism for 1-(2-hydroxyethylthio)-2-alkylisoindole.

The decomposition pattern suggests that the 2-mercaptoethanol adduct had possibly undergone a spontaneous intramolecular rearrangement with sulphur being displaced by oxygen. The stability of the OPA derivatives has been found to be affected by the thiol and the amine involved. Glycine, lysine, and ornithine derivatives are exceptionally unstable. The stability also seems to depend on the structure of the thiol used in the reaction. The stability is greatly increased by substitution of ethanethiol for 2-mercaptoethanol. The instability of the OPA derivatives according to some investigators, has been derived from the OPA itself (118, 119). A suitable ratio (2 or 3) of 2-MERC to OPA is observed to lessen the degree of instability. An excess of 2-MERC seems to enhance the decomposition of the adduct (121).

Coque *et al.* (122) reviewed the formation and instability of OPA derivatives of amino acids in the presence of 2-mercaptoethanol. They described the isoindole formation, which permitted a better understanding of the parameters affecting the stability of the reaction. They also reviewed the use of alternative thiols and o-phthalaldehyde-like reagents.

The physiological and biochemical significance of polyamines has necessitated the development of a series of analytical methodologies which have been reviewed by Seiler (123, 124). Skaaden *et al.* (125) reacted spermine, spermidine and cadaverine with OPA and ethanethiol. After a reaction of 90 sec, the stability of the derivatives was examined in the reaction medium, in ethyl acetate extracted and in different mobile phases with separation by HPLC. The stability of the ethanethiol derivatives was improved compared to the 2-mercaptoethanol derivatives.

OPA/2-MERC has become popular for pre-column or post-column derivatisation of amines and detection by HPLC (123, 124). However, the post-column procedures require special equipment and are time consuming to set up. In contrast, pre-column derivatisation has been developed to improve the efficiency and decrease the analysis time for resolution of amine mixtures.

Kucera and Umagat (119) designed and discussed a post-column system for the use with microbore columns. The system was applied to the reaction of amino acids and primary aliphatic amines, which were separated with fluorometric detection. The use of 3-mercaptopropionic acid as reducing agent was reported. Turnell and Cooper (126) described a method for estimating clinically important amino acids in serum or urine, using fluorescence detection of OPA/MERC derivatives and their separation by gradient

elution reversed-phase HPLC.

V - Fluorescamine

Fluorescamine (4-phenylspiro [furan-2-(3H),1'-phthalane])-3,3'-dione is a reagent capable of reacting with a wide spectrum of compounds that contain nucleophilic functional groups including primary and secondary amines, alcohols and thiols, but only the reaction with primary amines yields fluorescent products (127). It reacts almost instantly with these substrates while the excess reagent is hydrolysed to a non-fluorescent product (128, 129).

The mechanism of the reaction with the analyte involves the rapid, reversible addition of the primary amine across the double bond of fluorescamine to give a non-fluorescent intermediate which is transformed via a multistep rearrangement to the desired fluorescent product (130, 131). (Fig. 1.8.7.).

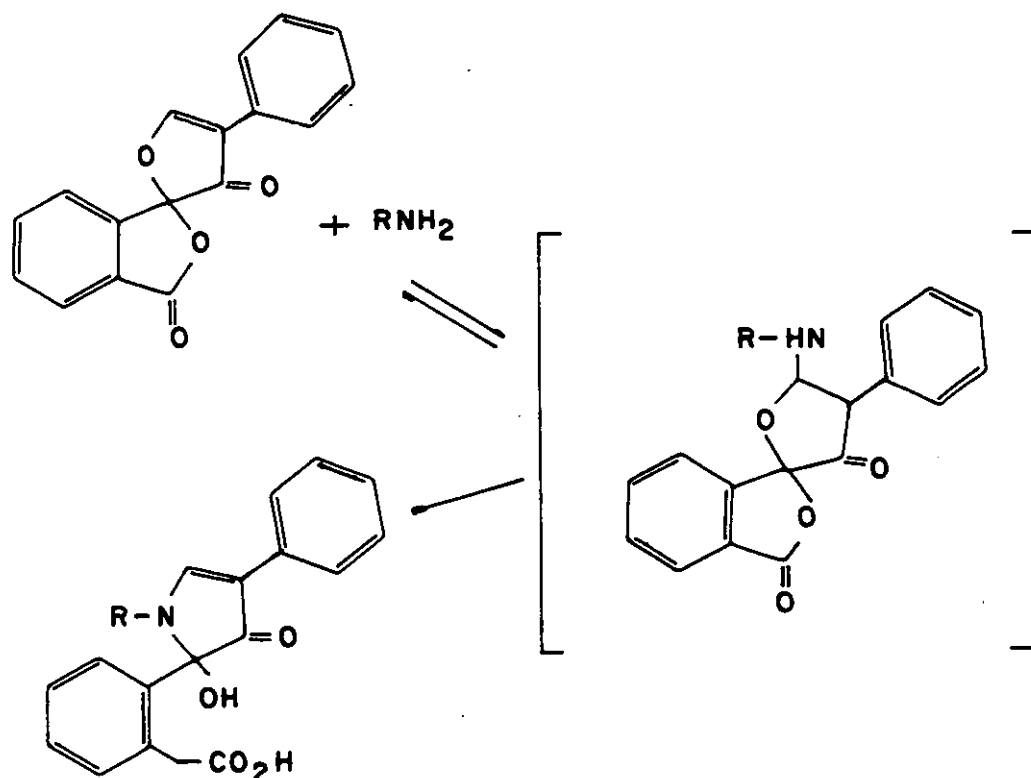


Fig. 1.8.7. - Fluorogenic reaction of a primary amine with fluorescamine.

The reaction is complete within a few seconds and the yield of the fluorophore is approximately 80-90%. The conversion to the fluorophore is carried out in aqueous-acetone mixture at pH 8-9, while the sample is shaken in a vortex mixer (131,132). The derivatives may then be directly chromatographed on a reversed-phase system. Since the reaction proceeds so rapidly, it is also ideally suited for post-column derivatisation after components have been separated. The excitation of the products usually occurs at 390 nm with fluorescence emission at 475 nm. The fluorescence intensity of fluorescamine adducts are the same with pH ranging from 4.5 to 10.5 and low in acidic conditions (127).

The fluorescence of the adduct is increased in mixed solvents than in pure ones. It is believed that water takes part in an additional energy wasting process with the excited state to form an excited state complex. The formation of this complex reduces the fluorescence quantum yield and decreases the sensitivity of assay. However, the removal of water from samples is not a practical procedure for routine work and the buffer system for the reaction is aqueous.

The dependency of the reaction on the pH is an important factor since the cyclisation of the lactone ring in the molecule is responsible for the loss of the fluorescence at pHs lower than 6 (131). Moreover, the protonation of the amine reactant interferes with the reaction. Castell *et al.* (127) studied the effect of the pH on the reaction using radioactively labelled amines. Two different profiles pointed out that pH is important in two ways - by lowering the available reactive species ($R-NH_2$) and by lowering the fluorescence of the adduct. Moreover, while the adduct is relatively stable giving maximum fluorescence from around 6 to 11, the reaction goes to completion in a pH around 8. The adducts are stable for several hours in the dark (128, 133).

Although fluorescamine is selective towards primary amines and much faster than either dansyl-chloride or NBD-halide reactions, it has several undesirable features. Its cost is much higher than the other two reagents. The fluorescence intensity of the product is remarkably influenced by pH. Reproducibility for replicate samples is worse than for dansyl and NBD-chloride. Also the stability of fluorophores is not as good as the others, so that the storage of products for more than few hours is not recommended.

Samejima (134) employed fluorescamine as fluorogenic label in determination of aliphatic amines and polyamines by HPLC. The best

separation was achieved by using a reversed-phase chromatographic system employing a chemically bonded ODS-Hypersil column with gradient elution. The limit of detection was of picomole order and the determinations could be made by measuring the peak-height ratio with respect to an internal standard.

Ingles and Gallimore (135) improved the separation of fluorescamine labelled amines when they chromatographed the derivatives as free carboxylic acids. Ethylamine was derivatised with fluorescamine and acidified with acetic acid. Extraction with chloroform gave rise to the crystalline free acid form in quantitative yield.

Fluorescence yields were not decreased in an acidic solution and the chloroform extracts of the derivatives were stable for several hours. Satisfactory results were achieved with reversed-phase silica column (Spherisorb ODS). Electrochemical detection was employed to identify the lactone product, also formed during the derivatisation since it does not show in the fluorescence detector. This procedure enabled the measurement of the rate of conversion of the free acid form of fluorescamine derivative to its non-fluorescent lactone form.

1.9. - THE PRESENT STUDY

The main purpose of the present work was to develop an assay for PTU at residue levels and also investigate its photodegradation pattern under ultraviolet irradiation.

The determination of PTU was carried out by HPLC with both UV or electrochemical detection on ODS-Hypersil column. To improve selectivity a derivatisation technique was adapted for PTU with *p*-nitrophenacyl bromide and resulting absorbing chromophore was monitored by reversed-phase HPLC with ultraviolet detection on a PS-DVB column. The structure of the derivative was characterised by spectroscopic methods.

The direct method was employed to evaluate PTU in sprayed lettuces and in fungicide formulations of Propineb.

During the study a number of fluorogenic labels, NBD-chloride, DNS-chloride, OPA and fluorescamine, were evaluated for the HPLC pre-column derivatisation of PDA (propylenediamine) which was a major breakdown product of PTU under UV irradiation.

CHAPTER II

2. EXPERIMENTAL

CHAPTER II

2. EXPERIMENTAL

2.1. - REAGENTS

Unless otherwise stated, the chemicals were used as received. Propylenethiourea and ethylenethiourea were kindly supplied by Bayer AG (West Germany). Propineb was kindly provided by the Laboratory of the Government Chemist.

Reagents

Boric acid, potassium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride were all A.R. grade from FSA Scientific Co. Loughborough (U.K.).

Propylenediamine, ethylenediamine, glycine were reagent grade from BDH Chemical Ltd. Poole-Dorset U.K.

2.2. - FLUORESCENT LABELS

NBD-chloride, NBD-fluoride, DNS-chloride, *o*-Phthalaldehyde, Fluorescamine were all reagent grade from Sigma Chemical Co. Poole-Dorset U.K.

2.3. - SOLVENTS

Water, methanol, acetonitrile and tetrahydrofuran were HPLC grade from FSA Scientific Co., Loughborough U.K. Benzene, methyl isobutyl ketone, dichlorometane, ethanol, petroleum ether, chloroform, hexane were SLR grade from FSA Scientific Co. Loughborough U.K.

2.4. - OTHER ITEMS

Alumina plates (layer thickness 0.2 mm)	E. Merck Darmstadt West Germany
Sand (40-60 mesh)	FSA Scientific Co. Loughborough U.K.
Glass fibre filter paper (GF/F) 5 μ m	Whatman International Ltd. Maidstone U.K.
Aluminum oxide	BDH Chemical Ltd. Poole-Dorset U.K.

The aluminum oxide was washed prior to use with methanol and the slurry filtered through a Buchner funnel. Al_2O_3 was then placed in an oven and heated at 135-140°C overnight and then stored in a closed container.

2.5. - INSTRUMENTATION

1. Ultraviolet irradiation apparatus

A multilamp reactor from Applied Photophysics Ltd. (model MLU 18) was employed. The reactor is designed for the external irradiation of a single container. The twin lamp modules are arranged symmetrically around a cooling fan mounted on a hexagonal metal base plate. (Fig. 2.1.). Five standard emission lamps are available for use with the reactor all being of identical size and electrical characteristics. For this investigation a 254 nm lamp (model 3020) that has no phosphor coating and emits 90% of its at 254 nm was used. This lamp must be used only with quartz glassware.

2. Measurement of Infrared Data

Infrared Spectra were recorded using a model 130 infrared spectrophotometer (Perkin Elmer Ltd, Beaconsfield, Bucks). Samples were prepared by grinding with potassium bromide and were pressed into discs.

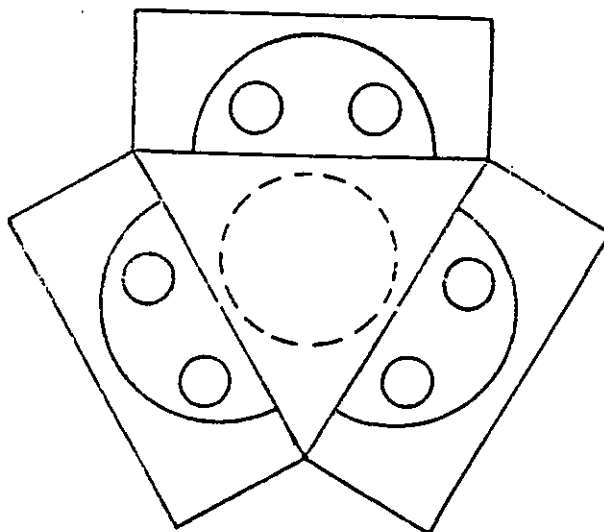


Fig. 2.1. - Arrangement of multilamp reactor using 3 lamp modules.

3. Measurement of Absorbance Spectra

Absorbance Spectra were measured using a UV 160 UV /visible spectrophotometer (Shimadzu Corporation, Kyoto, Japan), 1 cm path length quartz cells were used.

4. Measurement of Fluorescence Spectra

A fluorescence spectrophotometer (Perkin Elmer Ltd. Beaconsfield-Bucks) model 2000 was used to record excitation and emission. Silica cells of 1 cm path length were used for the measurements.

5. Mass Spectrometry

Mass spectral data were obtained on a Kratos MS 80/DS-55 double focussing instrumental with a sample resolution of 7500.

6. N.M.R. Spectrometry

Proton magnetic resonance spectra were recorded at 90 MHz on a Perkin Elmer equipment model R32.

7. Elemental Analysis

Elemental analysis was carried out at the Microanalytical Laboratory; Department of Chemistry, University of Manchester.

2.6. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY INSTRUMENTATION

The HPLC system consisted of a Pye Unicam high pressure pump model LC-XPS and a guard column filled with silica. A model 7125 Rheodyne injection valve (Cotati - California) fitted with a 20 μ l sample loop was used and the sample was introduced using a 10 μ l syringe (SGE, Australia). The columns used were 10 cm x 5 cm i.d. which had been packed in our laboratory with 5 μ m ODS-Hypersil (Shandon Southern Ltd.) and a 15 cm x 5 mm PLRP-S (polystyrene divinyl benzene column) supplied by Polymer Laboratory (Shropshire, U.K.). Three detectors were used, a Pye Unicam variable wavelength ultraviolet detector model PU 4020 (Cambridge-England), a fluorescence detector Perkin Elmer 2000 and an electrochemical detector from Kipp Analytica model 9205. The recording system consisted of a Perkin-Elmer chart record model 56 and a Hewlett Packard integrator model 3390 A.

2.7. PACKING AND TESTING A HPLC COLUMN

I. Packing procedure

A pneumatic pump Haskel (SAT/RTG), capable of generating 15000 psi was used. Appropriate fittings connected the packing column and HPLC column.

ODS-Hypersil (1.8 g) was dispersed in 30 ml of methanol and

shaken vigorously for five minutes. The slurry was quickly poured into the packing material reservoir and the HPLC column was connected above this reservoir using Teflon seals. (Fig. 2.2.).

A pressure about 6000 psi was immediately applied and upward packing was allowed to take place, first with 60 ml of propan-2-ol and then 80 ml of methanol. The packing reservoir assembly was then inverted and downward packing continued with 80 ml of the eluent which is to be used for the HPLC column. Pressure was slowly reduced to zero. After a short time, HPLC column could be disconnected and the excess packing material was then carefully removed.

II. Testing of the HPLC column

The efficiency of the column was determined by injecting 10 μ l of the standard test mixture containing acetophenone (0.0006% W/V), benzamide (0.0037% W/V), benzophenone (0.0007% W/V) using methanol/ water 50:50 as eluent for an ODS-Hypersil at 254 nm. For testing a polymer column 10 μ l of nortriptyline (0.002% w/v) was injected into the HPLC system at 263 nm using methanol/water 50:50 as eluent.

The column efficiency was calculated according to Equation 2.1.

TABLE 2.1. - Efficiency for an ODS-Hypersil and for a PLRP-S column.

COLUMN	COMPOUND	EFFICIENCY (N) OF THE COLUMN
ODS-Hypersil (10 cm x 5 mm)	Acetophenone	3182
Polymer (PLRP-S) (15 cm x 5 mm)	Nortriptyline	2325

2.8. DEFINITIONS

I. Column efficiency

Efficiency (N) of the column is given by Equation 2.1.

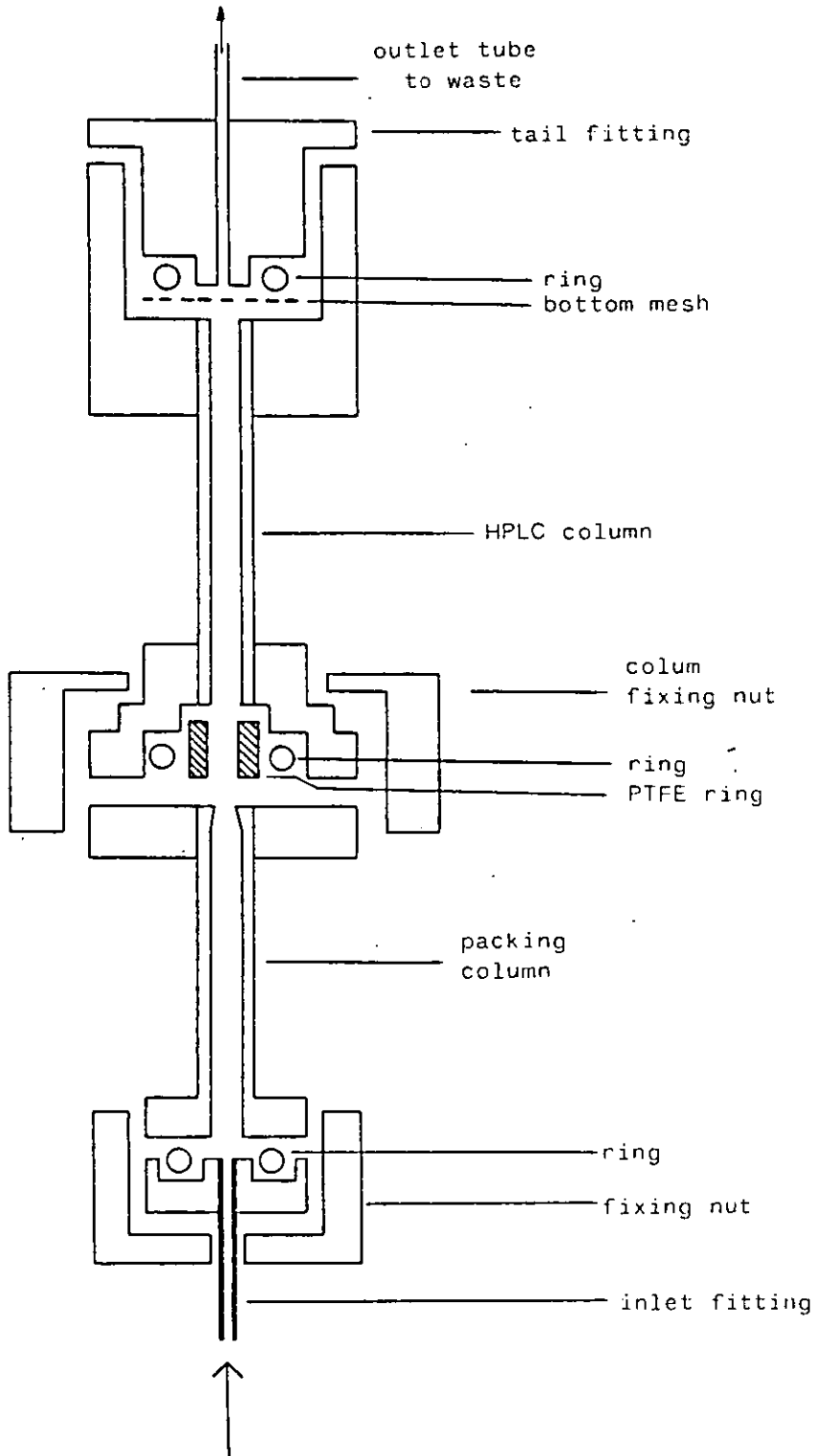


Fig. 2.2. - Details of packing chamber and HPLC column.

$$N = 5.54 \left(\frac{t_r}{W_{1/2}} \right)^2 \quad (\text{Eq. 2.1.})$$

Where

t_r = the retention time of the solute

$W_{1/2}$ = the peak width at half height

II. Capacity factor

Capacity factor k' is a measure of a degree of retention of a solute and is defined by Equation 2.2.

$$k' = \frac{t_r - t_o}{t_o} \quad (\text{Eq. 2.2})$$

Where

t_o = the retention time of unretained solute

t_r = the retention time of the solute

The dead volume for both columns employed throughout this study was determined using a sodium nitrate solution (0.039 g in 100 ml of methanol-water 50:50) and 10 μ l aliquot was injected onto the HPLC system with UV detection at 254 nm and elution with methanol-water 50:50.

III. Limit of detection

This is calculated as the amount of the analyte, which gives a signal equal to two standard deviations above the background level SB of the detector (136).

SB: is the standard deviation (Fig. 2.3) of background noise level of the detector. SB is determined by dividing the background detector output signal into a series of time segments less than one minute duration and summing the vertical displacement y_r of each segment on a fixed interval using Equation 2.3.

$$SB = \Sigma_{R=1}^{R=1} \frac{y_r}{n} \quad (\text{Eq. 2.3})$$

y_r = vertical displacement of the noise in y-axis

n = number of y_r values, usually between ten and fifteen.

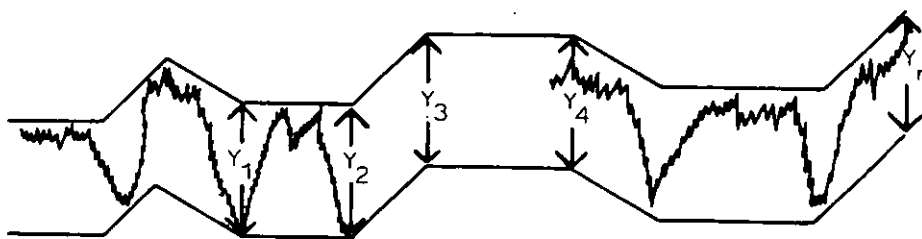


Fig. 2.3. - Calculation of standard deviation of (blank) noise level of detector from a chromatogram.

IV. Limit of determination

It is defined as the concentration of analyte which gives a background detector signal equivalent to the value of $10 S_B S$ (137). Sometimes it is also called as "Limit of quantification".

2.9. - GENERAL METHODS

2.9.1. - Degradation of propylenethiourea (PTU) under UV irradiation

A solution of PTU (500 ppm) in water was placed in a quartz tube (15 cm x 3.0 cm i.d.) and irradiated for 90 hours under UV light at 254 nm.

Every six hours an aliquot was taken and monitored by HPLC with fluorometric detection after derivatisation with fluorescamine (see section 2.9.6.).

2.9.2. - Fluorometric determination of PDA and EDA as NBD-chloride derivatives

Stock solutions of each diamine were prepared in distilled water at concentration of 0.01 mg/ml. An aliquot of amine stock solution (1 ml) plus 0.1 M NaHCO_3 (1 ml) were mixed in a reaction tube, followed by careful addition of an equal volume of 1% NBD-Cl solution in methyl isobutyl ketone. The mixture was mechanically stirred in a vortex mixture. The tube was immediately tightly closed, covered with aluminium foil, heated at 80°C for 30 minutes and allowed to cool. A 10 μl sample of the MIBK phase (top layer) was injected onto an ODS-Hypersil column.

The mobile phase methanol/water (50:50) was pumped at 1.0 ml/min. The fluorescence detector was adjusted to λ_{ex} 470 nm and λ_{em} 530 nm.

2.9.3.- Fluorometric determination of PDA and EDA as NBD-fluoride derivatives

An aliquot of amine stock solution 1 ml in phosphate buffer (2 ml) (pH 7.0 prepared with 0.066 M potassium dihydrogen phosphate and disodium hydrogen phosphate) in a 10 ml stoppered test tube was treated with 2 ml of a freshly prepared ethanolic 1% solution of NBD-F. Immediately after addition of the reagent the tube was tightly capped, covered with aluminium foil and heated at 50°C for 20 minutes. The mixture was then removed to a small test tube, cooled in ice. Aliquots (10 μ l) of the final solution were injected onto an ODS-Hypersil column eluted with methanol/water 50:50 with excitation at 470 nm and emission at 530 nm.

2.9.4.- Fluorometric determination of PDA and EDA as dansyl-chloride derivatives

An aliquot of 1 ml of amine stock solution (1 ml) was mixed either 0.1 M Na₂CO₃ solution (1 ml) (pH 8.0) or Li₂CO₃ (pH 8.0) and 0.1% dansyl chloride in acetone (1 ml). The reaction mixture was heated at 50°C for 60 minutes. After cooling, the mixture was extracted with ethyl acetate 3 ml and a 10 μ l aliquot of the organic phase was injected onto an ODS-Hypersil column and eluted with methanol/0.06 M phosphate buffer (pH 8.0)(50:50) pumped at a flow rate of 1.0 ml/min. The fluorescent derivatives were detected at 510 nm emission with excitation at 365 nm.

2.9.5. - Fluorometric determination of PDA and EDA as OPA derivatives

o-Pthalaldehyde (270 mg) was dissolved in 5 ml of ethanol (99.5%). 2-Mercaptoethanol (200 μ l) in OPA solution (5 ml) was added to borate buffer (0.4 M, pH 8.0) and volume adjusted to 50 ml with buffer. The reagent mixture was allowed to "age" for at least 24 hours prior to use.

An aliquot (1 ml) of each amine stock solution was reacted with OPA reagent (1 ml) at room temperature and injected onto the HPLC system after 2 minutes. An ODS-Hypersil column was used to separate the OPA derivatives. The mobile phase methanol/water (60:40) pumped at a flow rate of 1.0 ml/min. The fluorescent derivatives were measured at λ_{ex} 364 nm and λ_{em} 440nm.

2.9.6. - Fluorometric determination of PDA and EDA as fluorescamine derivatives

Fluorescamine solution was prepared daily by dissolving 20 mg in HPLC grade acetone (10 ml). Borate buffer (pH 8.0) was prepared by titrating 0.1 M boric acid with 6 N sodium hydroxide. Fluorescamine solution (1 ml) was added to a mixture of amine stock solution (1 ml) and borate buffer (1 ml) at room temperature in a vortex mixture. An aliquot (10 μ l) was directly injected to an ODS-Hypersil column. The eluent methanol-water delivered at 1 ml/min. The eluted fluorophores were measured at λ emission 455 nm after excitation at 340 nm. A calibration curve was plotted and the effect of MeOH concentration in the eluent on the capacity factor was determined.

2.9.7. - Fluorometric determination of glycine as fluorescamine derivative

To a mixture of 1 ml of glycine stock solution (0.01 mg/ml) and borate buffer (1 ml) (pH 8.0, 0.1 M) was added a fluorescamine solution (1 ml) at room temperature in a vortex mixture. An aliquot of 10 μ l was injected to an ODS-Hypersil column. The eluent methanol-water (30:60) was delivered at 1 ml/min. The eluted fluorophore was measured at λ emission 455 nm after excitation at 340 nm.

2.9.8. - HPLC analysis of propylenethiourea with UV detection

A sample solution was prepared by dissolving 0.01 g of propylenethiourea in 100 ml of distilled water. The separation was performed on ODS-Hypersil column using 100% water as eluent pumped at a flow rate of 1.0 ml/min and determined by UV detection at 233 nm. In order to improve the peak shape, THF (0.5 ml) was added to the mobile phase. The effects of THF and MeOH concentration on the capacity factors were determined for isocratic runs.

2.9.9. - HPLC analysis of propylenethiourea in Propineb formulations

Propineb (10 mg) was extracted with 10 ml of methanol. The suspension was centrifuged for 15 minutes (5000 rpm) and the supernatant was evaporated to dryness and diluted in methanol (10 ml).

An aliquot (10 μ l) was injected onto the HPLC system. The separation was carried out on an ODS-Hypersil column using methanol: water (5:95) as eluent pumped at a flow rate of 1.0 ml/min with UV detection at 233 nm. The amount of PTU in Propineb formulation was determined formulation was determined by comparison with standard solutions.

2.9.10. - HPLC analysis of PTU electrochemical detection

A propylenethiourea solution of 0.01 g/100 ml in distilled water was diluted to different concentrations for plotting a calibration curve.

An aliquot(10 μ l) of each solution were separated by HPLC using a coulometric detector, operating at a potential of 0.95V related to a reference eletrode $\text{Ag}^+/\text{AgCl}/\text{Cl}^-$. Methanol-water (5:95) containing 0.001 M sodium chloride was delivered at 1.0 ml/min onto an ODS-Hypersil column.

2.9.11. - HPLC analysis of PTU spiked onto lettuces

Extraction: Lettuces samples (30 g) were chopped and fortified with PTU (0.01 to 1.0 ppm). The samples were extracted with methanol (3 x 100 ml) and shaken for 10 minutes. The combined extract was filtered through a Buchner funnel which was washed with methanol. The solution was partitioned with hexane (3 x 100 ml) and the methanol layer (bottom layer) was evaporated to approximately 5 ml. The resultant slurry was poured onto a clean-up column (30 cm x 2,5 i.d.) plugged with glass wool and quartz sand (1 cm layer) and containing aluminium oxide (10 g).

The clean-up column was eluted with dichloromethane (160 ml) and the eluate was evaporated to dryness on a rotary evaporator. The residue was dissolved in methanol and analysed by HPLC according to the conditions described in section 2.9.9.

2.9.12. - Phenacyl halide derivatives of PTU

I - Synthesis: Phenacyl halides are strong irritant to eyes and mucus membrane and the reaction should be conducted in a fume cupboard.

Propylenethiourea (0.5 g) and p-nitrophenacyl bromide (5 g) were dissolved in 50 ml of ethanol and the solution was refluxed for two hours. The ethanol was evaporated to a small volume 10 ml, at 50^o C, on a rotary evaporator. After the addition of water (40 ml) the mixture was cooled at room temperature and acidified (to pH 5 or 6) by dropwise addition of

hydrochloric acid(1 M). The excess reagent was extracted into chloroform (2 x 20 ml). The aqueous solution was adjusted to pH 9.0 with sodium carbonate solution (1 M) and allowed to stand for fifteen minutes. The derivative present in the aqueous solution was then extracted into chloroform (3 x 40 ml), which was evaporated to dryness. The residue was recrystallized from ethyl-acetate/petroleum ether to give red crystals of 3-(p-nitrophenacyl)-6-methyl dihydroimidazo[2,1-b] thiazole in 83% yield with melting point 158-163°C. The structure was confirmed by mass spectra, NMR, IR and UV spectroscopy and elemental analysis. The product was soluble in chloroform ethyl acetate and hexane.

II - Spectroscopic characteristics of the phenacyl bromide derivative propylenethiourea

TABLE 1: Ultraviolet spectra

ρ-NITROPHENACYL BROMIDE DERIVATIVE

max (nm)	263	393
log (ε)	4.46	3.46

TABLE 2: Infrared spectra

ρ-NITROPHENACYL BROMIDE DERIVATIVE

C = N	1585 cm ⁻¹
C - N	1490 cm ⁻¹

TABLE 3: Mass Spectra of derivative

DERIVATIVE	ρ-NITROPHENACYL BROMIDE
Formula	C H N O S 12 11 3 2
Accurate mass	261.0571

TABLE 4: Proton - NMR Spectra

The spectra were measured at 90 MHz as CDCl_3 solutions

1.40	(3H d, CH_3CH)
3.39	(1H, dd, HCH)
4.08	(1H, dd, HCH)
4.61	(1H, m, $\text{CH}-\text{CH}_3$)
6.01	(1H, s, $\text{CH}=\text{C}$)
7.53	(2H, d, arom H O to NO_2)
8.19	(2H, d, arom H O to NO_2)

III - Elemental Analysis

Analysis for C, H, N, S.

	FOUND	EXPECTED FOR $\text{C}_{12}\text{H}_{11}\text{N}_3\text{O}_2\text{S}$
C	55.0	55.17
N	4.2	4.24
N	15.8	16.09
S	11.4	12.02

2.9.13. - Derivatisation of PTU with *p*-nitrophenacyl bromide at residues levels

Samples of PTU (0.01 mg) and an excess of *p*-nitrophenacyl bromide (0.5g) were refluxed in ethanol (50 ml) for two hours. The ethanol was evaporated on a rotary evaporator, at 50°C , to a small volume (10 ml). After addition of water (20 ml), the solution was adjusted to pH 9 with sodium carbonate (1M) and allowed to stand for fifteen minutes. The derivative was then extracted into chloroform (3 x 40 ml), which was evaporated. The residue redissolved in the eluent (1 ml) and a 10 μl aliquot was analysed by HPLC using a PLRP-S column with UV detection at 263 nm. The mobile phase used was acetonitrile: buffer 70:30 (pH 9.0).

CHAPTER III

3. RESULTS AND DISCUSSION

CHAPTER III

3. RESULTS AND DISCUSSION

3.1. - DETERMINATION OF PROPYLENETHIOUREA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

In view of the current economic importance of dithiocarbamates in world-wide agriculture and the potential health hazards associated with PTU, there is a need to develop a sensitive and reliable methodology for determining residue levels of PTU in crops and formulated pesticides and also to investigate its photochemical degradation pattern.

Since PTU is thermally unstable, previous methods for its determination using gas chromatography very often required two derivatisation steps leading to poor reproducibility and low sensitivity. HPLC techniques lack selectivity since PTU has a weakly absorbing chromophore. Thus, better selectivity and greater sensitivity can probably be achieved by derivatising PTU with phenacyl halides and analysing by HPLC with UV detection.

3.1.1. - Direct Methods for PTU Analysis

Direct quantification of PTU at low levels was based on the technique described by Van Damme *et al.* (87) originally applied to ETU, which employed a reversed-phase HPLC with UV detection at 233 nm.

The PTU was eluted from an ODS-Hypersil column with 0.5% THF in water. A preliminary approach to PTU analysis was performed by injecting an aqueous solution onto a packed ODS-Hypersil column.

In HPLC, the solvent used to dissolve the sample (analyte) can affect the peak shapes efficiency and response. The choice of solvent in this case was severely limited by the low solubility of PTU in organic solvents. The use of water as solvent to extract PTU from the sample has been claimed to give rise to unacceptable broad peaks (74). However, in the

present work it provided good separations with reasonable peak shapes. As a result for simplicity all determinations were performed using aqueous solution of PTU. Methanol and methanol-chloroform (74, 89, 91) have also been reported as extraction solvents and both resulted in good peak shapes.

PTU has a sharp absorbance at 233 nm and this was adopted as the wavelength for carrying out all the experiments. In previous works ETU had been detected at wavelengths ranging from 240 to 254 nm, (74, 85, 87, 89, 91), where UV detectors give weaker response due to lower extinction coefficients leading to poor sensitivity.

Some workers could not achieve reasonable retention times (87, 91) (stated as too short) since they had used substantial amount of methanol in the eluent (for example 65% in water). However, no problems were reported when small amounts of methanol or THF (87) were employed.

In order to prolong the life time of the main ODS-Hypersil column, a guard column was placed between the injector valve and the main column.

Initially the eluent used in the present study was just 100% water (t_r : 5.54 min and $k' = 5.08$), however, the peak tailed badly (Fig. 3.1). Therefore two different solvents were tested in the mobile phase to improve peak shape and resolution on adding THF (0.5%) PTU eluted with $t_r = 3.17$ min and $k' = 3.34$ and improved peak shape (Fig. 3.2). On adding 5% methanol to the water; PTU had $t_r = 2.97$ min and $k' = 2.26$ (Fig. 3.3).

The effects of adding a proportion of an organic solvent were then investigated (85, 88, 89, 90, 138) (Table 3.1. and 3.2).

TABLE 3.1. - Effect of THF concentration in eluent on retention time of PTU on ODS-Hypersil column

THF (conc) (%)	t_o	t_r	k'
0.5	0.91	3.17	3.34
1.0	"	2.60	2.58
1.5	"	2.24	2.10
2.0	"	2.19	1.90

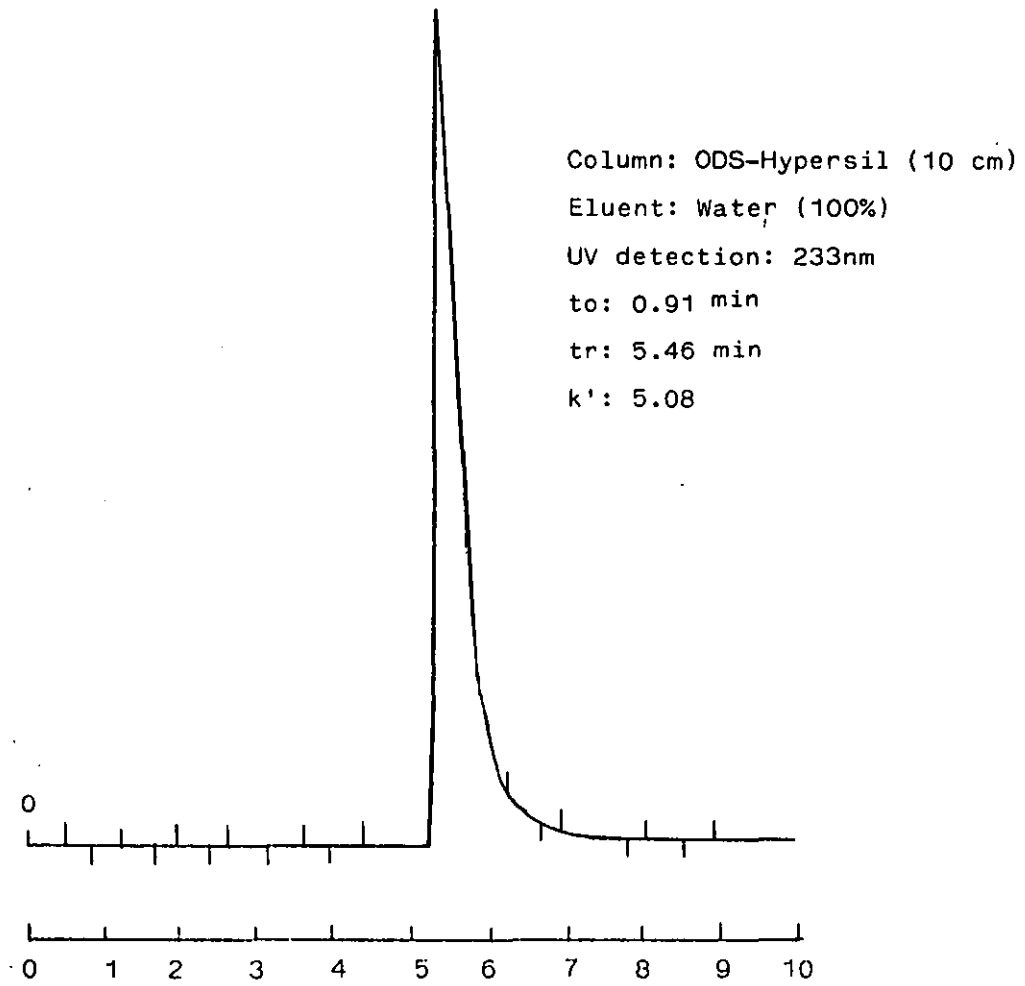


Fig. 3.1. - PTU eluting with 100% water.

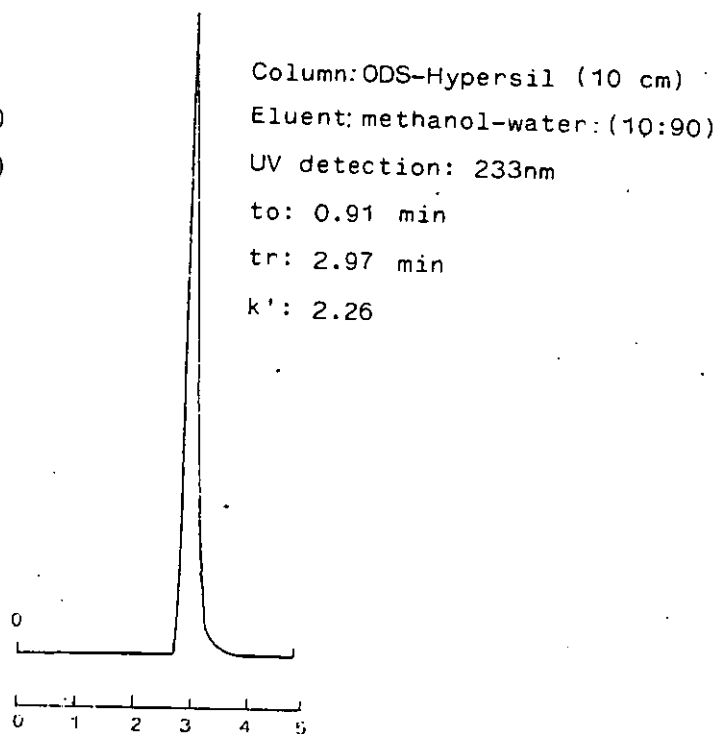
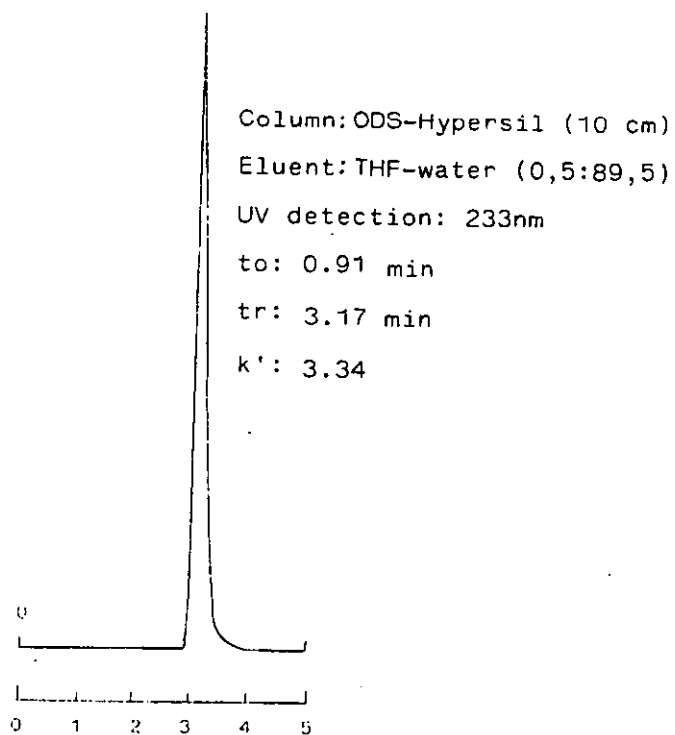


Fig. 3.2. - PTU eluting with 0.5% THF.

Fig. 3.3. - PTU eluting with 10% MeOH.

TABLE 3.2. - Effect of MeOH concentration on retention time of PTU on an ODS-Hypersil column

MeOH (conc) (%)	to	tr	k'
5	0.91	2.97	2.26
10	"	2.67	1.93
15	"	2.01	1.21
20	"	1.81	0.99

As expected the addition of an organic solvent to the eluent caused a decrease of capacity factors. Therefore for direct analysis of PTU the use of water-THF (99.5 : 0.5) as eluent was selected. This gave reasonable retention times and satisfactory peak shape. A retention time of 3.34 min is quite acceptable to give a good resolution of PTU from rapidly eluting peaks of polar extractives are often present in crop extracts.

A calibration curve was plotted based on the average of triplicate injections from 1 to 25 mg/kg. A good linear response (correlation coefficient = 0.999) was obtained in this range (Fig. 3.4.).

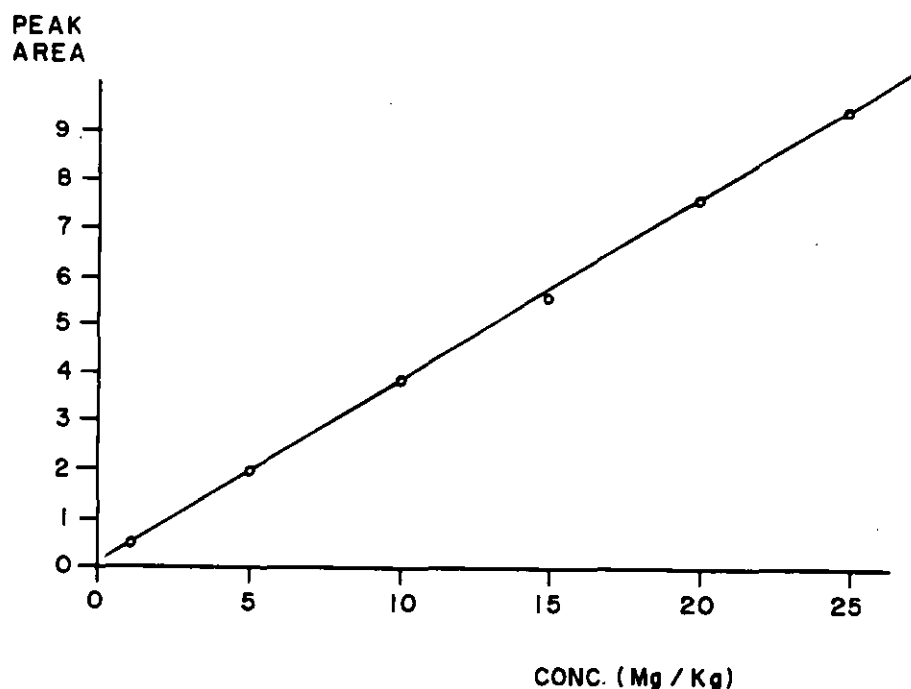


Fig. 3.4. - Calibration curve of PTU.

A similar linearity was achieved when solutions containing low PTU concentrations, less than 1 mg/kg, were chromatographed (Fig. 3.5.).

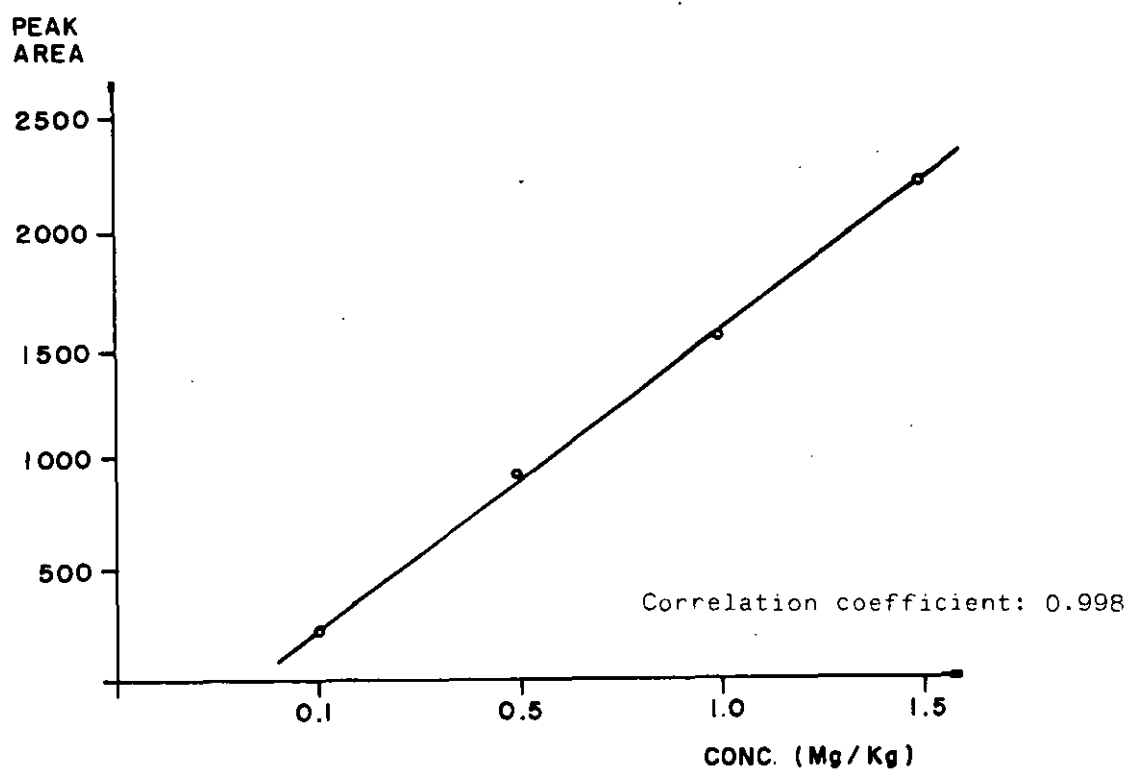


Fig. 3.5. - Calibration curve of PTU (0.1 to 1.5 mg/kg).

A limit of detection around 0.01 mg/kg was possible using this technique. Similar detection limits were reported by other investigators (89, 90).

3.1.2. - Determination of PTU in propylenebisdithiocarbamate formulations

PTU may occur in commercial formulations both as a manufacturing impurity and as a degradation product formed during storage (8). Thus, PTU residues in crops can result either from degradation or metabolism after

treatment with propylenebisdithiocarbamate fungicides or from PTU initially present in the commercial formulations.

High temperatures may degrade Propineb, therefore HPLC was used to avoid problems of thermal instability found in GLC techniques.

The determination could be performed at room temperature without formation of intermediate compounds attributed to the decomposition of Propineb.

A commercial formulation of Propineb (87,5%) was repeatedly extracted with methanol. The extracts containing suspensions of Propineb were centrifuged. The clear solutions were evaporated to dryness and the residues dissolved in water. This procedure was claimed to be very efficient since the dilution of the sample in water, after evaporation of methanol, suppressed a number of substances extracted by methanol but insoluble in water (87). The solutions were then injected onto an ODS-Hypersil column with UV detection at 233 nm and elution with water-THF (99.5:0.5) Fig. 3.6. The results were compared with standard solutions. The determination was repeated using water as the extraction solvent followed by filtration and direct analysis.

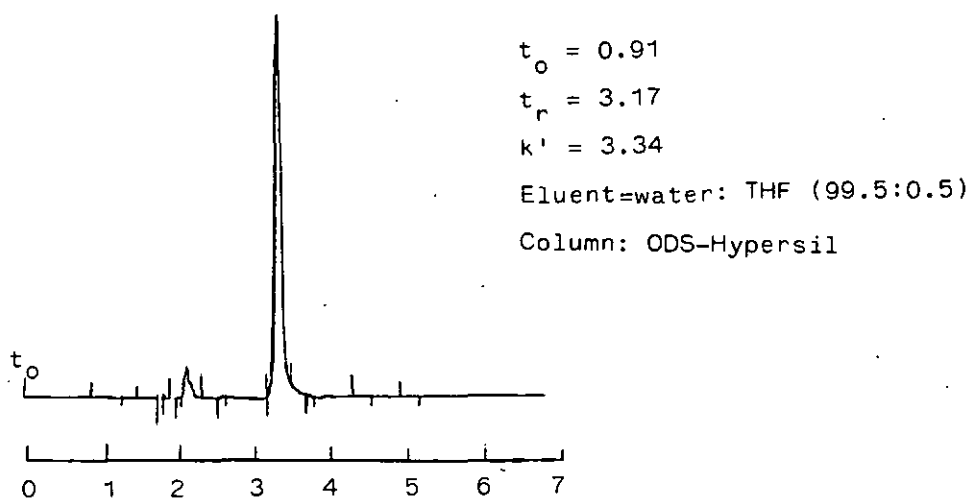


Fig. 3.6. - Analysis of PTU in Propineb formulation.

A considerable amount of PTU was found in Propineb, and methanol extracts gave higher concentrations (3.78%) than water extracts (Table 3.3.).

TABLE 3.3. - Quantification of PTU in propineb.

FUNGICIDE	EXTRACTION SOLVENT	PTU (%)	AVERAGE
Propineb	methanol	3.76, 3.78, 3.79	3.78
	water	3.41, 3.43, 3.43	3.42

One problem associated with investigations of PTU residues is whether Propineb is converted into PTU during the work-up of the samples. A small variability of results observed in this case was due probably to the different storage conditions and time of storage (91). Van Damen (87) reported that ethylenebisdithiocarbamates stored under normal conditions have shown an increase in ETU levels of 30 to 80% from the original amount after two years. PTU is the methyl analogue of ETU and can possibly undergo similar process.

3.1.3. - Extraction of PTU in lettuces samples

Before either the direct analysis of PTU at residue levels or analysis after a derivatisation step, there was a need to develop an extraction procedure which would reduce interferences from the crop components and give a cleaner extract.

The extraction technique used for determination of PTU in lettuce samples was based upon the GLC method for ETU reported by Otto *et al.* (139), applicable to a wide variety of crops, with some minor modifications. This procedure consisted of an extraction with a mixture of methanol and sodium ascorbate, hexane washings, clean-up on an alumina column, partition with dichloromethane and elution with ammonium chloride solution.

Some modifications were made in this methodology including:

1) Glass-fibre filter paper was used for filtration instead of Whatman n^o 42 as it gave clearer extracts.

2) The lettuces were extracted only with methanol without sodium ascorbate as in work with ETU found to be unnecessary (94).

3) The elution of PTU from the clean-up column was carried out with dichloromethane.

Initially the coarsely chopped lettuces were spiked with PTU (1.0 µ/g) and extracted with 3 different solvents methanol, ethanol and water. Methanol extracts showed higher recoveries for PTU decreasing in order of ethanol and water (Table 3.4.). Similar data for methanol, ethanol and water extraction were reported in the literature (71, 77, 89, 94, 138).

TABLE 3.4. - PTU recoveries using different extraction solvents. (Determined as section 2.9.11.).

EXTRACTION SOLVENT	RECOVERY (%)
methanol	86
ethanol	82
water	71

Hexane was employed to wash the methanolic extract to remove any non-polar compounds also extracted from lettuces since they could have a high retention time in HPLC. Hexane did not extract PTU from methanolic extracts (Table 3.5.).

TABLE 3.5. - PTU found in hexane wash. (Determined as section 2.9.11.).

METHANOL EXTRACT	HEXANE WASH
99.2	N.D.*

*N.D. = not detected.

The temperature used for the evaporation of the solvent from the crop extracts has not been reported in earlier work (84). Therefore there was a need to monitor the thermal stability of PTU aqueous solution at residue levels. Tests indicated that PTU was stable in the absence of

extracts up to 75 °C. Lower recoveries were observed when temperature increased above this level. This agrees with work done on ETU (94).

In order to remove insoluble and very polar compounds from the methanolic extracts before injection onto the HPLC system an aluminum oxide column was employed for the clean-up of lettuce samples. A PTU solution (1.0 mg/kg) was poured into an alumina column and eluted respectively with dichloromethane, methanol and chloroform to determine the best eluent (Table 3.6.). These eluents have been tested in earlier investigations of ETU (66, 140).

TABLE 3.6. - PTU recoveries alumina column with different elution solvents (80 ml).(Determined as section 2.9.11.).

ELUTION SOLVENT	RECOVERY (PTU)
dichloromethane	98%
methanol	94%
chloroform	67%

Therefore the method selected to determine PTU in lettuce sample involved extraction with methanol, washing of the extract with hexane, a clean-up on an alumina column, elution with dichloromethane and a concentration step. A blank extract showed no major interferences peaks from co-extractives with retention time close to PTU (Figs. 3.7 and 3.8). The recoveries from lettuces fortified with PTU as levels ranging from 0.01 to 4 mg/kg were consistent. The average value of triplicate analysis in fortified samples are given in Table 3.7.

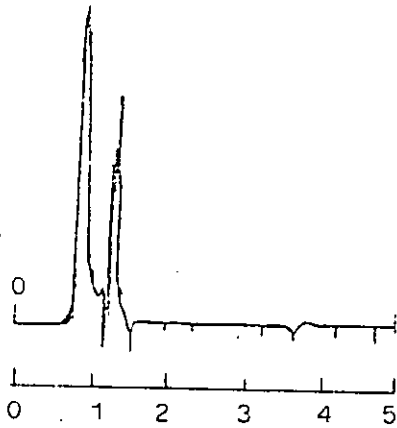
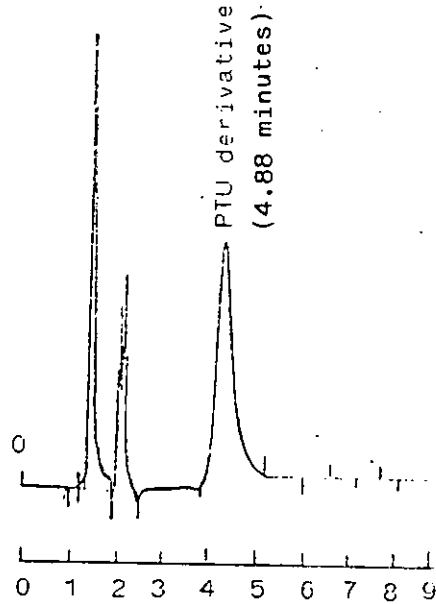


Fig. 3.7. - Blank from lettuces.

Fig. 3.8. - Fortified samples
(1 mg/kg) of PTU spiked lettuces.TABLE 3.7. - PTU recoveries for different fortified samples of lettuces.
(Determined as section 2.9.11.)

ADDED (mg/kg)	FOUND (mg/kg)	RECOVERY (%)
0.00	N.D.	-
0.01	0.006	60
0.1	0.07	70
0.5	0.35	70
1.0	0.80	80
2.0	1.60	80
4.0	3.53	88

The applicability of the method for determining PTU residues in stored products was examined with spiked lettuces maintained at 5°C. Aging did not affect the recovery obtained in 5 or 10 days. (Table 3.8.).

TABLE 3.8. - PTU recoveries after some storage time
(Level spike = 1 mg/kg)

DAYS OF STORAGE	PTU (RECOVERY)
0	92%
5	92%
10	91%

Within the time available for this work, it was not possible to carry out feeding and spraying studies on growing lettuces, although earlier work from Loughborough University using ETU has suggested that the method will be applicable to the determinations of residues (94).

3.1.4. - Determination of PTU by electrochemical detection

The present study was prompted by the speculation that electrochemical detection might be more sensitive and more specific than UV detection for residue analysis (141). Minor adaptations were made in Prince's (93) procedure to quantify PTU by high-performance liquid chromatography with electrochemical detection. Originally this method was applied to monitor worker's total exposure to EBDC's through ETU levels in their urine. As the matrix is not complex, no clean-up step was necessary. PTU aqueous samples were quantified by HPLC with electrochemical detector operating at potential of + 1.0 V using as mobile phase water-methanol (95:5) with addition of small amount of sodium chloride to the eluent to maintain the Ag/Cl reference electrode. The peaks obtained were symmetrical. (Fig. 3.9.).

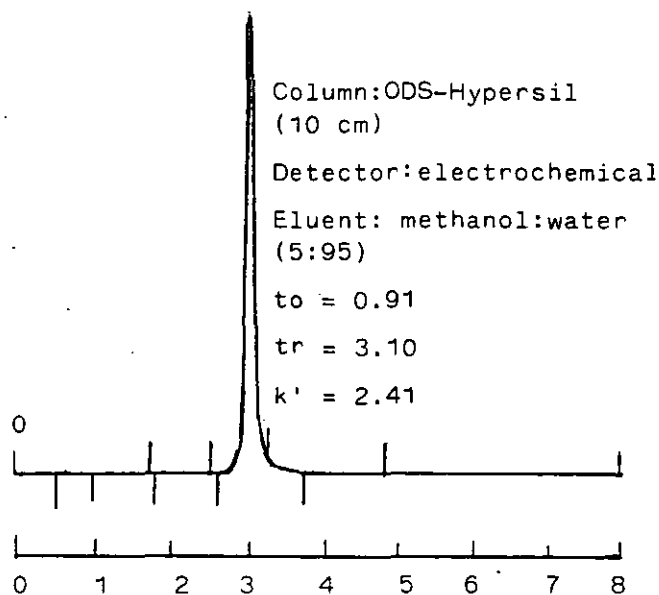


Fig. 3.9. - A typical chromatogram of PTU with electrochemical detection.

A calibration curve was plotted with an average of triplicate injections and a linear detector response was obtained. The detection limit observed was 0.01 mg/kg (Fig. 3.10.). Although electrochemical detectors have been claimed to be more advantageous than UV detectors since they can often achieve higher sensitivity and higher selectivity (66), in this particular example the sensitivity found was equivalent to that from UV detector (section 3.1.1.). However, the selectivity was so far better and less interfering peaks were observed.

It is necessary to point out that electrochemical detector was relatively unstable requiring a long time to stabilise the baseline when used at high sensitivity.

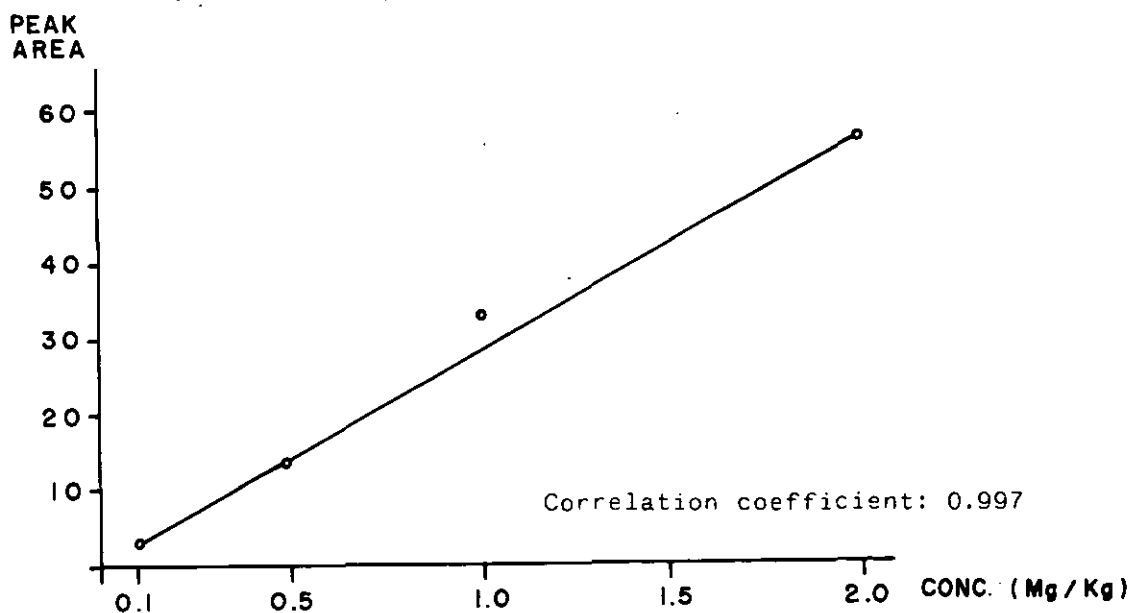


Fig. 3.10. - Calibration curve for PTU with electrochemical detection.

3.2. - FORMATION OF THE PHENACYL HALIDE DERIVATIVE OF PTU

Enhancement of sensitivity is very important for analysis at residue levels in complex matrices when many interfering substances may be encountered. Higher sensitivity and additional selectivity can possibly be achieved by employing derivatisation techniques to provide a suitable chromophore or fluorophore. The detection of PTU derivative could be more specific and could probably reduce any interferences from co-extractives in crop analysis. The derivatisation would be possible by introducing an ultraviolet absorbing chromophore with a longer wavelength maxima and higher extinction coefficient, a fluorophore or an electrochemically active group.

This approach has already been used for the determination of ETU, a compound closely chemically related to PTU. ETU presents two potential reactive sites, sulphur and nitrogen, where a derivatisation reaction could be performed. The thiol group can undergo tautomerism to give the structure $-N=C-SH$ (142). This thiol group may chemically act as weak acid and react with aryl halides or alkyl halides to yield S-substituted derivatives. However, these compounds still possess a basic amino group and for liquid chromatography there is a need to derivatise the amino group to reduce interactions with the silanol groups on the column packing.

Phenacyl halides, reagents widely used for the derivatisation of carboxylic acids (143, 144) were found to react in a two step reaction with the thiol group to firstly form S-phenacyl esters which under the conditions of the reaction cyclise with the amino (N-H) group of ETU to give bicyclic imidazo[2,1-c]-thiazole derivatives (145) (Fig. 3.11).

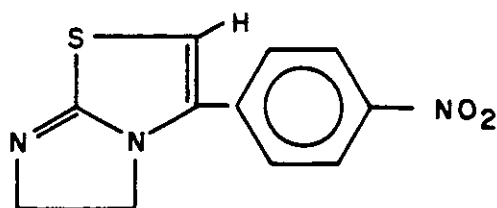


Fig. 3.11. - Bicyclic derivatives of ETU.

A number of different phenacyl halides have been investigated, *p*-nitrophenacyl bromide, *p*-phenyl phenacyl bromide and *p*-bromophenacyl bromide (145) and the first of these was found to be the most suitable for residues analysis. In a similar reaction, phenacyl halides have been found by Chadha *et al.* (146) to react with PTU to give *S*-substituted phenacyl ester derivatives in synthetic studies for the formation of thiazoles.

Apparently the extent of the reaction to give bicyclic compounds or phenacyl esters depended upon the solvent and reaction conditions (147). Under mild conditions the ester is formed but this cyclises on refluxing in ethanol.

The reaction of PTU with *p*-nitrophenacyl bromide was therefore studied under similar conditions to those used for ETU. The crystalline product was apparently the bicyclic derivative. However, PTU can potentially yield two isomeric products (A and B) depending on the direction of cyclisation although only one peak was observed on HPLC. Because of steric interaction between methyl and phenyl groups during the cyclisation step, isomer B might be anticipated to be the major product (Fig. 3.12.).

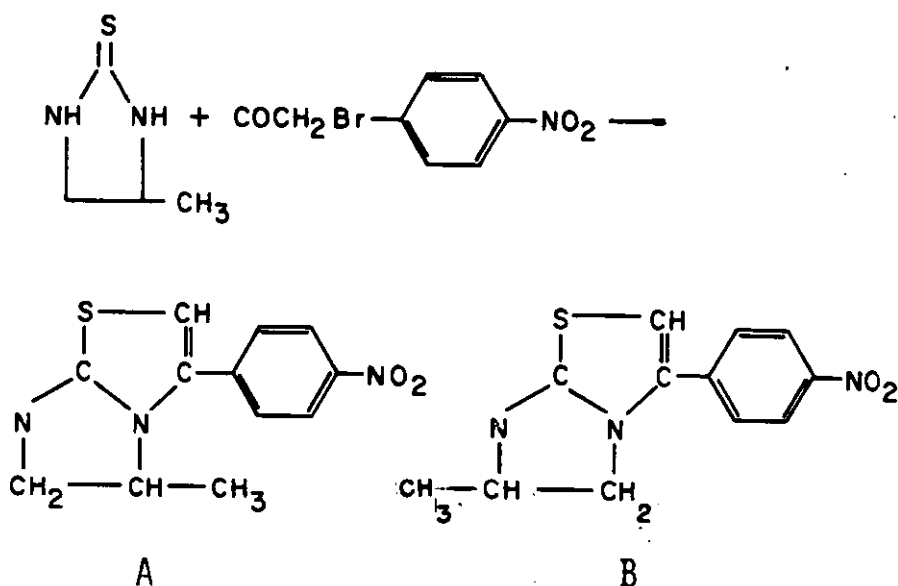


Fig. 3.12. - Isomeric derivatives of PTU.

In order to confirm the structure, the sample was purified by crystallisation (which would probably only give the major isomer) and the product examined spectroscopically. The infrared spectra of the purified and recrystallised sample (m.p. 158-160°C) contained the same characteristic bands found in the derivative of ETU (147), 1490 (C-N Stretching), 1585 (C=N and C-N), 2960, 2978 (CH stretching in CH) cm^{-1} . The lack of absorption in the region 1680-1700 cm^{-1} shows the absence of the carbonyl group present in IR spectra of phenacyl esters and confirmed the presence of the bicyclic structure.

The elemental analysis and exact of the molecular ion agreed with the proposed formula ($\text{C}_{12}\text{H}_{11}\text{N}_3\text{O}_2\text{S}$). The comparison between the NMR spectrum of PTU and *p*-nitrophenacyl derivative enabled the couplings to be assigned as shown in Fig. 3.13.

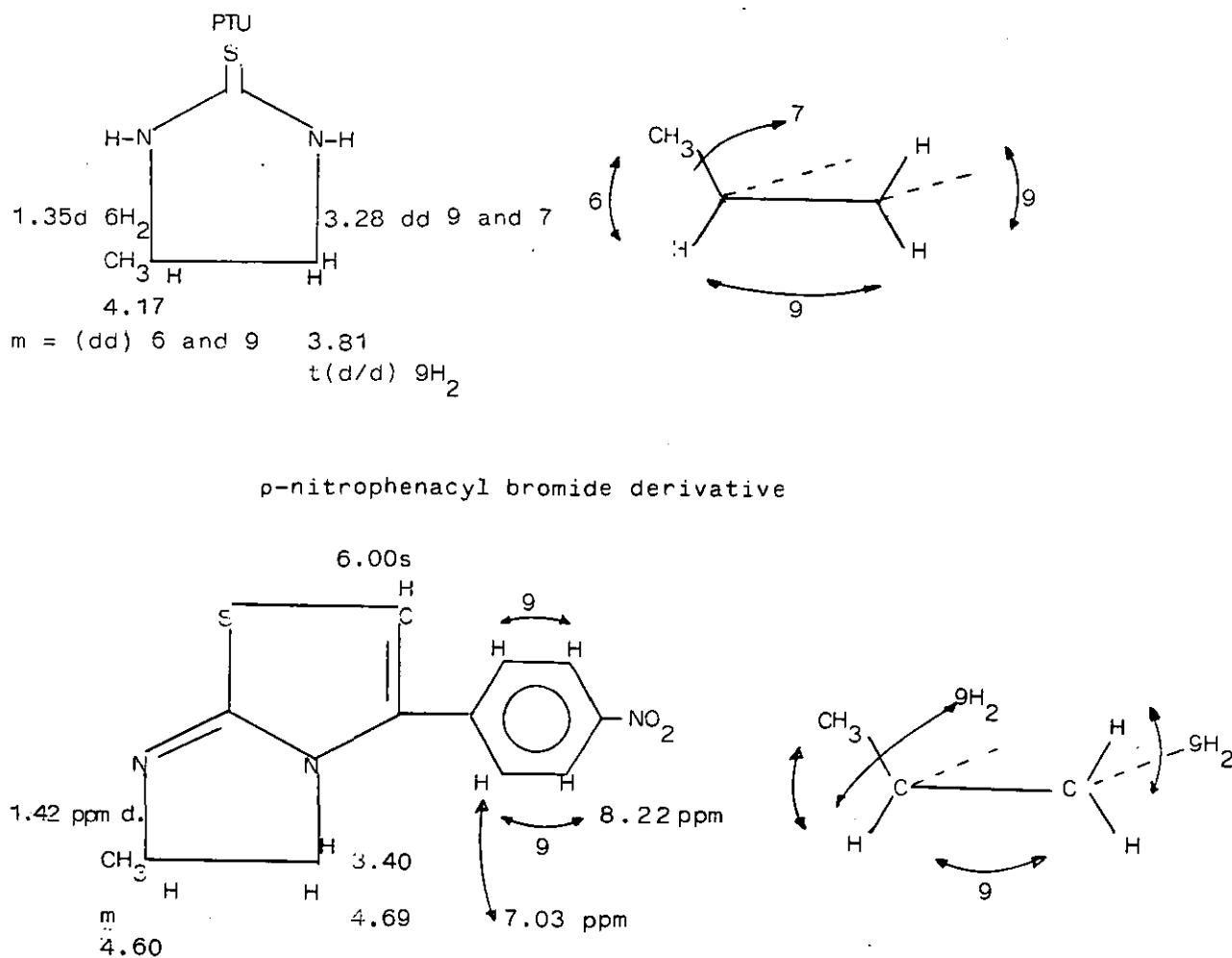


Fig. 3.13. - Analysis of NMR Spectra.

However, it is not possible from these values to positively confirm the major isomer as B as the spectra of isomer A would be expected to be very similar. If the crude reaction product was examined a second much smaller doublet was observed in the NMR spectra for the methyl group (1.35 ppm $J = 6\text{Hz}$) which could be attributed to a second isomer but this represented less than 5% of the major isomer and none of the other peaks in the spectrum appeared to be split.

3.2.1. - Analysis of *p*-nitrophenacyl derivative of PTU by HPLC

The separation of the phenacyl halide derivative of PTU was investigated on an ODS-Hypersil column using methanol-water as eluent (50:50). The retention times were satisfactory, however, the peaks tailed badly, possibly due to the strong interaction of uncapped silanols on silica surface of the column with the tertiary amino group of the derivative. In order to get a good peak resolution, the derivative should be eluted as a free base. Therefore the pH of the mobile phase was increased to 9. Better peak shapes were obtained but the column lifetime was severely limited. As a result the separation of phenacyl halide derivative was performed in a polystyrene-divinyl benzene column (PS-DVB), which has claimed to be very resistant to pH extremes from 1 to 13 (149).

The derivative presented symmetrical peaks with no interferences from the excess of the reagent using 70:30 acetonitrile: buffer (pH = 9) as eluent. (Fig. 3.14.). Better peak resolution and longer retention times were obtained. Even at high concentrations of methanol in the mobile phase some tailing was present.

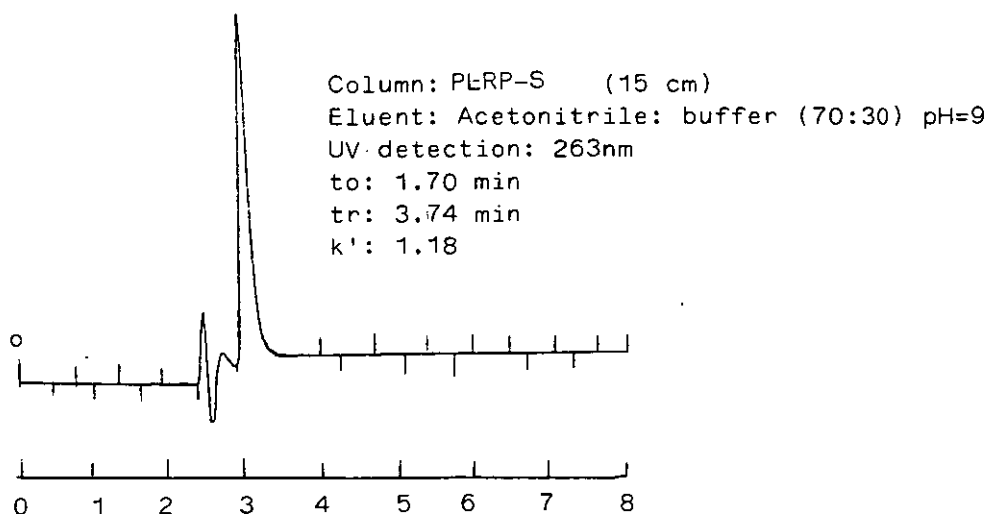


Fig. 3.14. - Typical chromatogram of PTU phenacyl derivative.

The effect of acetonitrile concentration of the retention time of PTU is shown on Table 3.9.

TABLE 3.9. - Effect of acetonitrile concentration on the capacity factor of PTU derivative on a PS-DVB column.

ACETONITRILE CONC (%)	to	tr	k'
50	1.70	5.94	2.49
60	"	4.38	1.56
70	"	3.74	1.18
80	"	3.07	0.80

As expected the capacity factors decreased when acetonitrile concentration increased.

A series of ethanolic solutions containing 0.1 to 4 mg/kg of PTU were derivatised with *p*-nitrophenacyl bromide and analysed by HPLC with UV detection at 263 nm. By working at longer wavelengths more selectivity will be achieved, since PTU has a weakly absorbing chromophore at 233 nm. A calibration curve was plotted (Figure 3,15). A limit of detection of 0.01 mg/kg was found. However, this is similar to that found by the direct HPLC method.

Although the derivatisation of PTU with phenacyl halides can provide a detection limit as low as 0.01 mg/kg, but the preparation of the derivative slows down the analysis and increases the risks of errors. It should be pointed out that the derivatisation method is attractive as long as it is specific, reduces interferences and it is reliable but not for its sensitivity. Therefore the direct method is recommended for determination of PTU.

3.3. - DERIVATISATION OF PRIMARY AMINES WITH FLUOROGENIC LABELS

PTU is supposed to degrade to propylenediamine (PDA) under UV irradiation and a methodology to monitor the possible formation of PDA was necessary.

A number of approaches to pre-column derivatisation of propylenediamine (PDA) with fluorogenic labels were studied based on the need to prepare a stable derivative with high fluorescence to monitor propylenethiourea (PTU) under UV irradiation.

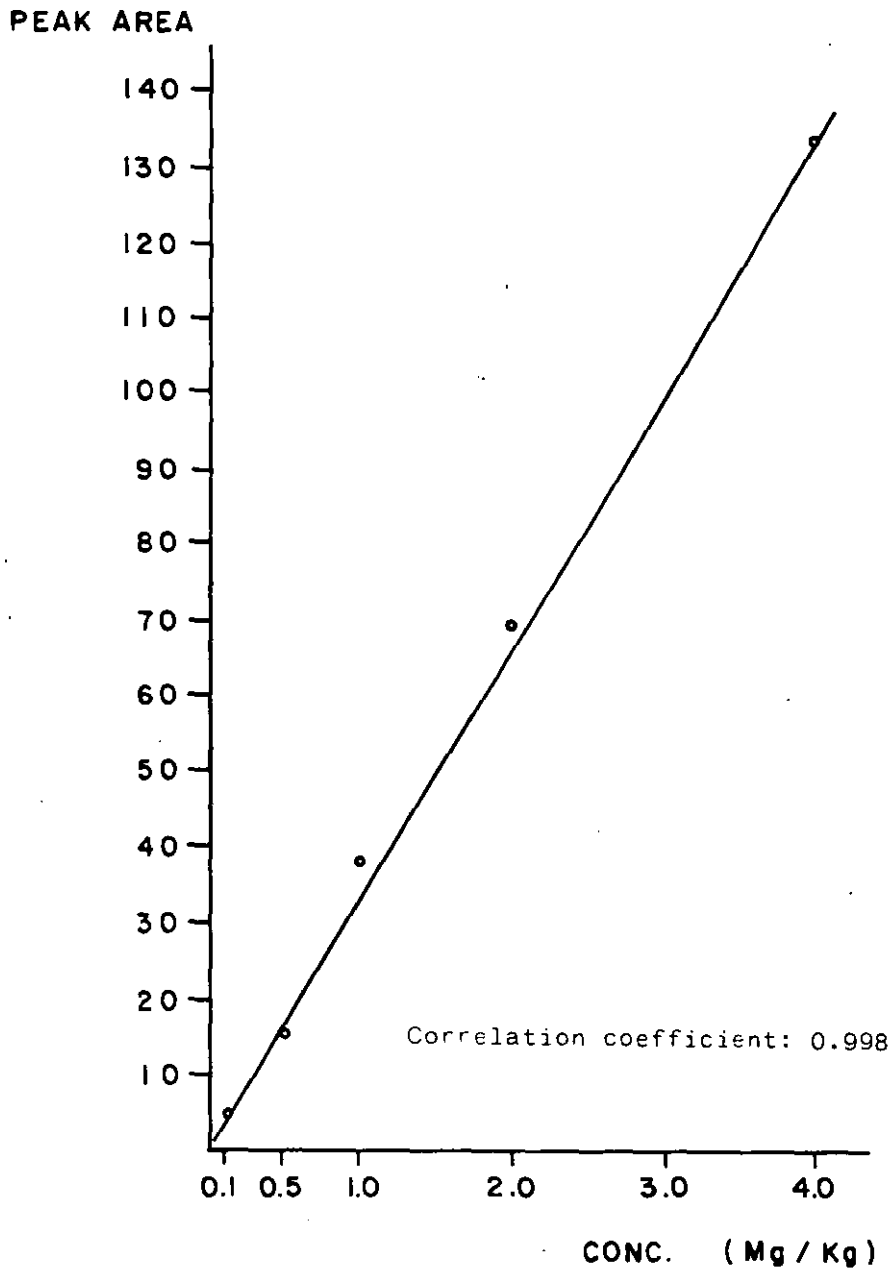


Fig. 3.15. - Calibration curve of phenacyl derivative (0.1 to 4 mg/kg).

By virtue of the non-bonded electron pair on nitrogen, amines are subject to electrophilic attack i.e. acylation and alkylation reactions. Alkylation has not been widely used for derivatisation (101). On the other hand acylating agents like, DNS-chloride, OPA and fluorescamine have been widely used to yield fluorescent adducts from primary amines (see Chapter I). Arylation have been also used to derivatise amines by the nucleophilic displacement reactions with aromatic substrates. NBD-chloride and NBD-fluoride are typical example of this technique (95).

The present study investigated the derivatisation reactions of propylenediamine with these fluorogenic labels.

3.3.1. - Derivatisation of PDA with NBD-chloride

The preparation of derivatives was apparently easy. Initially the amines were dissolved in 0.1M NaHCO_3 and reacted with NBD-chloride solution in (1%) methyl isobutyl ketone at 80°C . A total time of 30 min was required before the conversion to NBD-derivatives was completed. The coupling reaction is faster in polar solvents such as alcohols and ketones, and 80°C was reported to be the optimum temperature for the coupling reaction, avoiding the water phase and excessive evaporation of the organic layer while not requiring a long reaction time for the adduct formation (98).

The NBD-chloride adducts were separated by HPLC on a reversed-phase column. The analysis revealed the formation of two components (Fig. 3.16.).

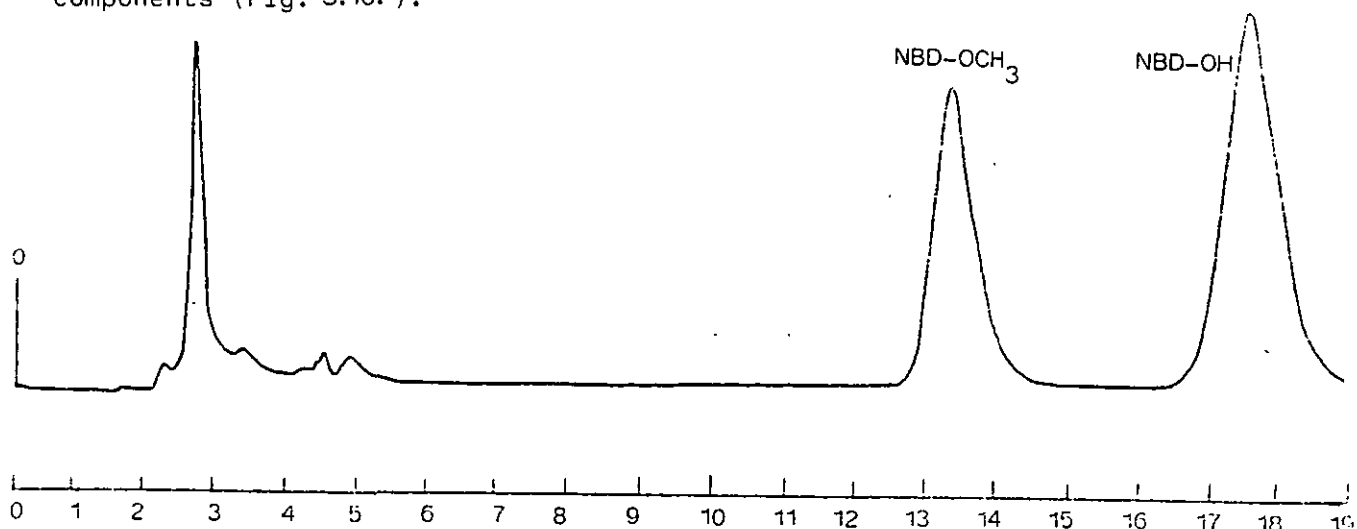


Fig. 3.16. - Chromatogram of reaction of PDA with NBD-chloride.

Reagent blank reaction and hydrolysis products showed also high fluorescence and apparently yielded the same compounds (Fig. 3.17.) with the same retention time so that it appeared that the reaction of both PDA and EDA was not occurring.

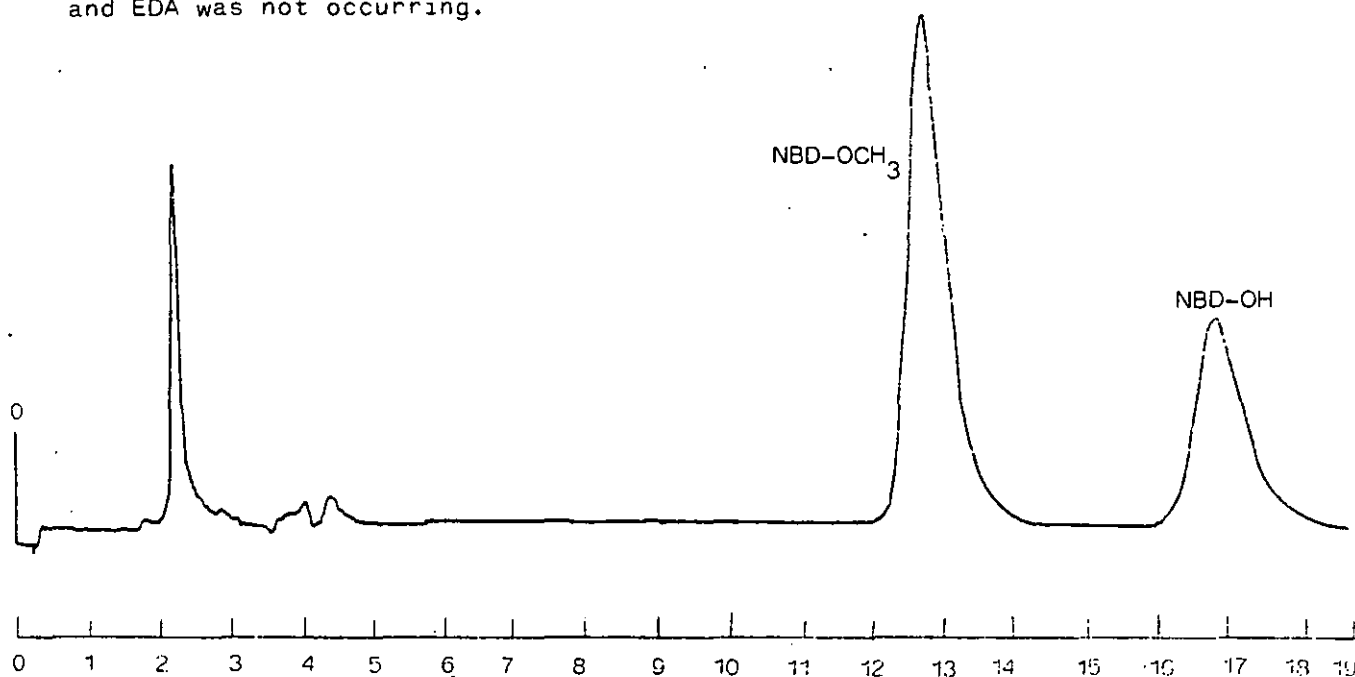


Fig. 3.17. - Blank chromatogram of the reaction with NBD-chloride.

A number of tests were carried out to improve the reaction conditions and explain the apparent lack of reaction. The derivatisation was performed at different temperatures (40, 50, 60, 70, 80°C) at pH 9.0 in methyl isobutyl ketone. The reaction and fluorescence decay were accelerated with the increase of temperature. Higher temperatures gave higher fluorescence intensity and higher blank fluorescence.

No significant changes were observed when different organic solvents, methanol, ethanol, acetone and acetonitrile were tried. The emission spectra of the product compounds were constant varying the concentrations of methanol or MIBK. Tests involving reaction mixture of PDA and NBD-chloride at different pH values showed the fluorescence intensity remained constant between pH 2 to 9. The reagent blank fluorescence was claimed to be suppressed by adjusting the medium to around pH 1 with hydrochloric acid (106). Similar procedures have been tried experimentally but even at low pHs the blank remained highly fluorescent. Two compounds were tentatively identified in previous works (150) as 7-nitro-4-benzofuranol (NBD-OH) and 4-methoxy-7-nitrobenzofurazan NBD-OCH₃ (Fig. 3.18.) formed by the reaction between NBD-chloride and the solvent.

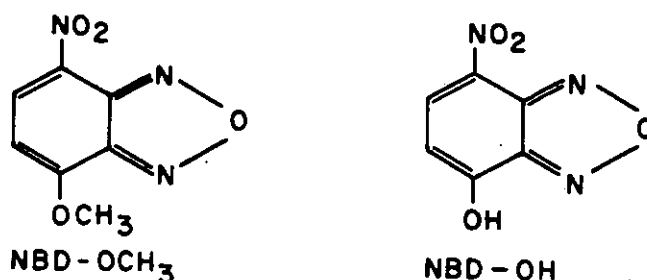


Fig. 3.18. - The side products of the reaction of amines with NBD-chloride.

It appeared that NBD-OCH₃ is an intermediate compound which reacts further to give NBD-OH. A close examination of the supposed derivative showed that the reaction rate is not related to the concentration of NBD-chloride adduct as would be expected. The reaction proceeded very poorly for propylenediamine and this conclusion is supported by the reported reaction rates that decrease in the order secondary amines, imino acid, primary amines and amino acids (104). Earlier work on fluorogenic derivatisation confirmed the suitability of NBD-chloride for secondary amines (106) but its unsuitability for primary amines are often not mentioned.

3.3.2. - Derivatisation of PDA with NBD-fluoride

The fluorogenic reactions of amines with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole are claimed to be superior in terms of reactivity and fluorescence yield to the reaction with analogous 7-chloro and 7-bromo derivatives (106). The 7-fluoro moiety in *p*-position to a nitro group in the aromatic ring is supposed to be more reactive than the 7-chloro moiety (151). The reagent blank fluorescence with NBD-F can be suppressed by adjusting the pH to 1 with hydrochloric acid (106).

In previous papers (108, 152), the fluoro analogue was reported to react 10 times faster than NBD-chloride with amines, amino and imino acids. Pre-column labelling of amines with NBD-fluoride for HPLC at picomolar levels was also reported (152,153). The derivatisation reaction of NBD-F with PDA and EDA was carried out in 50% ethanol-0.1M borate buffer (pH 8.0) based upon the method developed by Ahnoff *et al.* (150) with slight

modifications. The mixture was heated for 20 min at 60°C. To stop the reaction, the solution was cooled in ice-water and acidified with hydrochloric acid to pH 1. The compounds formed were separated by HPLC reversed phase on an ODS-Hypersil column eluting with methanol/water (50:50). Both reaction mixture with amine and reagent blank gave rise to the same peaks (Figs. 3.19.,3.20.).

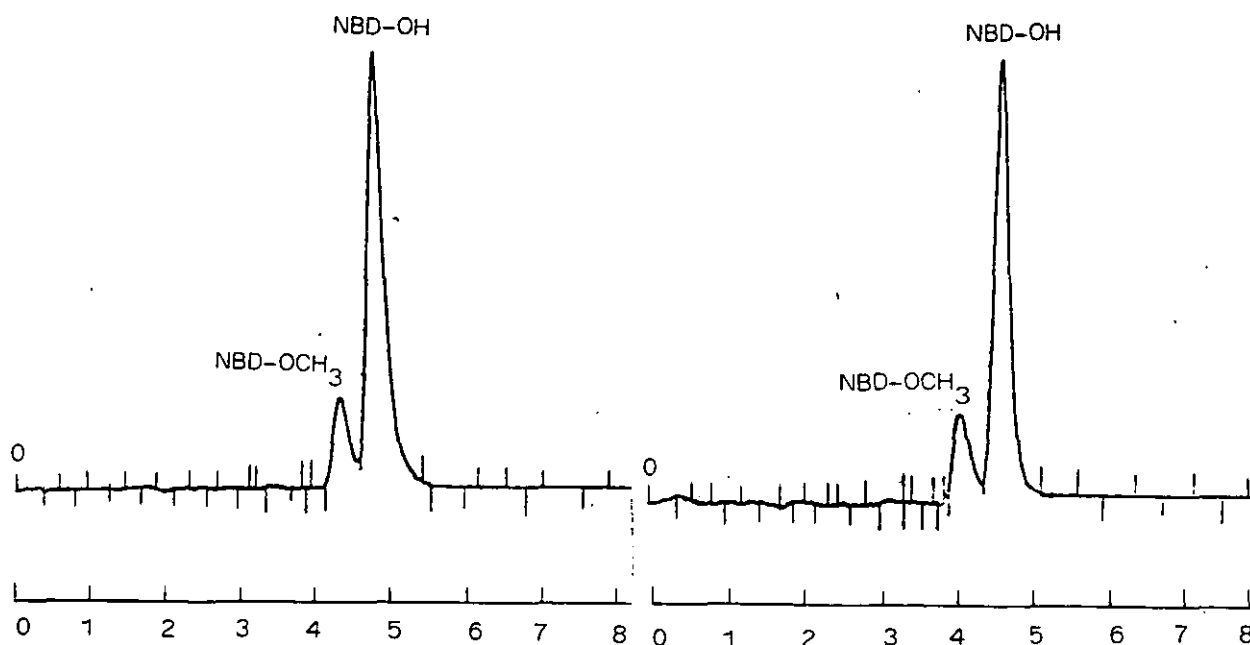


Fig. 3.19. - Chromatogram of PDA reaction with NBD-F.

Fig. 3.20. - Blank chromatogram of NBD-F reaction.

Thus it again appears that the solvent has reacted with NBD-F to yield 4-methoxy-7-nitrobenzofurazan (NBD-OCH_3) and 7-nitro-4-benzofurazan (NBD-OH).

A series of experiments with different pH, temperatures, buffers and solvents were carried out aiming to achieve the formation of adducts. Preliminary studies were performed to determine the dependence of reactions on temperatures and solvents. A reaction mixture containing PDA with NBD-F at pH 8.5 was diluted in borate buffer. The mixture was heated at 60°C for 20 minutes either in methanol, ethanol, acetonitrile or MIBK.

Virtually no differences were observed in the reaction rates. Solutions containing more than 50% methanol or ethanol were not studied because of formation of precipitate in such media. Buffers were also tested but no difference in the reaction rates were found. Finally the effect of temperature and pH was checked with the same reaction conditions as above. The mixture were heated at 60°C in borate buffer with pH ranging from 1.0 to 10. Results were disappointing. The fluorescence intensities of side-products (NBD-OCH₃ and NBD-OH) of the reaction mixture and of the reagent blank remained constant, even when the solutions were adjusted to pH 1 with HCl. This is in contrast to the reported decrease in fluorescence intensity of the reagent at low pH (101,152,153).

3.3.3. - Derivatisation of PDA with Dansyl-chloride

Dansyl-chloride is claimed to be very reactive to primary or secondary amines, imidazoles and phenols under slightly basic conditions to form a sulfonate ester or a sulfonamide. The reaction of the specific group towards DNS-chloride is highly influenced by pH. The labelling of most amines is optimal at pH 9.0-10.5. Hydrolysis of the reagent is faster at high pH.

The reaction of DNS-Cl and propylenediamine (PDA) was performed with tenfold molar excess of reagent in a mixture of acetone and aqueous sodium carbonate according to the method developed by Hayman et al. (114). The adducts were formed by leaving the reaction mixture overnight at room temperature or for shorter periods of time at elevated temperature (40 to 60°C). The organic soluble compounds were removed from the reaction mixture by direct extraction of the aqueous solution with solvent as hexane or ethyl acetate. The organic phase was then separated by HPLC with fluorometric detection and with emission maxima at 510 nm and excitation maxima at 365 nm. Smaller amounts of DNS-chloride were insufficient for dansylation of primary amines at least 1% solution was necessary possibly owing to the fast hydrolysis of DNS-Cl (110).

On dansylating PDA, the hydrolysis product formed during the reaction, dansyl sulphonic acid (dansyl-OH), was highly fluorescent in the pH ranging from acidic to alkaline. It appeared that no reaction took place, only the formation of side-products like dansyl-sulphonic acid (see Fig. 3.21.).

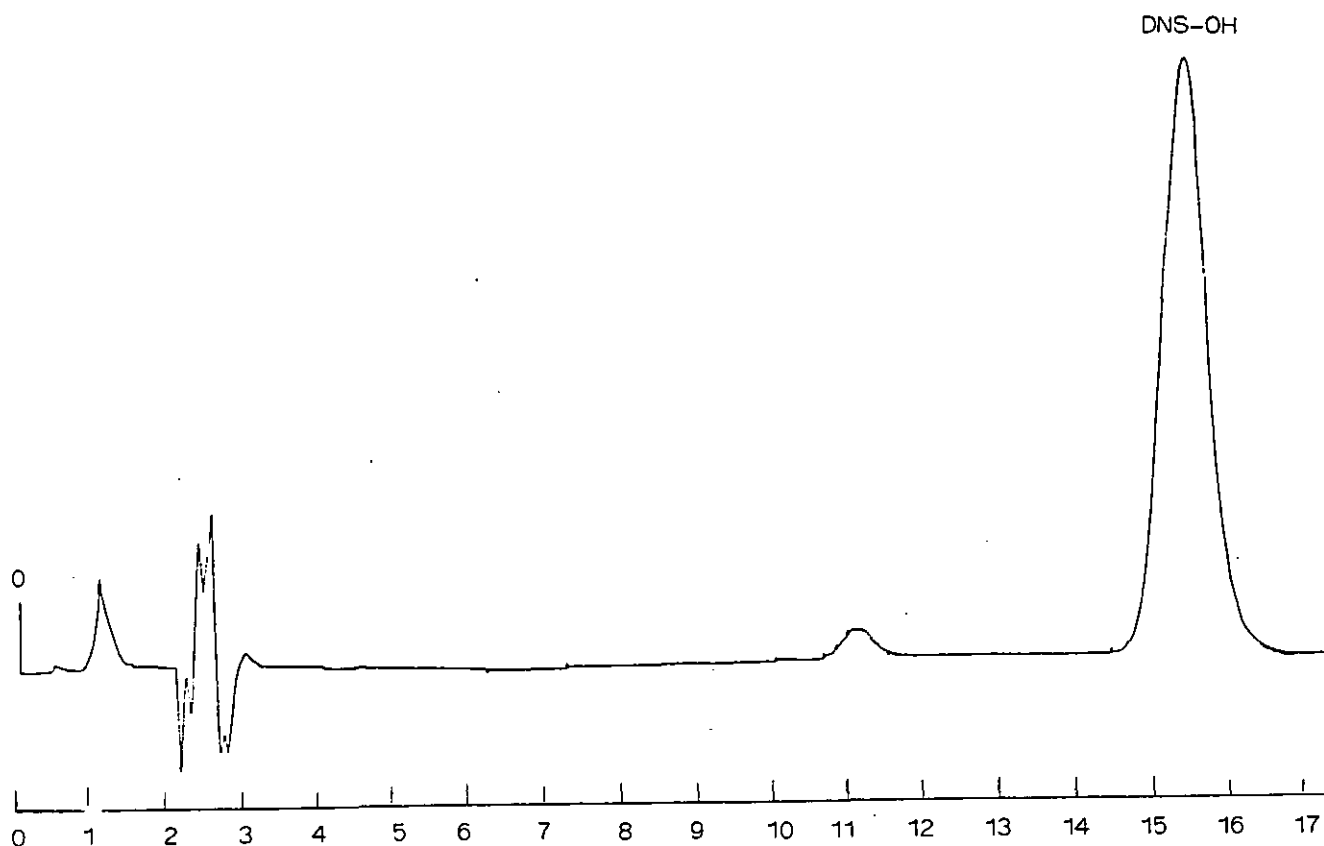


Fig. 3.21. - Chromatogram of the reaction of PDA with DNS-Cl.

A recent report (116) recommended conditions for suppressing the side reactions 0.15% DNS-Cl in acetonitrile - 0.04M lithium carbonate solution pH (9.5) for 35 min at room temperature (22-23°C) in the dark, followed by addition of 2% methyl ammonium chloride to destroy the excess reagent. A similar technique was examined in the dansylation of PDA but the attempt was unsuccessful and the side product was not eliminated.

Increases in the pH temperature and proportion of organic solvent in the reaction mixtures seemed just to increase the formation of the side-products. Various organic solvents and buffers were tested and the effect on the reaction was not significant. Acetonitrile and acetone were reported to be the best for dansylation of PDA. The reaction apparently proceeded in the same way at different buffers. Tests under pH ranging from 1 to 9 just accelerated the formation of dansyl sulphonic acid. In contrast Newton *et al.* (154), Eckerman *et al.* (115) found no difficulties in forming dansyl derivatives of mixtures of diamines such as ethylenediamine and propylenediamine for HPLC.

Dansylation has another disadvantage since the reaction can occur with amines and phenols, any co-extracted material or impurities containing these groups might procedure interferences in HPLC (95). Ammonia from the

air and from the solvent will readily react with dansyl-chloride to form a sulfonamide. Although these products may pose no problems if separated by chromatography they still consume the reagent and therefore may affect the yield of the desired compound.

EDA was also reacted with DNS-Cl. Similar results to PDA were obtained. For all above reasons the labelling of amines with DNS-Cl was discontinued.

3.3.4. - Derivatisation of PDA with *o*-phthalaldehyde (OPA)

The reaction of OPA and amines occurs rapidly and quantitatively in the presence of a strong reducing agent such as 2-mercaptoethanol, to give fluorescent adducts with excitation at 340 nm and emission at 455 nm. OPA is not fluorescent itself, this of course minimises interferences and reduces the need for separation of the derivative from the reagent OPA is soluble and stable in water unlike fluorescamine. It offers tenfold greater sensitivity than fluorescamine and is considerably less expensive.

Although OPA is more suitable for post-column derivatisation, has been used for the pre-chromatographic reaction and analysis of several amines, amino acids and catecholamines by several workers and it is discussed in a detailed review by Imai (104). Despite the widespread analytical application of OPA, the isoindole formed by the derivatisation reaction is relatively unstable and decomposes to non-fluorescent species (120, 121, 126, 155). The relative fluorescence intensity depends upon the structure of the thiol and amine (127).

The derivatisation of propylenediamine was carried out using borate buffer in the presence of either ethanethiol or 2-mercaptoethanol to yield strongly fluorescent adducts. The OPA should be added in 2-3 orders of magnitude higher amounts according to the procedure described by Lindroth (156). The use of borate buffer was preferred to phosphate buffer since the latter quenches the fluorescence of OPA adducts (117, 120). The isoindoles were separated by HPLC on an ODS-Hypersil column with fluorometric detection. Contrary to expectations, increase in fluorescence intensity with time. This problem was supposed to be overcome by reacting the amines with OPA immediately before the chromatography a standard time was chosen for the reaction. In this case it was practically impossible to optimise the reaction since the fluorescence intensity only reached a plateau after several hours. The major causes of variability in a pre-

column derivatisation system with OPA is supposed to come from errors in the mixing ratio of sample and reagent and changes in reactivity of OPA reagent (the use of different thiols) (125). Therefore a series of studies was carried out to determine the possible reasons for the increasing fluorescence intensity. The long term instability of OPA derivatives over a period of several hours was initially examined, 10 minutes after the reaction, then each 60 minutes and finally each 24 hours. The fluorescence quenched just after 3 days. The mobile phase and reagent were freshly prepared daily throughout the tests.

The effect of OPA concentration of the fluorophore was also investigated. The amount of OPA was 2-3 orders of magnitude higher than the amine. For a fixed reaction time, the fluorescence response of the amine derivative were nearly independent of the reagent composition as long as at least 200 times excess of reagent was maintained when the excess was allowed to fall below this limit, non-linear responses were obtained.

The reaction fluorescence time profile and stability with different mercaptans has been investigated. Simons and Johnson (121) demonstrated that ethanethiol could replace 2-mercaptoethanol to yield a more stable product of the same general structure. However, with PDA ethanethiol showed similar reaction pattern to 2-mercaptoethanol with increasing fluorescence with time.

Kucera and Umagat (119) suggested in their study that 3-mercaptopropionic acid exhibited better stability than ethanethiol or 2-mercaptoethanol due to the stabilising effect of the carboxylic group which also affects the fluorescence intensity of the isoindole ring. To the this affirmation PDA was reacted with OPA using 3-mercaptopropionic acid, however the same instability as with the other two mercaptans was observed. The same reactions were tested with ethylenediamine. Similar results to those obtained for propylenediamine (PDA) were achieved.

In spite of instability the OPA-adducts were highly fluorescent and gave good separation in HPLC with better peak shape than the other labels. (Fig. 3.22.).

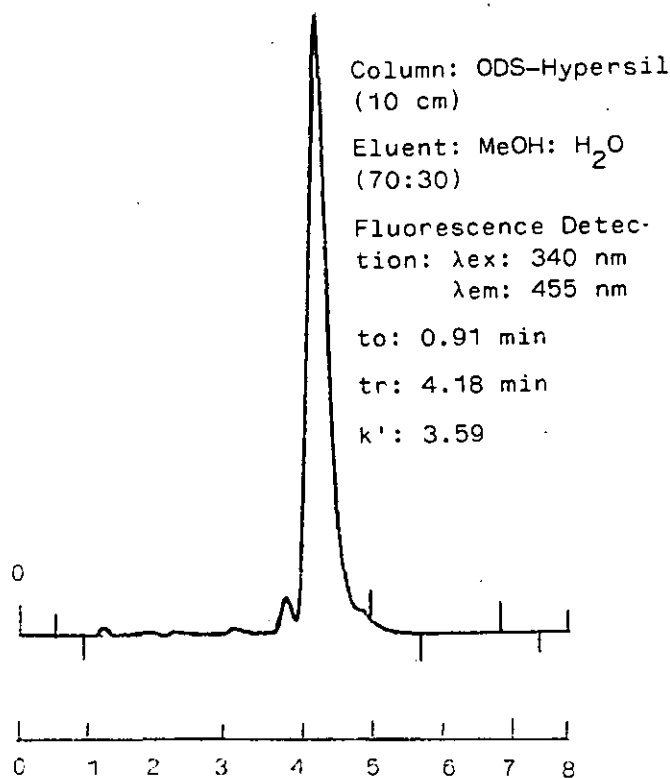


Fig. 3.22. - Typical chromatogram of OPA reaction with PDA.

3.3.5. - Derivatisation of PDA with fluorescamine

Fluorescamine reacts instantaneously with primary amines to yield highly fluorescent adducts (127, 129, 131, 132). Although the reagent degrades rapidly in water, the degradation products are non-fluorescent. Fluorescamine is very selective towards primary amines and has been used for pre-column derivatisation of amines,; however this reaction can involve the formation of two compounds.

Propylenediamine (PDA) was derivatised with fluorescamine with a molar ratio of reagent to primary amine of at least 3.6:1 (134) to ensure completion of the reaction. The best separation of the product was achieved using a reversed-phase HPLC system employing a chemically bonded octadecylsilane column.

Both phosphate and borate buffers were compared under the same conditions of molarity (0.1M pH 8,0) and mixing ratio of organic solvent (Table 3.10.).

TABLE 3.10. - Effect of buffers on retention time of PDA derivative

BUFFER	RETENTION TIME
phosphate	1.85
borate	2.05

A marked decrease in the retention time was observed with phosphate buffer. This might be explained by the interference of ionic strength (134). As it was desirable to increase the retention time borate buffer was preferred.

Tests were performed with different solvents for the reagent. Retention time decreased in the following order acetone, methanol and ethanol (157). Since fluorescamine is water miscible in non-hydroxylic solvent, acetone showed better performance in the reaction. The fluorescent intensities were constant in pH ranging from 4 to 10. (Table 3.11.).

TABLE 3.11. - Influence of pH in the fluorescence intensities.

pH	PEAK AREA
10	1680
9	1675
8	1678
7	1686
6	1673
5	1669
4	1630
3	1510
2	1350
1	1130

The adducts formed were stable for several hours at low temperature in the dark (128,133) (Table 3.12.).

TABLE 3.12. - Stability of PDA Derivative with time.

TIME (HOURS)	PEAK AREA
6	319.400
12	320.810
18	331.500
24	332.130
30	322.990
36	319.600

When the reaction mixture of PDA was injected onto HPLC system two peaks were observed with a ratio of (5:1). It appears that two reaction products are reversibly related and exhibited the same excitation and emission wavelength (Fig. 3.23.).

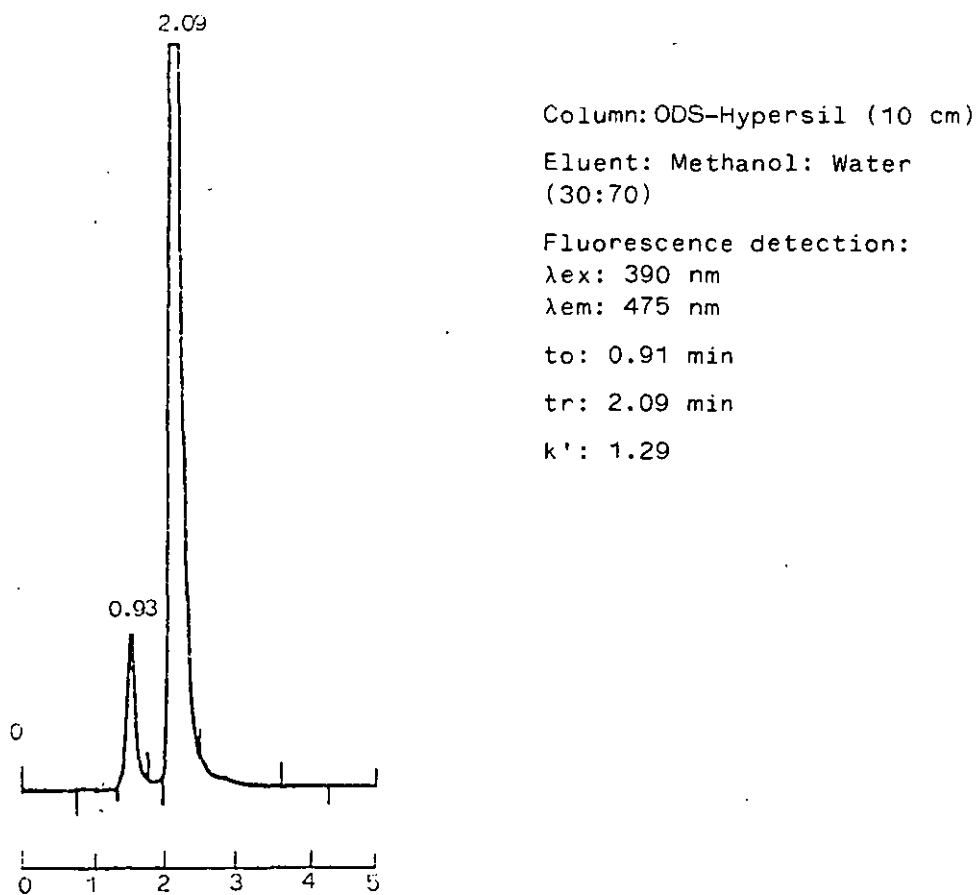


Fig. 3.23. - Typical chromatogram of fluorescamine reaction with PDA.

One component with longer retention time can be characterised as the fluorescamine derivative of PDA and the second compound is presumably a lactone formation, a side product of the reaction. The lactone formation was inversely proportional to the concentration of amine. On increasing the amine concentration the lactone formation decreased to a minimum (Fig. 3.24.).

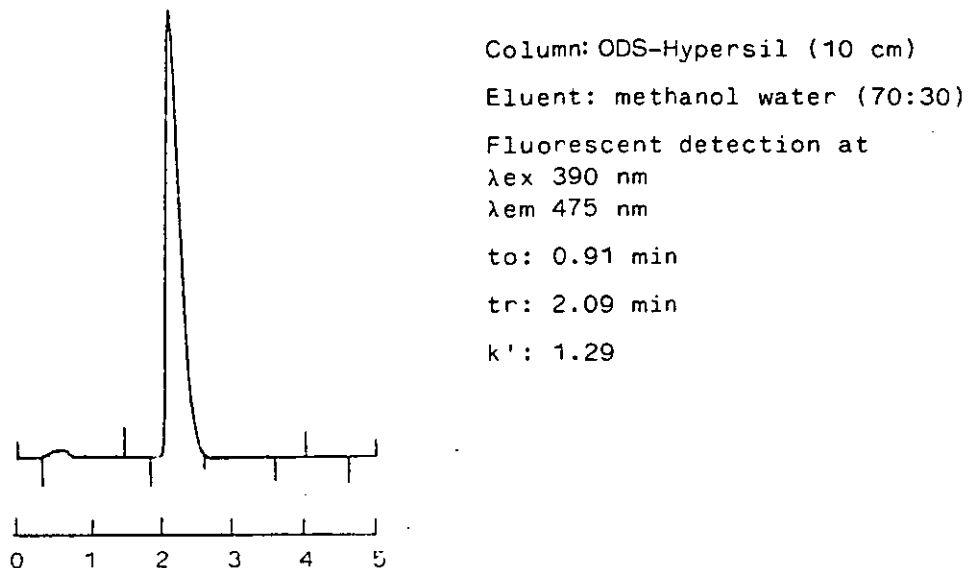


Fig. 3.24. - Reaction of fluorescamine with PDA (The lactone formation does not interfere).

McHugh *et al.* (158) found that amino acids derivatised with fluorescamine yielded two fluorescent compounds which were in equilibrium. The expected adduct and a lactone formed between the free amino acid carbonyl group and proximal hydroxyl (Fig. 3.25.).

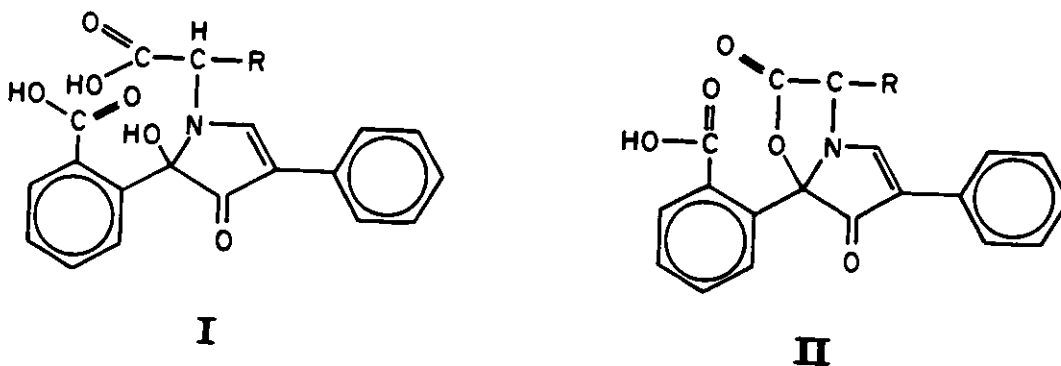


Fig. 3.25. - Structure of lactone formed between the free amino acid and proximal hydroxyl.

Ingles *et al.* (135) reported results by HPLC in acid solvents when the derivative chromatographed was the free carboxylic acid instead of sodium salt. The samples were reacted with fluorescamine, then acidified with glacial acetic acid to pH 4,0 and extracted with chloroform. Similar approach was examined with PDA, but the results were disappointing, peaks were broad and the resolution was poor, however, the retention time was longer (Fig. 3.26.).

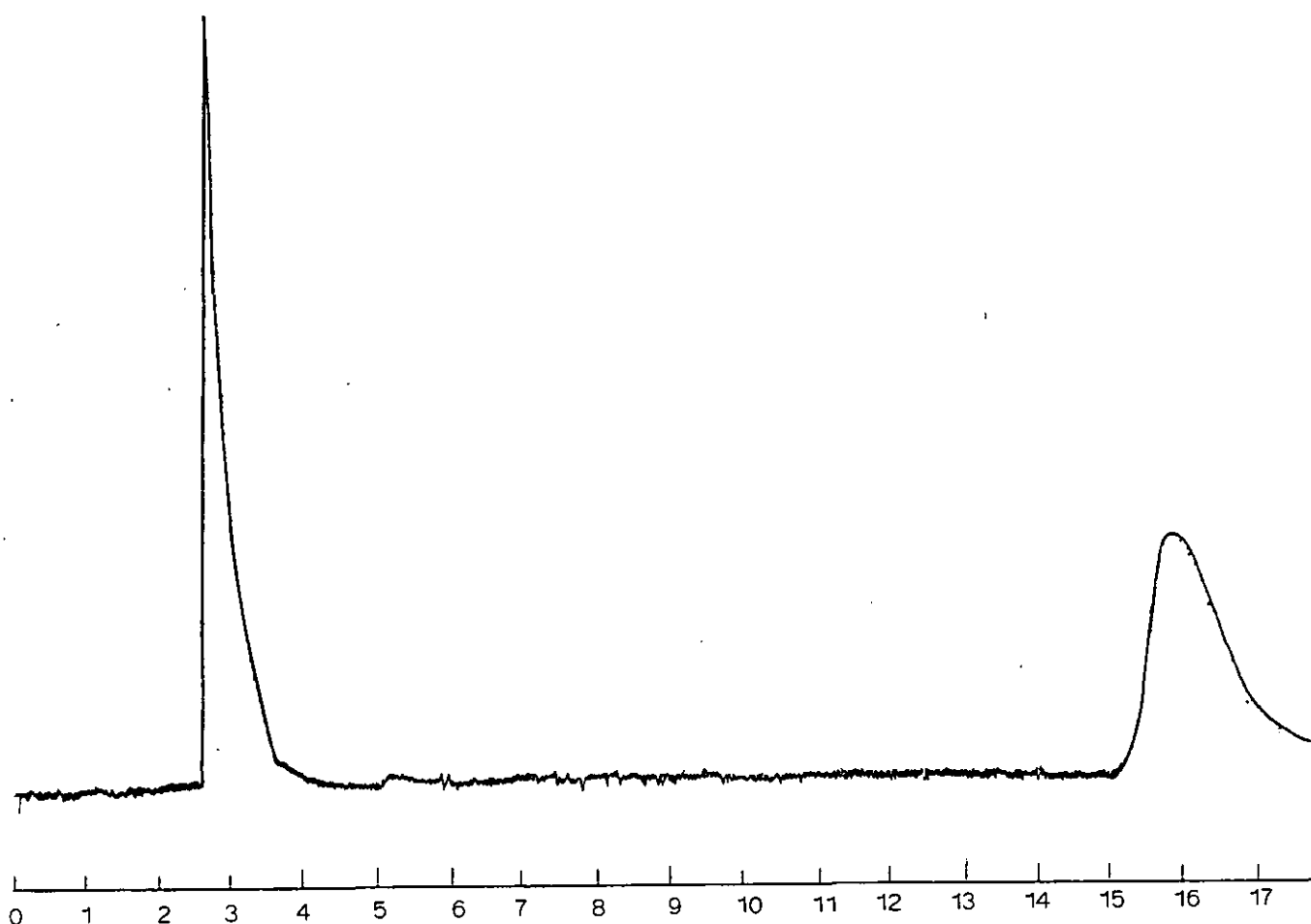


Fig. 3.26. - Free amino acid form of fluorescamine reaction.

Lactone formation should not occur with peptide derivatives since the free carboxylic acid group is situated at some distance from the primary amine. McHugh (158) confirmed this by reacting fluorescamine with 1-alanyl-2-alanine and obtained just one component. The protonation or suppression of lactonisation can be achieved by esterifying the amino acid prior to derivatisation, which decreases the attractiveness of fluorescamine.

As long as the lactone formation did not interfere in the reaction rates and the results were reproducible, the derivatisation of PDA could be carried out with fluorescamine. The calibration curve based on the second major peak suggested that the reaction was not affected by the lactonisation and can be performed quantitatively with good reproducibility (correlation coefficient = 0.998) Fig. 3.27.

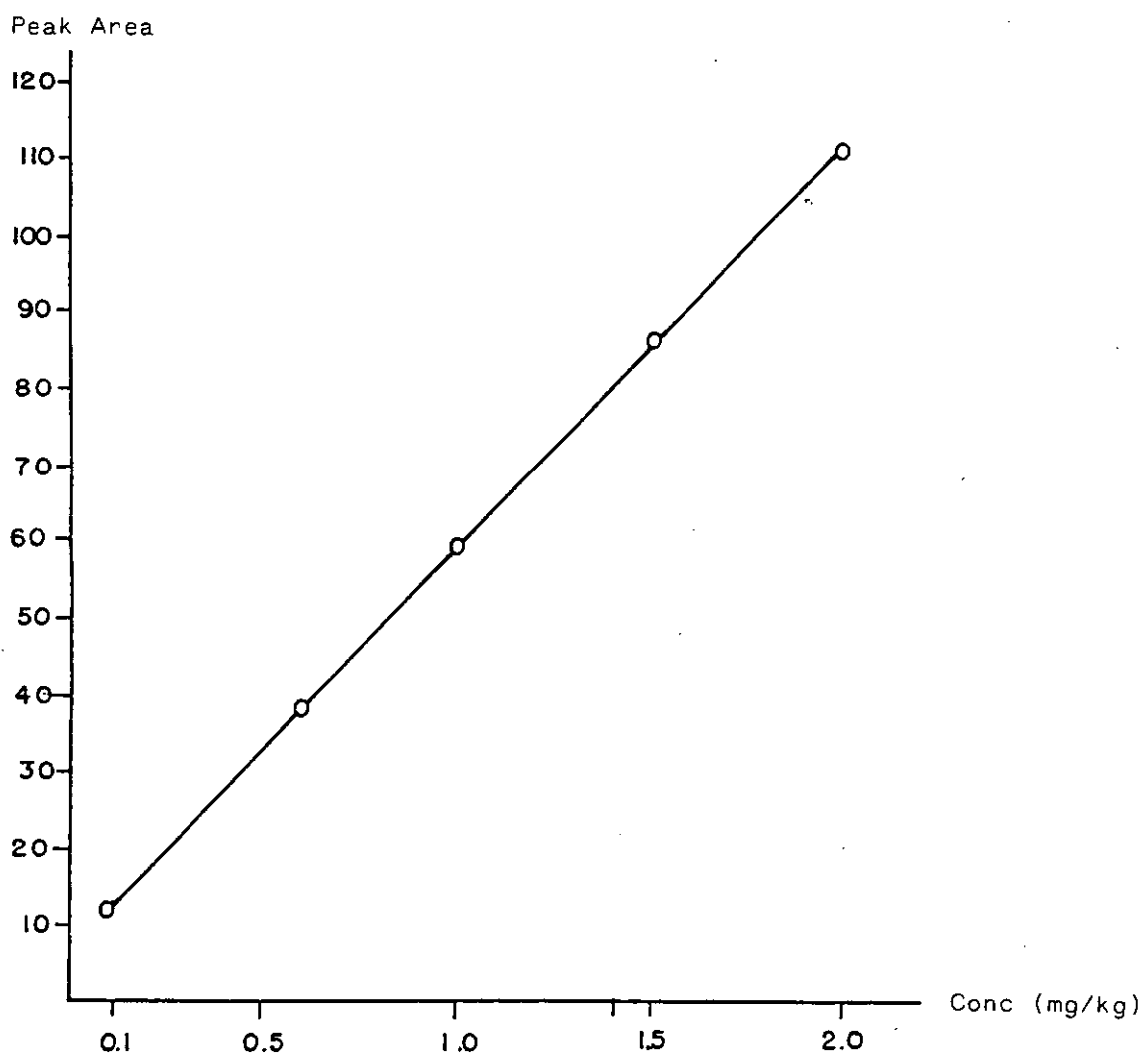


Fig. 3.27. - Calibration curve of fluorescamine derivative of PDA (0.1 to 2.0 mg/kg).

Different reaction conditions, variation of pH, concentration of fluorescamine and organic solvent produced some changes in the absolute peak area, but did not suppress the lactone formation. A linear relationship was obtained between fluorescence intensity and the concentration of the adduct formed. Ethylenediamine (EDA) a degradation product of ETU was also derivatised with fluorescamine and similar results to PDA were observed (Fig. 3.28.).

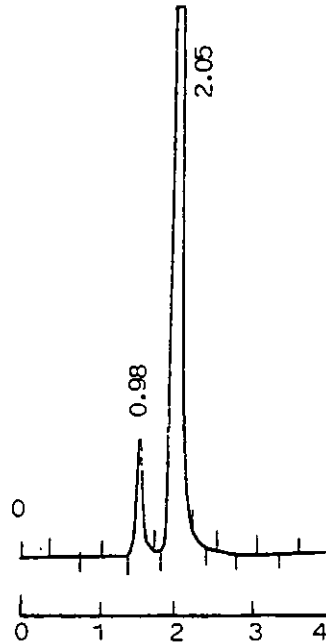


Fig. 3.28. - Chromatogram of EDA fluorescamine derivative.

A mixture of fluorescamine adducts of PDA and EDA could not be easily separated as both have very similar retention times 2.09 for PDA and 2.05 for EDA (Fig. 3.29.).

The separation would have been desirable as method to distinguish residues of ethylenebisdithiocarbamates and propylenebisdithiocarbamates.

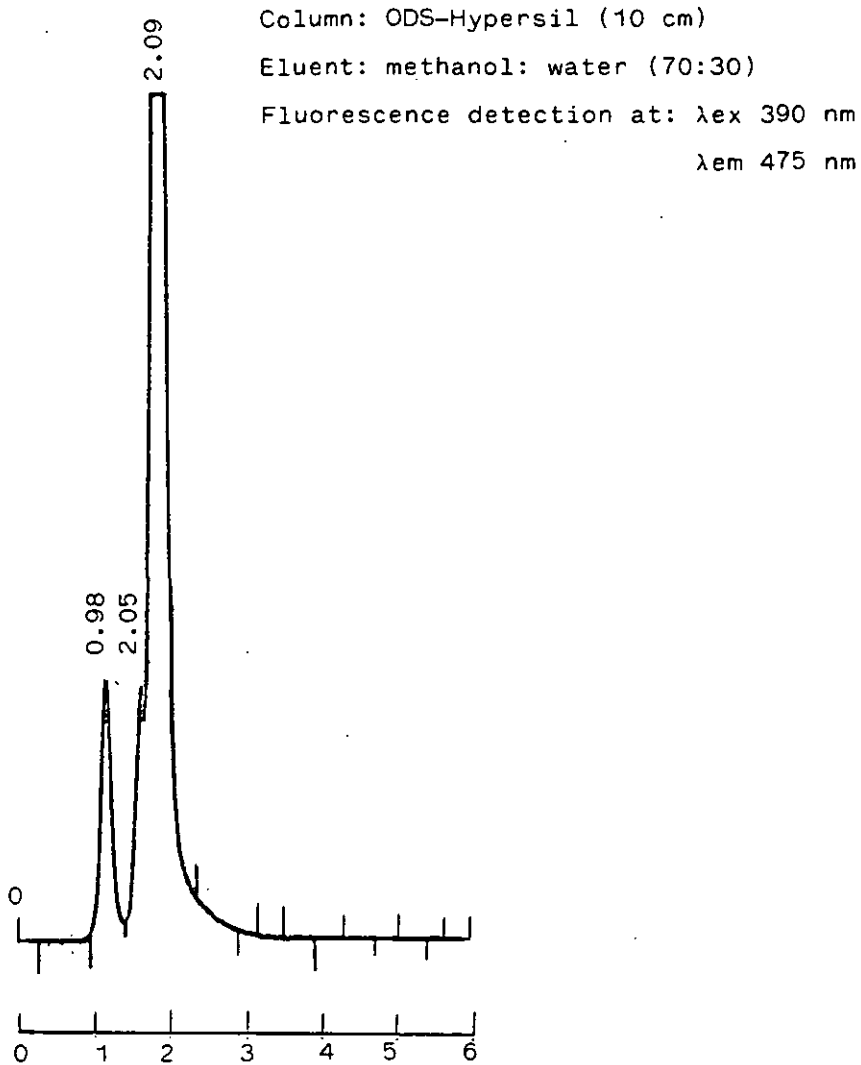


Fig. 3.29. - Separation of a mixture of PDA and EDA.

Attempts were made to obtain resolution by changing the eluent in a series of isocratic runs were performed with 30, 40, 50 and 60% of methanol but the retention times were very similar (Table 3.13.), with borate buffer (0.1 M pH 8).

TABLE 3.13. - Effect of MeOH conc on PDA derivatives

MeOH %	to	k' (PDA)	k' (EDA)
30	0.91	1.29	1.25
40	"	1.05	1.02
50	"	0.87	0.83
60	"	0.74	0.69

From these results the mobile phase selected was methanol-water (30:70). A detection limit of 0.01mg/kg for PDA was achieved with good reaction conditions.

This method of fluorescamine derivatisation was then used to monitoring the degradation of PTU under UV irradiation.

3.3.6. - Degradation of propylenethiourea under UV irradiation

The widespread application of the ethylenebisdithiocarbamates fungicides all over the world has arisen a considerable interest in their degradation. In particular, concern is increasing over residue levels of the major degradation product ETU. Similarly PTU is produced from Propineb.

Photodecomposition might be expected to be the major degradation reaction of PTU. One of the targets of this investigation was to find out whether PTU degrades to PDA (propylenediamine) under UV irradiation. Samples with high concentration of PTU (500 mg/kg) was irradiated under UV light at 254 nm for specific intervals of time (from 0 to 90 hours). The

concentration of PDA was determined after derivatisation with fluorescamine by using HPLC fluorometric detection (Fig. 3.30.).

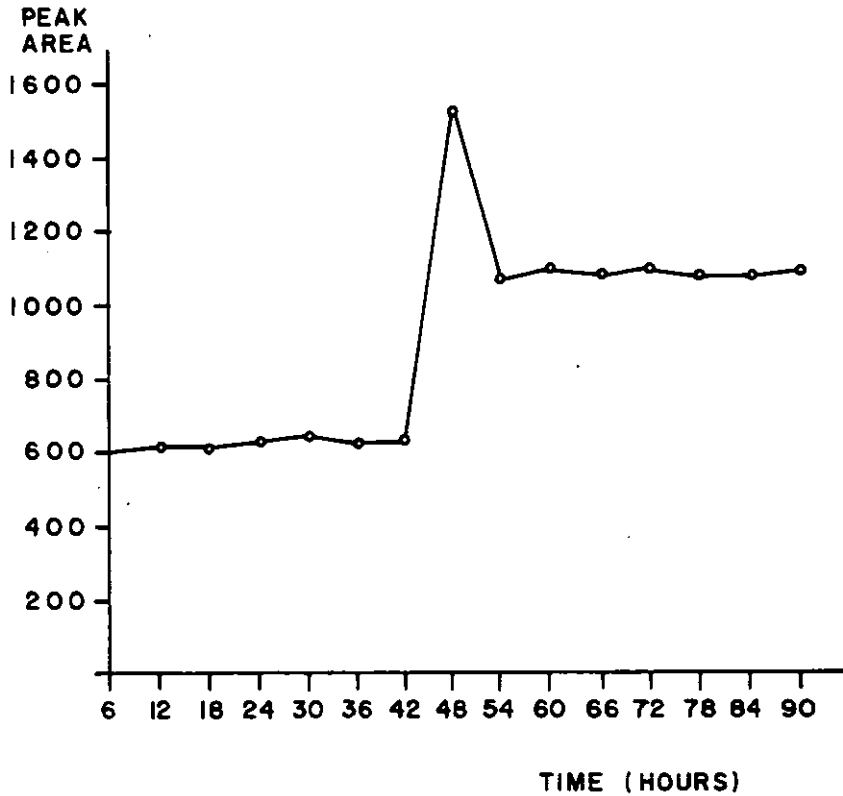


Fig. 3.30. - Degradation pattern of PTU to PDA.

Little degradation appeared to be occurring until 48 hours when the level of PDA increased but no further change was observed.

Simultaneously a PDA solution was also irradiated under UV light and monitored by HPLC after derivatisation with fluorescamine (Fig. 3.31.).

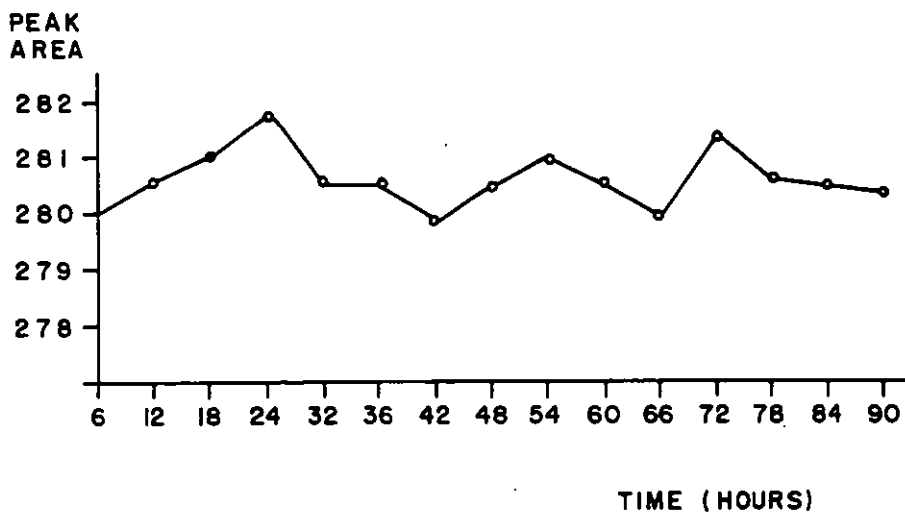


Fig. 3.31. - Behaviour of PDA under UV irradiation.

This procedure verified that propylenediamine was virtually stable under these conditions (Fig. 3.31.). However these results are not conclusive and give only tentative support to the formation of PDA from PTU photochemically.

As ETU has been reported to form glycine on degradation, its derivative was examined by HPLC and eluted earlier. The derivative of glycine eluted at 1.33 min and PDA at 2.09 min (Fig. 3.32.). No peak was observed in the chromatogram of PTU derivatisation at this retention time.

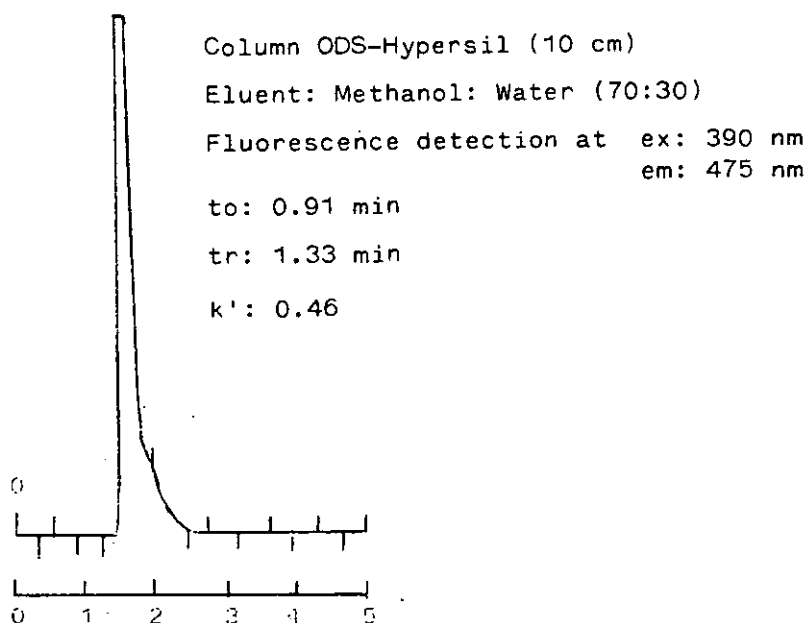


Fig. 3.32. - Derivatisation of glycine with fluorescamine.

There are few published data concerning PTU degradation and PDA has not yet been reported as degradation product of PTU. Because of the limited time available only concentrated samples of PTU were irradiated under UV light. Further studies should be carried out in diluted samples.

CHAPTER IV

4. CONCLUSION AND RECOMMENDATIONS

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Many different approaches to PTU analysis by HPLC were tested. Initially PTU was determined by HPLC on an ODS-Hypersil column with UV detection and this was found to be a simple, fast and reliable determination. Thus, this methodology was applied to the analysis of PTU in commercial formulations of Propineb as well as PTU on spiked lettuces.

Electrochemical detection of PTU was also studied since this kind of detector was claimed to be more sensitive and selective than UV detector. Both of them possessed the same detection limit (0.01 mg/kg) but the electrochemical detector should be more selective. To improve sensitivity and enhance selectivity, PTU was derivatised with *p*-nitrophenacyl bromide to give 3-(*p*-nitrophenacyl bromide)-6-methyl-5,6-dihydroimidazo[2,1-*b*]thiazole, but only the same limit of detection was achieved (0.01 mg/kg). Within the time of this thesis was not possible to extend this study to crops.

The photodegradation of PTU under UV light was studied. To monitor the possible formation of PDA there was a need to find a suitable methodology. Many reagent labels such as NBD-chloride, NBD-fluoride, Dansyl-chloride, OPA and fluorescamine were investigated. Fluorescamine proved to be the best reagent for derivatisation of propylenediamine, although in the reaction two products were formed. One of them was characterised as a lactone and the other as the fluorescamine derivative. The lactonisation did not interfere in the adduct reaction and a linear calibration curve was obtained. PDA was monitored by HPLC with fluorometric detection after derivatisation with fluorescamine. However, when applied to photochemical reaction, the results were inconclusive and little conversion of PTU to PDA seemed to be occurring.

OPA also showed a good performance, forming isoindoles which were highly fluorescent with a good peak shape and resolution, however the reaction was unstable and the yield increasing with the time.

CHAPTER V

5. REFERENCES

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