



University
of Glasgow

McKenzie, John (1989) *The effects and residues of maleic hydrazide within the potato crop*. PhD thesis.

<http://theses.gla.ac.uk/2551/>

Copyright and moral rights for this thesis are retained by the author

A copy can be downloaded for personal non-commercial research or study, without prior permission or charge

This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the Author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the Author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

THE EFFECTS AND RESIDUES
OF MALEIC HYDRAZIDE WITHIN
THE POTATO CROP

JOHN MCKENZIE B.Sc.

Thesis presented for
the degree of Doctor of Philosophy
January, 1989

Agricultural Chemistry,
Chemistry Department,
University of Glasgow

ACKNOWLEDGEMENTS

I am very grateful to Dr. H. J. Duncan

for his supervision of the work described in this thesis. His advice and encouragement throughout the project were of great value to me.

I wish to thank staff and colleagues in the Agricultural Chemistry Section, both past and present, who have been of valuable assistance in many ways. In particular, I thank all friends who helped with the manual labour during field trials.

In addition, I thank Messrs M. Beglan and R. Munro of Glasgow University Chemistry Department and the Photographic Section, Huntingdon Research Centre, Huntingdon, Cambs., for their photographic work.

The financial support of the Potato Marketing Board in the form of a post-graduate studentship is gratefully acknowledged.

I am also indebted to Uniroyal Ltd. for their co-operation, discussion and provision of potato samples for analysis and the supply of Fazor for field trials.

I gratefully acknowledge the typing skills and effort of K. Day and L. Wood.

Finally, I would like to thank my father and mother, Mr. and Mrs. A. McKenzie, for the use of their property for field trials, but especially for the support and assistance during the span of this thesis.

	<u>CONTENTS</u>	<u>PAGE</u>
ACKNOWLEDGEMENTS		I
CONTENTS		II
SUMMARY		VI
A list of abbreviations for names (other than chemicals) used in this thesis		IX
CHAPTER ONE :	The effects and residues of maleic hydrazide within the potato crop	1
1.1	Potato production and use	1
1.2	Traditional potato storage methods	2
1.3	Low temperature storage and why there is a market for chemical sprout suppressants	3
1.4	The association of high temperature storage and chemical sprout suppressants	4
1.5	Chemical sprout suppression using IPC and CIPC	4
1.6	Chemical sprout suppression using TCNB	5
1.7	Problems known to occur with volatile sprout suppressants	6
1.8	Recent developments in potato storage	7
1.9	The introduction of maleic hydrazide into the UK potato industry	10
1.10	A summary of the growth regulant properties and biochemical effects of maleic hydrazide	10
1.11	The health risks of maleic hydrazide in food	11
1.12	The aims of this thesis	15
CHAPTER TWO :	Field trial assessment of maleic hydrazide as a sprout suppressant with growth regulant activity	19
2.1	Introduction	19
2.2	Uptake of MH through the potato leaf cuticle	20
2.3	Time of application of MH to a potato plant	21

2.4	Translocation of MH within potato plants	23
2.5	Sprout inhibition of MH treated tubers	24
2.6	Growth regulation properties of MH and the effect on potato tubers	25
2.7	The effect of MH on the yield of a potato crop	26
2.8	Groundkeeper control with MH	26
2.9	Deformities of tubers caused by MH	27
2.10	MH effects on reducing sugars in tubers	27
2.11	Cultivar behaviour towards MH	28
2.12	Aims of this chapter	28
2.13	The Glasgow University 1985-86 field trial materials, methods and results	29
2.14	The PMB 1985-86 field trial materials, methods and results	30
2.15	The Glasgow University 1986-87 field trial materials, methods and results	32
2.16	Discussion : the use of MH as a sprout suppressant and an assessment of its growth regulant activity	34
CHAPTER THREE :	Analysis of maleic hydrazide residues in Fazor treated potato crops	61
3.1	Introduction	61
3a.1	Total MH : materials and methods	63
3a.2	Total MH : procedure	64
3a.3	Validation of method for total MH	66
3a.4	Total MH : results and discussion	68
3b.1	HPLC residue analysis : materials and methods	80
3b.2	Validation of method for HPLC MH	81
3b.3	HPLC residue analysis : results and discussion	82

3c.1	Fractionation of MH in field treated potato crop	88
3c.2	Fractionation of MH into pools in a field treated potato crop : materials , methods and results	89
3c.3	Discussion of fractionation results	90
CHAPTER FOUR :	The distribution of maleic hydrazide within a potato crop	102
4.1	Introduction	102
4.2	The concentration of MH in individual tubers	103
4.3	The internal distribution of MH within tubers	108
4.4	The concentration compared with tuber size grades and correlation between free MH and total MH	111
4.5	MH residue levels after processing	120
4.6	The distribution of MH within a potato crop	129
CHAPTER FIVE :	An investigation of the metabolite of maleic hydrazide found in potato tubers using radiotracer analysis	131
5.1	Introduction	131
5.2	The techniques used in ^{14}C radiolabelled analysis	133
5.3	The aims of this chapter	134
5.4	the synthesis of MH	134
5.5	The synthesis of (4,5 - ^{14}C) MH	135
5.6	The purity of the synthesized ^{14}C -MH	136
5.7	The activity of the synthesized ^{14}C -MH	137
5.8	The efficiency of the autoradiography	138
5.9	The application of the synthesized ^{14}C -MH to the potato plant	138
5.10	Residue analysis of the ^{14}C -MH treated potato tubers	139

	5.11	Radiolabelled trace analysis of the ^{14}C -MH treated potato tubers	139
	5.12	Results and Discussion	144
CHAPTER SIX :		A study of conjugation of maleic hydrazide with glucose or glutathione	155
	6.1	Introduction	155
	6.2	Acid and alkaline hydrolysis of potato juice extracts	157
	6.3	A study of MH cleaved from a glucose conjugate by a specific enzyme (beta-D-glucosidase)	159
	6.4	The extraction of potato protein as an enzyme source	162
	6.5	A study of conjugation between MH with activated glucose (UDPG)	164
	6.6	A study of conjugation between MH with reduced glutathione	165
	6.7	Results and discussion	167
CHAPTER SEVEN :		The effect of maleic hydrazide on wound healing in tubers	182
	7.1	Introduction : The process of wound healing	182
	7.2	Histological examination of cut tubers treated with MH	184
	7.3	An assessment of wound healing by the rate of water loss from cut potato discs treated with MH	188
	7.4	Discussion : The effect of MH on wound healing in tubers	192
CHAPTER EIGHT :		Conclusions and discussion of the effects and residues of maleic hydrazide within the potato crop	204
REFERENCES			209

This thesis describes the observations of field trials and analytical investigations of the residues of maleic hydrazide (1,2 - dihydro - 3,6 - pyridazinedione) known as MH. MH is marketed in a formulation called 'Fazor' and was introduced to the United Kingdom under PSPS (Pesticide Safety Precaution Scheme) in 1985. It is targeted at sprout suppression of ware tubers in store with the added benefits of increased marketable yield and reduced groundkeepers.

Two field trials were conducted to investigate the sprout inhibition of MH and its effect on yield. Both field trials used a popular ware seed (cv. Maris Piper) grown under typical growth conditions and treated with Fazor (5 kg/500 litres/hectare) under optimum conditions. Treated potatoes were observed to have typical characteristics of a break in apical dominance. Every eye on the tubers was active and formed spikelets that developed into growths similar in appearance to a small cauliflower head. It was also noticed that sprout suppression was not complete. A proportion of potatoes had inadequate suppression. Some showed no characteristics of MH, typical of an untreated potato. The inadequate sprout suppression was investigated by analysing the MH distribution and the comparison of sprout suppression. MH treated plots were found to have slightly lower yields than untreated plots. This reduction in yield was not shown to be statistically significant.

Two methods to calculate the residues of MH were adapted. The first used the action of zinc in concentrated sodium hydroxide to produce hydrogen, which reduced and hydrolysed MH to hydrazine. The hydrazine was distilled and caught in an acid scrubber containing a colour reagent. The colour reagent and hydrazine combined to produce a coloured chromophore. The colour intensity was proportional to the amount of MH. This method was used to calculate the total MH. The second method calculated the amount of free MH (not metabolised or conjugated) by a methanol extraction, cleanup of the concentrate and analysis by high pressure liquid chromatography (HPLC).

The total MH was roughly made up of three factions :-

- (1) Non-extractable MH
- (2) Methanol extractable MH
 - (a) free MH
 - (b) metabolised MH

The uptake of MH over a four week period, after spraying Fazor, was assessed by harvesting at weekly intervals for four weeks and analysing the tubers by the above methods. The distribution of MH between individual tubers was investigated by calculating the MH in whole tubers. The distribution within individual tubers was calculated by analysing the skin, the outer flesh and the inner core. The distribution of MH between grades was calculated. The carryover of MH into processed foods was calculated by analysis of crisps and boiled potatoes from field treated plots.

It was found the MH accumulated in the potato tubers before one week and remained static up to four weeks. However, there was a fraction, a possible metabolite, that increased in concentration, whereas the free MH concentration decreased. Sprout suppression was found to be dependant on the total MH. Small tubers had lower concentrations than larger tubers. MH was found throughout the whole tuber with a slightly lower concentration in the core. MH was also found in fried and boiled potatoes and was identified as free MH.

The metabolites of MH were investigated by applying radiolabelled MH to greenhouse grown potato plants. The potatoes were extracted by methanol and the concentrated extracts separated by thin layer chromatography (tlc). A metabolite was detected after exposing the plates to X-ray photographic plates. However, this metabolite was not successfully identified. This was due to the combination of low specific activity of the radiolabelled material, and the low quantity of metabolite produced.

Hydrolytic enzymes were used to cleave possible conjugated metabolites in the potato juice concentrates. The action of a beta-D-glucosidase was found to increase the free MH. This would appear to suggest that a metabolite of MH is a conjugated product between MH and a glucose molecule. However, a conjugate could not be synthesized between uridine diphospho glucose (UDPG) and MH with soluble potato protein. There was no evidence to suggest that a metabolite is produced between MH and glutathione.

It was suspected that MH would inhibit wound healing because it inhibits cell division. Cut potatoes treated with various concentrations of MH were examined histologically to assess cell division and suberin production. The rate of water loss from potato discs treated with MH was calculated. If the rate of water loss does not slow down because of chemical inhibition of the process, potatoes will lose weight and condition in store.

No difference between control potatoes and MH treated potatoes was found at the beginning of a storage season. At the end of storage (four months), MH treated tubers had better wound healing than controls.

A list of abbreviations for names used in this thesis.

ac	acre
a.i.	active ingredient
AOAC	Association of Official Agricultural Chemists
AR	analytical reagent
$^{\circ}\text{C}$	degrees Celsius
c.v.	cultivar
2-D	two dimensional
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
EPA	Environmental Protection Agency
equ.	equation
ext.	extract, extraction, or extractible
$^{\circ}\text{F}$	degrees Fahrenheit
FID	flame ionisation detection
FDA	Food and Drug Administration
g	gram
gal.	gallon
GC	gas chromatography
h	hour
ha	hectare
HPLC	high pressure liquid chromatography
i.e.	that is
i.d.	internal diameter
Inc.	Incorporated
i.r.	infra red
kg	kilogram

l	litre
lb	pound
LD50	lethal dose required to kill 50% of population
Ltd.	Limited
m	metre
M	Molar unit
M ²	square metre
mg	milligram
min	minute
ml	millilitre
mm	millimetre
N	normal unit (concentration)
nm	nanometre
No.	number
O.S.	ordinance survey
PMB	Potato Marketing Board
pp	pages
p	page
ppm	parts per million
PSPS	Pesticide Safety Precaution Scheme
R.B.	round bottom
RH	relative humidity
RNA	ribonucleic acid
rpm	revolutions per minute
SAX	strong anion exchange
SCX	strong cation exchange
SE2	super elite 2

sp.	species (singular)
spp.	species (plural)
t	tonne
tlc	thin layer chromatography
UK	United Kingdom
uv	ultra violet
Vol	volume
v/v	volume for volume
w	weight
%	percentage
<	less than
>	more than
ug	microgram
ul	micro litre
"	inch
*	multiplication symbol

THE EFFECTS AND RESIDUES OF MALEIC HYDRAZIDE WITHIN THE POTATO CROP1.1 Potato production and use

The potato crop and its processed products are considered as a substantial part of European diet. The importance of the potato crop and its processed products can be highlighted by the statistics collected and presented by Young (1981) and by the Potato Marketing Board (Anon [1985a], Anon [1985b]). These reports have shown that between the years 1955 and 1985 the annual level of UK production has remained at roughly 6.5 to 7 million tonnes. In a typical season (1977/78) 6.7 million tonnes were produced. Of this 59% were sold for consumption as fresh potatoes, 13½% were used in processing, 9% were used as seed, ½% were exported and 17½% represented waste, losses, sales for stockfeed and the operations of the Potato Marketing Board (PMB).

In the report by Young (1981) the potatoes were processed into the products listed below in order of decreasing tonnage :-

- (1) Crisps (400,000 tonnes)
- (2) Chips (377,000 tonnes)
- (3) Dehydrated products (98,000 tonnes)
- (4) Canned whole (15,000 tonnes)

For the same season (1977/78) Young (1981) reports the average human consumption in the UK was almost 19 kg per person per year of fresh weight equivalents. Between the years 1978 and 1984 the UK average guaranteed price ranged from £44.25/tonne to £125.76/tonne (Anon [1985a]).

It is obvious from these data that potatoes and processed potato products are an important part of the UK national diet and in the economy of the agricultural sector and associated industries.

When potatoes are harvested they can remain dormant up to 15 weeks. However, this dormancy period depends on the temperature of storage, cultivar, growing conditions in the field and the maturity of the crop at harvest (Anon [1985c]). If there is no storage facility the fresh potatoes have to be sold before sprouting because consumers will not buy a potato that has sprouts, a wrinkled appearance and a rubbery feel due to water loss. With no storage facility there will be a glut on the market with low prices and massive wastage.

Today's fresh potato market and the producers of processed potatoes demand a storage period up to nine months. This provides a supply of potatoes and processed products from home grown potatoes for most of the year. The rest of the year is supplied by early production in specialised areas of the UK, including Ayrshire in Scotland, the South West of England, and imports mainly from the Mediterranean area.

1.2 Traditional potato storage methods

Traditional potato storage in the UK was in non-specialised barns, pits, or piled in the field with straw for insulation. These methods had a lot of wastage due to rodents, rots, temperature damage and sprouting (Anon [1985c]). In recent years, purpose-built stores have been constructed with insulation, temperature monitoring, refrigeration and ventilation.

The approved methods of ware potato storage, with respect to sprout control, are low temperature storage and chemical sprout suppressants.

1.3 Low temperature storage and why there is a market for chemical sprout suppressants

Most of the information summarised in this section can be referred to in the PMB published pamphlet 'Potato Storage' (Anon [1985c]).

In normal bulk storage situations low temperatures allow storage of potatoes for long periods. Cold storage is the only approved method of storing seed. Storage at 4°C will reduce or effectively stop sprouting, but can cause side effects of increased reducing sugars and sweetening, disease and damage. These problems are outlined below.

The increase in reducing sugars causes dark coloured fried products, i.e. crisps and chips. The dark colour is caused by a reaction between the amino group on amino acids and the carbonyl on the reducing sugar, known as the Maillard reaction. The products are then unacceptable for sale to the public. The increased sugars cause a sweetening, which is reversible by conditioning the tubers at higher temperatures. Long-term storage also produces a form of sweetening known as senescent sweetening, which is not reversible and depends on cultivar, maturity of the tubers and the length of storage. The increase in sugars is not a problem for canned potatoes because the general public accept the characteristic sweet taste.

Disease can occur at lower temperatures, particularly gangrene (Phoma exigua), and skinspot (Pholyscytalum pustulans). Lower temperatures also reduce the wound healing process. This is known to increase disease because of a decreased physical barrier to infection (McGee [1984]). Cold potatoes are more brittle, more susceptible to bruising and are more likely to be damaged at store emptying and in transport to processing factories and sale outlets.

1.4 The association of high temperature storage and chemical sprout suppressants

Commercial ware stores and potato processors would prefer to reduce the problems associated with cold storage, outlined in section 1.3, and store tubers at higher temperatures with chemical sprout suppressants. This practice cuts down the reducing sugars and sweetening, disease and damage. Ware potatoes are usually stored at 7 to 10°C with a chemical sprout suppressant.

The cost of refrigeration at 4°C also inhibits small ware producers. They can easily store potatoes at ambient barn temperatures and apply sprout suppressants themselves or by contract.

1.5 Chemical sprout suppression using IPC and CIPC

IPC (1-methylethylphenylcarbamate, known as prophan), has been known to suppress potato sprouts since the early 1950's (Rhodes et al, [1950], Dettweiler [1952]). See figure 1.1 for the structure.

CIPC (1-methylethyl [3-chlorophenyl] carbamate, known as chlorprophan), has also been known to suppress sprouting since the 1950's (Marth and Schultz [1950], Sawyer and Dallyn [1957]). See figure 1.1 for the structure.

There is far more CIPC used in the UK than IPC, however, there are some formulations that contain mixtures. Most of the information and discussion applies to CIPC because of its far greater use in practice. IPC would probably behave similarly.

Much of the information summarised in the following pages can be referred to in the PMB published pamphlet 'Potato Storage Chemicals' (Anon [1985d]).

CIPC is applied at a rate of 10-20 g per tonne as a fog or granules and can be effective up to 150 days after application.

It is known to prevent cell division as a mitotic poison and therefore prevents sprouting. It also prevents wound healing and is only to be used after the curing period. However, it should be applied before any signs of eye movement. Treatment after initial eye activity has begun will only partially control sprouting.

CIPC is also known to absorb onto wood and cement and can contaminate buildings for subsequent years. This contamination can inhibit the germination of other crops stored as seed.

Potatoes treated with CIPC should not be sold for consumption for up to three weeks after treatment.

1.6 Chemical sprout suppression using TCNB

TCNB (1,2,4,5 tetrachloro-3-nitrobenzene, known as tecnazene), is a chlorinated nitrobenzene. See figure 1.1 for the structure. The chlorinated nitrobenzenes have been known to inhibit potato sprouting since the 1940's (Brown [1947]).

TCNB has been the most effective and is the only one approved for sprout suppression on seed (Brown and Reavill [1954]). It is approved for control of dry rot (Fusarium spp.) and partial control of skinspot (Polyscytalum pustulans) silver scurf (Helminthosporium solani) and gangrene (Phoma exigua) in seed tubers.

TCNB delays dormancy and is usually applied as a dust as the potatoes are going into store. It does not inhibit wound healing and may improve the wound healing process (McGee [1984]). TCNB will not inhibit sprouting if dormancy has already broken.

Potatoes should not be sold for consumption for up to six weeks after treatment.

1.7 Problems known to occur with volatile sprout suppressants

CIPC and TCNB need to be adsorbed onto the potato skin, probably on the eye and its surrounding area, to be effective. If the temperature in the store or in localised areas is higher than normal the greater concentration of active chemical will be present in the atmosphere instead of the potato surface, therefore decreasing its effectiveness. If there are draughts then the volatile chemicals will be depleted by adsorbed chemicals desorbing and being lost with the draughts (Dalziel [1978] Coxon and Filmer [1985]).

Because of inadequate ventilation in potato piles and boxes, CIPC may not be distributed uniformly by forced ventilation fogging through ducts constructed under the pile (Corsini et al. [1979]) or by fogging machines between aisles of potato boxes (Duncan et al. [1986]). This can mean large concentrations on some tubers and less active concentrations on other tubers.

The volatile chemicals may also be prevented from accumulating at the active sites by physical barriers and by their physico-chemical properties. CIPC may be absorbed onto soil on the potato, decreasing the concentration for sprout suppression. In particular, deep set eyes may be filled with soil and are known to have problems of inadequate suppression of sprouting by volatile chemicals. CIPC and TCNB are not appreciably soluble in water, therefore films on damp potatoes can also act as a physical barrier to these chemicals reaching the active site of the eye.

The use of TCNB and CIPC is also associated with a condition known as internal sprouting. Internal sprouting is a condition where the sprouts grow through the tubers and cannot be detected until the potato is cut during processing for crisps or chips on the production line. The theory behind this condition is that TCNB breaks the apical dominance of the individual eyes causing all the possible sprouts from one eye to be active. If CIPC is then applied it only inactivates central sprouts but not the peripheral sprouts of the eyes. These peripheral sprouts are physically barred from sprouting out of the tuber by the bulbous rosette caused by TCNB and therefore grow outwards or downwards through the tuber flesh.

Small growers who use CIPC depend on outside contractors to fog CIPC and are therefore at the mercy of the availability of the contractors. Late applications of CIPC can mean that a larger percentage of the potatoes are unsaleable, and small decreases in total quantity for sale can substantially decrease the profit margin.

There is also a general apprehension of using CIPC and TCNB because they are classed as carbamates and organochlorines respectively. Both of these chemical groups are notorious for being toxic and should be carefully controlled and used with caution. However, it is suspected that these chemicals are added in excess to ensure adequate suppression.

1.8 Recent developments in potato storage

- 1.8.1 Irradiation of the potato crop with gamma rays has been proven as an effective sprout inhibitor since Sparrow and Christianson (1954) irradiated potato tubers (c.v. Katahdin) with a Co⁶⁰ source (Sawyer and Dallyn [1957]). Irradiation is well reviewed by Josephson and Peterson (1983).

There have been suggestions of irradiation causing after-cooking blackening, after tastes, and an immediate increase in reducing sugars (Burton et al. [1959]).

Muir et al. (1987) confirms that there is an initial increase in reducing sugars. This increased level was shown to drop down to a more acceptable level after 200 days of storage at 8°C. Higher irradiation doses give higher initial sugar levels.

There are a number of pilot schemes in Japan, Holland, Canada and the UK to assess irradiation's commercial viability. It will be interesting to note the outcome of this proposed method. The commercial viability depends on two main factors. Firstly, the high capital cost of the building containing the irradiation source and the production line capable of handling vegetables may inhibit any commercially viable plants. Secondly, the costs of transporting vegetables, including potatoes, from the field to the irradiation housing may be too high with little profit benefit. Conversely the development of mobile irradiation sources is still in the early stages and cannot be commented on at this stage.

Other problems do exist, such as : will it be acceptable to the public? There will be no residual radiation. However, key flavour components may be altered. This may lead to a lower quality product compared to other storage methods.

There is also the education of the buyers and consumers at large who may be wary of this method of food storage. However, this problem should disappear with education and promotion.

- 1.8.2 Twenty naturally produced volatile chemicals were assessed by Beveridge et al. (1981a). Benzothiazole and 1,4-dimethyl naphthalene proved as effective at 100 mg/kg as TCNB at 120 mg/kg in controlling sprouting over a twelve week period. Carvone, pulegone and borneol were also as effective at 500 mg/kg. A small field trial assessed the potential viability for ware and seed storage. The opinion was that benzothiazole and 1,4-dimethylnaphthalene should be assessed in larger field trials because of the promising results.

The mixed isomers of dimethylnaphthalene (DMN) had initially been shown to control sprouting by Meigh et al. (1973) and this was confirmed by Beveridge et al. (1981b). They both prescribe its use as a sprout suppressant, especially seed, with similar effects to TCNB.

There has been more interest in DMN than benzothiazole. There has been little progress with DMN since its discovery as an effective sprout suppressant. The slow progress has been attributed to contention between individuals and private companies as to who actually has the rights to make and market the chemical. The difficulties in promoting and marketing this chemical may prove too costly to overcome with low returns since the chemical may only be targeted at a particular small area, that is sprout suppression in the agricultural sector.

1.9 The introduction of maleic hydrazide into the UK potato industry

Maleic hydrazide (1,2-dihydro-3,6-pyridazinedione, known as MH) has been extensively used in the USA as a growth regulant sprout suppressant for potatoes since 1950 (Zuckel et al. [1950]). See figure 1.1 for the structure.

As shown in figure 1.2 there are three possible tautomeric forms of MH. The generally accepted structure is form II (6-hydroxy-3(2H) - pyridazinone). MH is a white, nonvolatile crystalline solid. MH is slightly acidic and can exhibit phenolic properties.

The potassium salt of MH is formulated as a water soluble granular formulation marketed as 'Fazor'. It is produced by Uniroyal Inc., and marketed by Chiltern Farm Chemicals Limited.

Fazor has had limited UK clearance under the Pesticide Safety Precaution Scheme (PSPS) since 1985. It is also cleared and marketed as an onion sprout inhibitor. The use, and limitations are reviewed in Chapter 2, sections 2.1 to 2.11.

1.10 A summary of the growth regulant properties and biochemical effects of maleic hydrazide

Maleic hydrazide was first highlighted by Schoene and Hoffman (1949). The growth regulant properties on tomato plants were described as a pronounced, but temporary, inhibiting effect on plant growth with an associated loss in apical dominance and very little harm to the plant. It was also found to inhibit corn seedling growth but not germination.

The first mode of action studies identified MH as having an anti-auxin effect (Leopold and Klein [1952]), and an anti-gibberellin effect (Brian and Hemming [1957]).

Audus (1963) thoroughly reviewed the effects of MH and classified MH as a growth inhibitor "... that doesn't cause such a dislocation of the growth processes that death or serious damage results".

It is now accepted that MH does not primarily affect the hormonal control of plants, these effects are secondary. The primary mode of action of MH is on the metabolism and control of the nucleic acids.

Peterson and Naylor (1953) noted amino acid disruption and suggested that MH induced plants to use protein as a respiratory fuel or as an inhibitor of protein formation.

Coupland and Peel (1971) found a large portion of radiolabelled MH was translocated, accumulated in the roots of willow and was associated with the RNA and subsequent effects on cell metabolism via abnormal protein and enzyme synthesis.

Nooden (1972) observed the effect of MH on corn and pea roots and noted chromosomal breakage, inhibition of cell division and retardation of plant growth. He suggested the mechanism of MH was as a pyrimidine antagonist or analogue.

It is because of this association with the nucleic acid fraction that there is concern over its level in food products.

1.11 The health risks of maleic hydrazide in food

Because of the chromosomal aberrations observed in plants treated with MH there is concern over MH effects in animal and other biological systems.

There is an excellent review, by Ponnampalam et al. (1983), of the environmental and health risks of MH. The final conclusion of this review is that MH has some potential toxicity for rats and mice, however, they do recognise that it is difficult to extrapolate rodent studies to humans. The difference between species may be tremendous. The review also recommends an extensive survey on possible human health effects and biotransformations that alter MH or degrade MH into a more toxic chemical.

Cradwick (1975) suggested that MH could be incorporated into RNA. Appleton et al. (1981) have proved MH is incorporated into RNA in yeast cells. The possibility of MH incorporation into DNA was not excluded.

Ponnampalam et al. (1983) describes experiments on MH toxicology. Acute oral LD50 of technical grade MH was greater than 4 g/kg for rabbits. Acute oral LD50 of the diethanolamine salt of MH was 2.35 g/kg and 6.95 g/kg to rats. In subchronic cases, a dairy cow was fed 8.8 lb of MH in her diet over an eight month period with no apparent physiological or pathological effects. Two dogs were fed 1 g of sodium salt of MH for one month with no adverse effects on the liver, spleen, kidneys or bone marrow. No ill effects were produced in rats fed dietary levels of 0.5-5.0% sodium salt of MH for 11 weeks, but the diethanolamine salt at a 1% level killed 21 out of 24 animals in 11 weeks. Rats fed with sodium salt of MH (0.6-6.0%) for two years had no difference in breeding performance. However rats fed potatoes, treated with MH in the field, had decreased fertility. This would suggest a possible metabolite produced within the potato plant. It may be inappropriate to identify MH as the active agent of any health problems.

Peddie et al. (1986) explains that the technical grade MH has traces of hydrazine. The hydrazine concentration is reduced in the sodium and potassium salts but not in the diethanolamine salt formulations.

The decreased toxicity of the sodium and potassium salts may be due to the lower concentrations of hydrazine. If hydrazine is a degradation product of MH in some plant systems then this may explain the above observations of decreased fertility of rats fed with treated potatoes. MH has a stable ring structure in some plant systems (Smith et al. [1959], Nooden [1970], Frear and Swanson [1978]), however, Biswas et al. (1967) suggested that MH may degrade releasing hydrazine, although this was not convincingly proved. Newsome (1980a) found no detectable residues of hydrazine in potatoes treated with MH.

The triethanolamine and MH salt was found to have low toxicity (Germane and Kemenis [1973]), any toxicity was attributed to the triethanolamine molecule.

When lactating cows were fed MH treated silage (Kashafutdinov and Tsarev [1973]) there was no general toxic effects, the MH was excreted in the urine and secreted in the milk.

Because of MH being present in such a large part of the UK diet and its toxicity, mutagenicity and carcinogenicity not being fully understood, further studies should be carried out to extrapolate laboratory animal studies to human cases.

MH is listed under 'Dangerous properties of Industrial Materials' (Anon [1984]). It is listed as not carcinogenic in adult rats or mice following oral or subcutaneous administration. MH contaminated with 0.4% hydrazine, as an injection, caused hepatomas in newborn mice. The listing also mentions that in 1980 the FDA and WHO reconfirmed MH as not mutagenic. MH may be a potential human mutagen but evidence supporting a serious human risk is weak. The listing also states that MH is not teratogenic in rats orally dosed with 400-1600 mg/kg/day on day 6 to 15 of gestation.

A recent study (Hunt et al. [1985]) was carried out to investigate application exposure to MH. This study investigated MH in the urine of farm workers after foliar application of MH to flue cured tobacco and found MH in the urine after 24 hours since application. The peak of clearance was between 6 to 12 hours. However, the method of detection was the non-specific method by Lane (1963) giving no clues of metabolites. The report by Hunt et al. (1985) was used to reinstate MH in the USA after the Environmental Protection Agency (EPA) issued a 'Rebuttable Presumption against Registration' in 1977.

There is insufficient evidence to accept or condemn MH use. However, the evidence is insufficient to provide a clear picture of its hazard risks. The governing bodies that regulate agrochemical use have accepted the doubt and have issued a limited registration under the PSPS. MH has to be re-registered annually.

1.12 The aims of this thesis

The aims are :-

- (a) To review MH use on the UK potato industry and to consider its use with respect to current sprout suppression methods.
- (b) To assess the methods used to quantify the residues in potatoes and to use these methods to investigate the distribution of MH within the crop and its processed products.
- (c) To identify metabolites of MH in potato tubers.
- (d) To assess MH effects on the wound healing process of potatoes.

The chapters are arranged, as described below :-

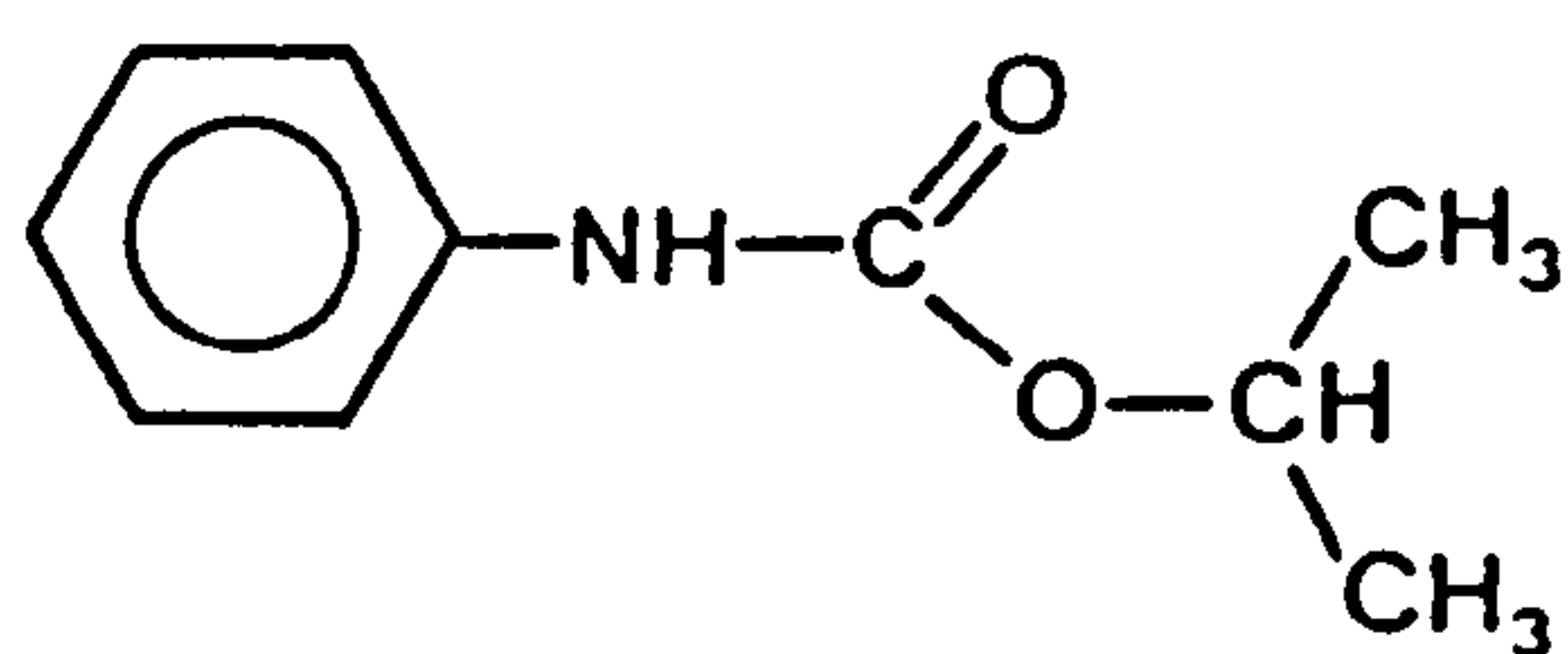
- Chapter 2 : review the literature of MH use on potatoes and assess the growth regulant effects of MH on tubers.
- Chapter 3 : assess two residue methods and the implications of residue date.
- Chapter 4 : because of observed irregular sprout suppression, the distribution of MH within the potato crop and the carry-over of MH into processed foods was investigated.
- Chapter 5 : radiolabelled MH use to determine the structures of possible MH metabolites.
- Chapter 6 : acid and alkaline hydrolysis and enzymic bioassays used to determine the structure of possible MH metabolites.

Chapter 7 : assess the effects of MH on wound healing
 properties

Chapter 8 : discuss the effects and residues of
 MH within the potato crop

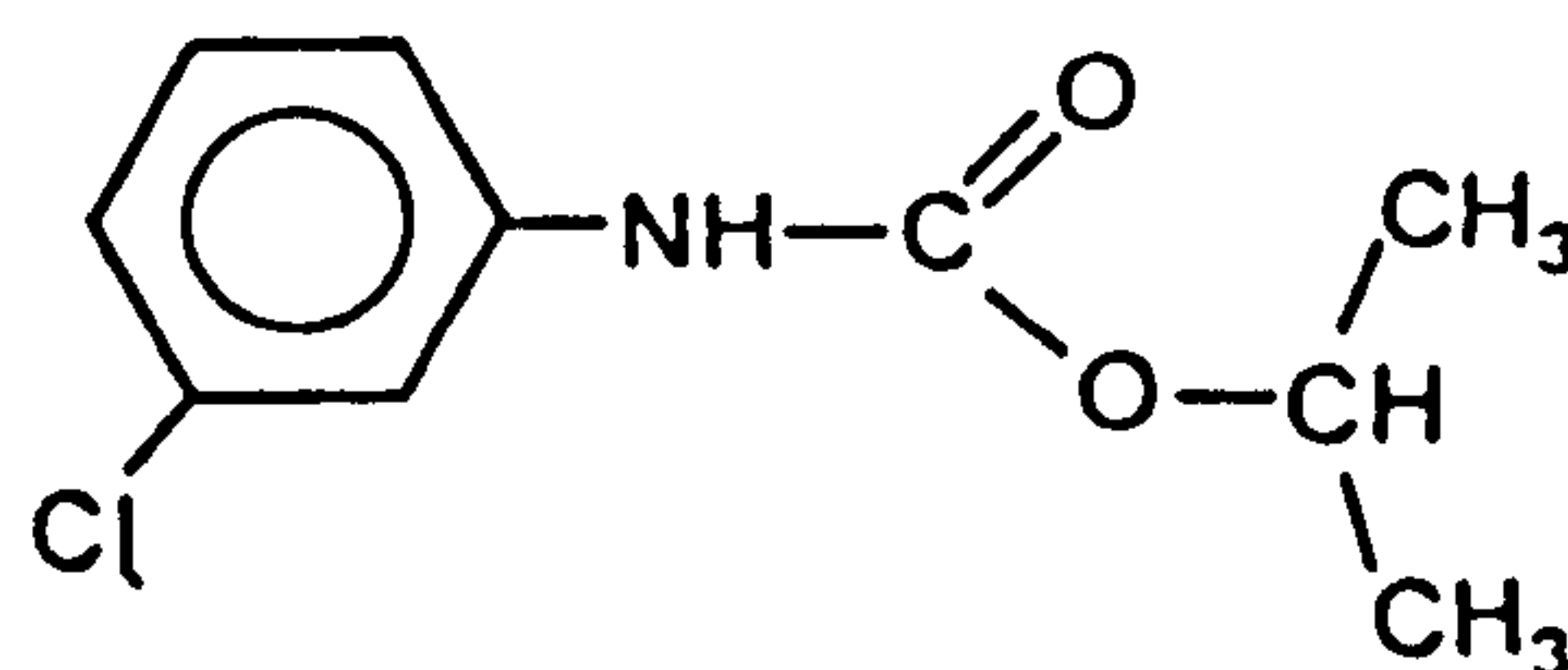
Tables, graphs, photographs and figures are arranged at the rear of each chapter for easy access. Chapters three and four are exceptions. The relevant results are at the rear of each sub-section. Sub-sections are 3a, 3b, 3c, 4.1, 4.2, 4.3, 4.4, 4.5 and 4.6.

Figure 1.1 Currently used sprout suppressant chemicals



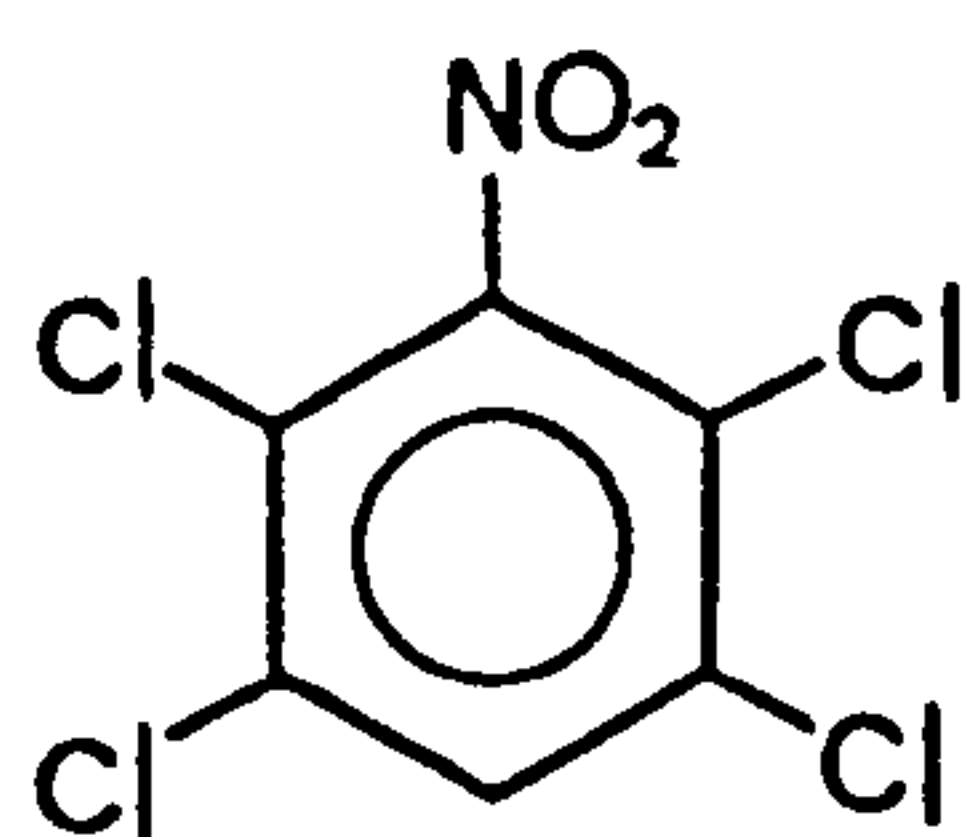
IPC

(1-methylethylphenylcarbamate)



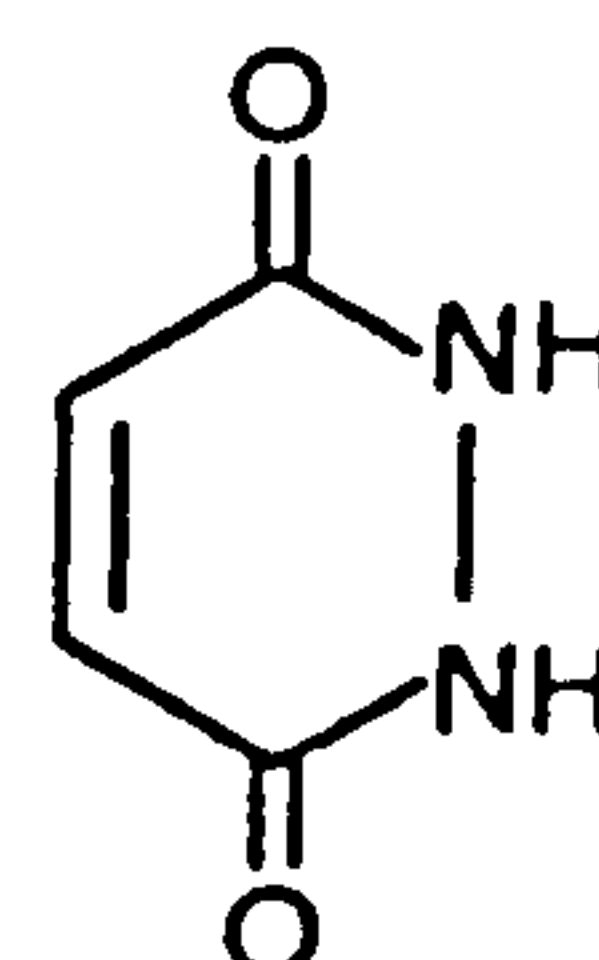
CIPC

(1-methylethyl(3-chlorophenyl)carbamate)



TCNB

(1,2,4,5-tetrachloro-3-nitrobenzene)

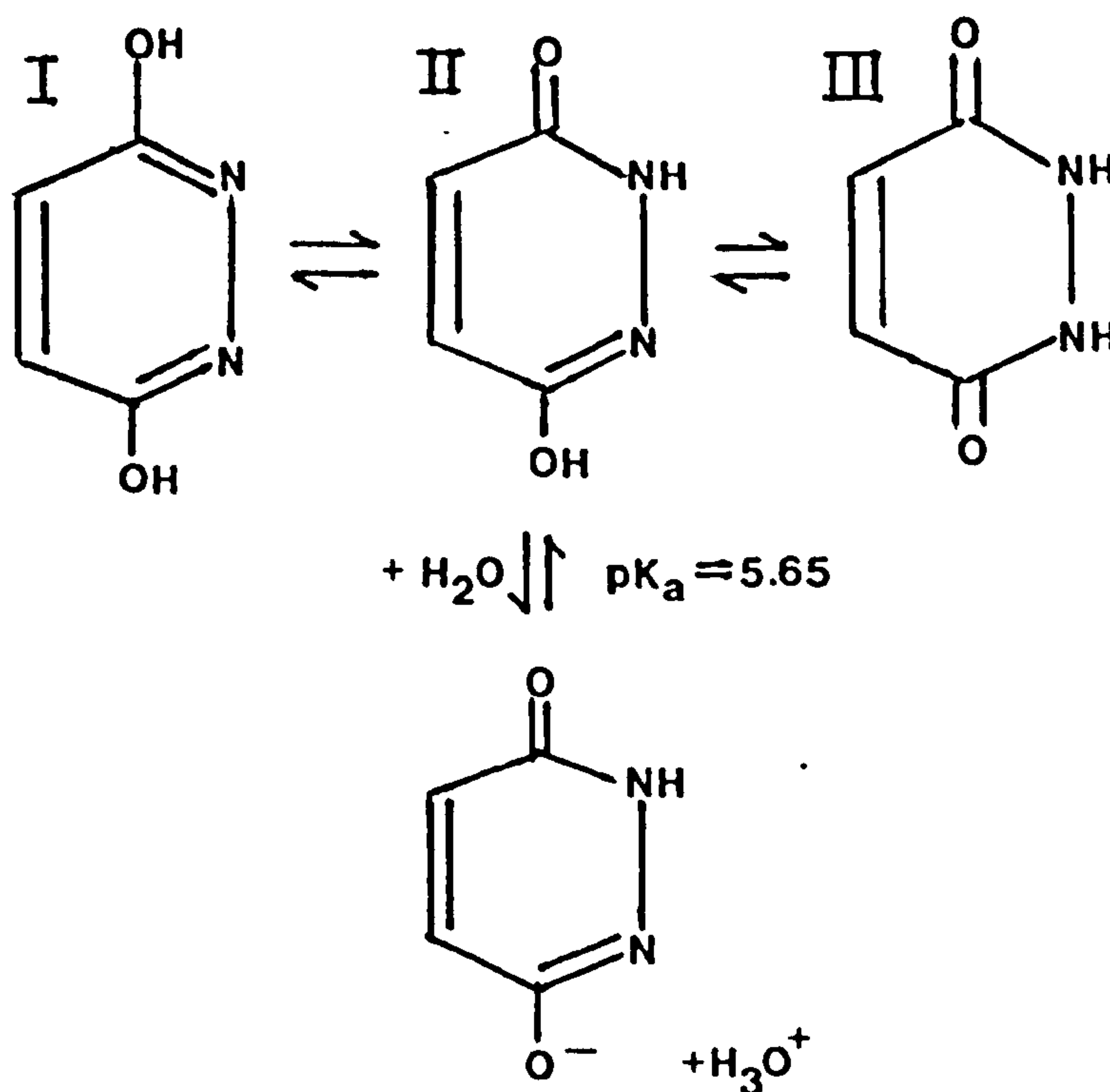


MH

(1,2-dihydro-3,6-pyridazinedione)

Figure 1.2 MH chemical and physical properties

Tautomeric forms exists as II (Miller and White [1956])



Empirical formula $\text{C}_4\text{H}_4\text{N}_2\text{O}_2$.

Molecular weight 112.09 g.

Melting point 296-298 °C (Weast [1979]).

Solubility at 25 °C (Ponnampalam et. al. [1983]).

2.4 g/100 g dimethylformamide

1.0 g/100 g of solvent in water, ethanol, acetone and xylene.

Formulation solubility.

Diethanolamine salt 70 g/100 g water

Sodium salt 20 g/100 g water

FIELD TRIAL ASSESSMENT OF MALEIC HYDRAZIDE AS A SPROUT SUPPRESSANT WITH
GROWTH REGULANT ACTIVITY

2.1 Introduction

This chapter intends to review MH in field trials conducted at Glasgow University and the PMB research station at Sutton Bridge in the 1985-86 season and the 1986-87 season.

MH has been used in the USA for many years as a potato sprout suppressant and to improve the ware crop quality. Its use as a sprout suppressant was first recognised by Zuckel (1950). It is still being marketed as 'Royal MH 30' and is thought to treat 25% of the USA ware crop.

A water soluble granular formulation containing 80% of the potassium salt of MH has been developed by Uniroyal Inc. It has had limited clearance since 1985, and is marketed in the UK as 'Fazor' by 'Chiltern Farm Chemicals Limited'.

Uniroyal marketing literature claims it to be a cost-effective growth regulant with one rate of use and one time of application giving the following benefits :-

"More uniform crop - fewer very small tubers.

More uniform tubers - few misshapen tubers caused by secondary growth.

Inhibition of sprouting - no sprouting during normal storage periods, even at higher temperatures.

No conditioning of tubers for processing - high storage temperatures mean less sugar accumulation.

Reduced shrinkage - more marketable crop through reduced sprouting and shrinkage in store.

Less volunteers - fewer small tubers to remain in the field as volunteers and any which do remain are inhibited from sprouting.

An easy-to-use product with many of the handling advantages of a liquid."

The success of a foliar applied growth regulant such as Fazor depends on a number of factors that should be reviewed and effects discussed before any study of field trial results.

2.2 Uptake of MH through the potato leaf cuticle

The uptake of MH was thoroughly investigated by Smith et al. (1959). Losses before absorption through plant cuticles may be due to water wash off but not volatility or chemical breakdown. Plants absorb MH at different rates; e.g. tomatoes, nutgrass, and quackgrass absorb slower than tobacco, johnson grass, and potatoes. Light intensity and day length do not affect uptake. However, uptake was less at 50°F than 90°F. The most important factor was humidity. Absorption at 100% RH was two to three times faster than at 50% RH. It was also noted that the diethanolamine and choline salts were taken up faster than the potassium salt even with the surfactant sorbitol (1:1 v/v) added.

The alkali metal salts are preferred because they have a lower toxicity compared to the diethanolamine salt. Mukhorina (1973) showed that the diethanolamine salt had a weak toxic effect on blood, liver, and nervous systems of mammals, but the sodium salt had low toxicity and no cumulative effects. Peddie et al. (1986) explained that the diethanolamine salt formulation has traces of hydrazine. However, hydrazine was not present in the potassium formulation and hydrazine was thought to be the cause of toxicity.

Smith (1955) found that 50% of a 2500 ppm solution was absorbed through the cuticle after 25 hours under optimum conditions. However, Franklin and Loughheed (1964), calculated 50% of a 2500 ppm solution was absorbed through the cuticle and translocated into the tubers within 55 hours. The difference was explained thus : Smith's conditions were optimum and the MH was measured as a disappearance from the leaf surface and not the amount accumulated in the tubers.

2.3 Time of application of MH to a potato plant

The application of MH at the recommended time will cause MH to be absorbed through the cuticle. Application times vary; just after full bloom (Franklin and Thompson [1953]), at blossom fall (Sawyer and Dallyn [1958], Rakitin et al. [1973]), one to two weeks after bloom and six weeks prior to harvest (Rao and Wittwer [1955]), ten to fifteen days after bloom in greenhouse, and fifteen to twenty five days in the field (Moll [1973]).

Uniroyal Ltd. marketing material recommend :-

"Spray Fazor when it is known that there will be 24 hours without rain wash-off, or irrigation, but do not use under drought conditions when the temperature exceeds 26°C (80°F). Also, do not use if plant is under stress, i.e. disease, insect attack, to ensure maximum uptake.

Do not apply haulm dessicants within two weeks of application.

Do not use on seed or first earlies."

The successful application of MH depends on green actively transpiring foliage to ensure absorption and translocation. This depends on some degree of subjectivity in getting the timing right.

Uniroyal Ltd.marketing material recommend application when :-

"Flowering : The last few flowers may still be apparent but most of the blossom will already have fallen.

Haulm : A few of the lowest leaves may be turning yellow and the field may have lost its 'brightness'. The haulm may be starting to fall over but will still be actively growing and predominantly green and free from disease or insect attack.

Tubers : The smallest tubers required to reach marketable size will be 25 mm long and starting to expand. A few sample plants should be dug to examine tuber size and number before deciding to spray. Tubers less than 25 mm long will stop growing after application, so only the tubers which are longer than this at application will be harvested."

There have been other application methods assessed such as tuber dipping in MH solutions which have proved effective as sprout suppressants. Chan and Karanov (1983), applied 10^{-2} M as a dip, Pudova et al. (1985) applied 0.1% as a dip and both were effective. However, Marshall and Smith (1951) found dipping ineffective and found adequate effects with toothpicks soaked in MH and stuck in tubers. This is clearly impractical on a large scale.

The concentrations applied have varied from Kennedy and Smith (1951) using 0.001%, 0.01% and 0.1% solutions, to Franklin and Thompson (1953) using 1250, 2500 and 5000 ppm (0.125, 0.25 and 0.5%). Both papers concluded that an increase in the concentration applied increased the effects, i.e. more sprout inhibition and less weight loss.

The recommended rate is 5 kg Fazor in 350 to 500 litres, per hectare (4.5 lb per 35-50 gal per acre).

2.4 Translocation of MH within potato plants

MH is xylem and phloem translocatable, and can radiate out after application to the leaves, (Smith et al. [1959], Crafts et al. [1958], Clor et al. [1962]), and accumulates in areas of active growth.

In tobacco, MH was described as rapidly translocatable (Frear and Swanson [1978]). In potatoes, Rakitin et al. (1973), required three to four days before detection in tubers and ten to fifteen days to get sufficient concentration to suppress sprouting. Franklin and Loughheed (1964) applied 2500 ppm three weeks after full bloom to two cultivars. They found a stimulation of sprouting after 2 to 4 hours with 4 ppm in the tubers. However they found satisfactory inhibition at 50°F for six months storage, after 24 hours with 6 ppm in the tubers. After 48 hours 17 ppm was detected with a resultant increase in sprout control, and a maximum of 36 ppm after seven days.

Smith et al. (1959) applied radiolabelled MH to tomato plants by micropipette, 35% of that applied left the leaves after 24 hours, 70% of the activity had left the leaf after four days. The labelled material was found to accumulate in the growing tips.

2.5 Sprout inhibition of MH treated tubers

MH has been recognised as a sprout inhibitor since Zuckel (1950).

Paterson et al. (1951) applied 500, 1000 and 2500 ppm solutions of MH to two cultivars from one to seven weeks pre-harvest and then stored effectively for seven months at 7°C and 13°C. They noticed sprout inhibition, loss of apical dominance through increased activity in all eyes, reduced shrinkage, a greater uniformity in size and shape of tubers, and the possibility of groundkeeper control. They also noted that 2500 ppm MH applied four to seven weeks pre-harvest controlled sprouting more effectively than 245-T (2,4,5-trichlorophenoxy acetic acid) and MENA (methyl ester of naphthalene acetic acid) applied post-harvest. Kennedy and Smith (1951) applied 0.75, 1.50 and 3.00 lb per acre on 7 cultivars at three dates pre-harvest and stored them at 50°F. They observed the above effects plus the more MH applied the more reduction in sprout growth. There was less of a decrease in weight loss compared to controls, and no effect on reducing sugars. Matlob (1979) also observed the above effects and that sprout inhibition was more effective at lower store temperatures.

Franklin and Thompson (1953) applied 1250 and 5000 ppm MH to a potato crop. The tubers were harvested on different dates and stored for six months at different temperatures. It was noted that sprout growth was inversely proportional to the concentration of MH applied, but proportional to the maturity and the storage temperature. However, Isenberg (1954) and Franklin and Loughheed (1964) found that low concentrations of MH applied actually stimulated sprouting with no mention of abnormal sprouting effects.

In store the tubers treated with MH show characteristic signs after dormancy breaks. These signs are of 'spikelet' formation that may develop into a growth similar in appearance to a small cauliflower head. However, weak spindly sprouts have also been noted (Kennedy and Smith [1951]). There is also a characteristic break in apical dominance causing all eyes to be active and show spikelets (Paterson et al. [1951], Hansen [1960]).

2.6 Growth regulation properties of MH and the effect on potato tubers

MH has been accepted as a cell division inhibitor (Nooden [1972], Coupland and Peel [1971]) allowing cellular expansion but not division (Greulach and Haesloop [1954]).

Uniroyal Ltd. marketing literature states that sprout growth and formation is inhibited, but if MH is applied to the foliage at the correct stage of formation and growth of the tubers then tuber initiation and newly formed tuber growth will be halted allowing tubers greater than 25 mm to expand to a greater yield of a marketable grade.

Struckmeyer et al. (1981) described how the treated potato tissue did expand more than the untreated tissue, and the cortical tissue had greater expansion than the perimedullary tissue.

2.7 The effect of MH on the yield of a potato crop

Bishop and Schweers (1961) applied 3 lb per acre and 6 lb per acre (considered a double dose), and found no significant differences in total yield. Timm et al. (1959) applied from 1000 to 6000 ppm MH to five cultivars 2 to 3 weeks before harvest and reported that total yield was not influenced by application times nor application rates. Chung (1983) applied from 1000 to 3000 ppm to a potato crop and agreed that total and marketable yield were not influenced by the concentration applied. Bevis and Jewell (1986) applied 3 kg of Fazor in 490 litres of water per hectare to a potato crop of 20% crop senescence two weeks prior to dessication. They reported no effects on total yield, nor on the proportion of small tubers to total yield, compared to the untreated. Finally Uniroyal Ltd. field trials applied 3 kg of Fazor in 300 to 400 litres per hectare at a number of different sites and found no decrease in total yield caused by MH (Peddie et al. [1986]).

There have been reports of reductions in yield (Cibes et al. [1955], Hansen [1960]). Denison (1950) explains that early application, or applications of high concentrations can reduce yield. Baijal and Kumar (1978) applied MH to 40, 50 and 60 day old potato plants. They associated early application with reduced total yield. Davis and Groskopp (1981) found a reduction in total yield but no decrease in the yield of the US#1 grade (greater than 170 g). Weiss et al. (1980) found an increase in US#1 grade. However early application reduced yield.

2.8 Groundkeeper control with MH

Because of MH effects on sprouts of tubers it has been recognised as a controller, and limiter of groundkeepers (Paterson et al. [1951], Kennedy and Smith [1951]). Fazor has been shown to reduce groundkeepers to less than 14% compared with an untreated plot, and to reduce sprout numbers and sprout lengths (Peddie et al. [1986]) although the effects were greater in the ware grade than in the smaller tubers.

There have been doubts whether this suppression is consistent (Thomas and Smith [1983]) and whether the MH applied in August can sustain its groundkeeper control until May and June (Lutman [1986]).

2.9 Deformities of tubers caused by MH

MH was thought to hold promise as a possible means to increase tuber set. Barnard and Warden (1950) found that MH applied at tuber set increased the tuberisation. Kennedy and Smith (1951) also found increased tuber set, but there were a lot of deformed small tubers and an injury similar to growth cracks in larger tubers, at harvest. Deformities occurred less with lower concentrations and later applications. Weiss et al. (1980) agree that early application can increase misshapes.

Denison (1950) confirmed that high application rates increase the number of small misshapen tubers. Poapst and Genier (1970) describe the high application rate abnormality as a netted, furrowed appearance similar to water loss.

Kennedy and Smith (1951) also noticed plants that had early applications, showed signs of chlorosis, stunted plants and stolons, and an early abscission of flowers.

2.10 MH effects on reducing sugars in tubers

Reducing sugars are known to accumulate due to prolonged periods of cold storage and at the late stages of storage. The condition due to prolonged cold storage is reversible, the late storage condition known as senescent sweetening is not reversible.

The reducing sugars react with amino acids in the potato juice at high temperatures, such as deep fat fryers, to produce a dark coloured product. The potato processors, such as the potato crispers and chip producers, prefer to have a light coloured fried product.

Instead of storage at low temperature, higher temperatures may be used with a chemical sprout suppressant only if the chemical passes the safety regulations and it does not have other unsatisfactory side effects, for instance, the production of a dark fried product caused by induced high sugar levels including the reducing sugars.

A number of workers found MH to have no effects on reducing sugars and colour of fried products (Kennedy and Smith [1951]), Highlands et al. [1952], Zaehrig et al. [1966]). Paterson et al. (1951) found MH to lower reducing and non-reducing sugars. Salunke et al. (1953) also found less reducing sugars in treated tubers. Payne and Fults (1955) explain that if MH is applied early there is a decrease in reducing sugars but an increase in sucrose. Moll (1973) described early application or optimal application as lowering the hexose content whereas, late spraying increased the hexose content.

2.11 Cultivar behaviour towards MH

Effects on sprouting, yield, and misshapes are not totally dependent on MH. Sprouting effects are also dependent on cultivar (Franklin and Loughed [1964], Kennedy and Smith [1953]). Yield differences and misshapes are also dependent on cultivar (Rao and Wittwer [1955], O'Keefe [1974]).

2.12 Aims of this chapter

This chapter is intended : (1) to assess the merits of MH (as Fazor), in field trials, (2) to discuss its use as a sprout suppressant for ware storage compared with existing chemical sprout suppressants, and (3) to assess its use as a growth regulant.

This section details three separate field trials : (1) a field trial to assess MH sprouting characteristics, and to yield some field treated tubers for residue analysis (see section 3c.2), (2) an assessment of Fazor efficacy compared with existing chemical sprout suppressants, covering collaborative work between the PMB research station at Sutton Bridge and The Agricultural Chemistry section at Glasgow University, (3) an assessment of Fazor growth regulating properties.

2.13 The 1985-86 Glasgow University field trial materials, methods and results

This field trial was initiated to observe the sprouting characteristics of MH treated potatoes and to provide material for residue analysis outlined in section 3c.2.

The crop husbandry is detailed in Table 2.1

2.13.1 The observed sprouting characteristics

By January 1986 the treated tubers were observed to have less sprouts than the untreated tubers - photographs 2.1 and 2.2. The treated tubers did not have complete suppression. The characteristic signs of MH treated tubers are displayed in photograph 2.3, these are a break in apical dominance with all eyes active and showing multiple spikelet formation. These spikelets develop into sprouts similar in appearance to small cauliflower heads - photograph 2.5. This was also displayed in field treated tubers by Hegazy et al. (1978). Photograph 2.4 shows the clear difference in sprouting after four months storage.

After six months storage, photograph 2.5 shows the typical appearance of MH treated tuber. The controls, photograph 2.6, have longer normal sprouts. Abnormal sprouts were observed after six months storage from treated plots - photograph 2.7.

Some of MH treated potatoes showed normal sprouting characteristics. This aspect is investigated in future chapters. Some of the potatoes from the untreated plots showed characteristics similar to treated potatoes, this was probably due to spray drift.

2.13.2 Residue analysis of field treated potatoes

The results are tabulated in Table 2.2.

The MH content of the treated plots was analysed by the method explained by Lane (1963). Full results are detailed in section 3c.2.2.

The untreated plots had less than the limit of detection of MH. All the treated plots were above the minimum concentration required for successful sprout suppression (quoted by Uniroyal Ltd. representatives, in private communication, to be between 10 and 15 ppm).

2.14 The PMB 1985-86 field trial materials, methods and results

In 1986 the PMB conducted trials with Fazor to assess sprout control, compared with CIPC and TCNB. They also assessed the yield and wastage of MH treated potatoes with untreated control potatoes. At four different sites seed was planted and grown in parallel with other potato crops; a hectare of MH treated, and three hectares of untreated control. A hectare of control was stored with either TCNB (5% TCNB granules at 2.5 kg/t) applied at loading or CIPC (5% CIPC granules at 0.5 kg/t) four to seven weeks after loading.

All potatoes were stored at 13°C for two weeks then held at 8 +/- 1°C.

The crop husbandry details are outlined in Table 2.3.

2.14.1 Residue analysis of field treated potatoes

Samples from all of the treated sites were sent to Glasgow University's Agricultural Chemistry section for analysis. The residue method used was the adapted method of Lane (1963) described in full in section 3a.1.

The results are tabulated in Table 2.4.

The results show that two of the plot samples had less than 10 ppm. However, they had adequate suppression for six months storage at $8 \pm 1^{\circ}\text{C}$ (photographs 2.8,9,10,11 and 12).

There were no untreated potatoes available for residue analysis or storage comparison.

2.14.2 The PMB storage results

The PMB trial potatoes were still in good conditions by the end of April 1986, after six months storage. These potatoes had negligible sprouting (photographs 2.8,9,10,11 and 12). There were no controls provided as comparison.

Refer to Table 2.5 for the weight loss data. The mean weight loss showed that there is greater water loss from the control than MH, CIPC and TCNB treated potatoes. However, the MH treated potatoes have greater water loss than the CIPC and TCNB treated tubers.

Refer to Table 2.6 for the sprout control data. The mean results showed that MH treated potatoes have less weight of sprouts than the controls, but more weight of sprouts than CIPC and TCNB.

Refer to Table 2.7 and 2.8 for yield data. The mean results showed a slight increase of 0.8 t/ha in MH treated plots. However, the treated plots had less in ware size (40-80 mm) and more in sub-ware size (40 mm). Of the ware size there were more misshapes and growth cracks.

2.15 The Glasgow University 1986-87 field trials materials, methods and results

This field trial was initiated to assess the growth regulant properties of MH treated potatoes.

The yield variations within grades were noted between treated and untreated potatoes. The sprouting was also assessed.

The crop was also used for residue analysis variations within grades (Section 4.4).

The crop husbandry is detailed in Table 2.9.

2.15.1 Comparison of yields between MH treated potato crop and untreated potato crop

The potato plots were graded over 3.0 cm and 4.5 cm grids and classed as bull (<3.0 cm), seed (>3.0 cm, <4.5 cm) and ware (>4.5 cm). It should be clarified that MH is not to be used as a seed sprout suppressant. Seed in this case is used to name a size of potato. The weights of each grade from each grid were measured with a 12 kg spring balance. The results are detailed in Table 2.10.

Because of no obvious differences, a statistical analysis was carried out on what was termed the marketable yield (seed plus ware) and the total yield (bull plus seed plus ware). The statistical analysis required a comparison of means of two small samples (Bailey [1981]). The Null hypothesis was that there was no difference in yields, marketable and total, between treated and untreated grades. Therefore, the population means were equal and it was assumed that the true variances of the two populations to be compared were the same.

The yield data is treated as two small samples containing n_1 and n_2 observations with variances of S_1^2 and S_2^2 and with means of X_1 and X_2

$$\text{Mean } \bar{x} = \frac{1}{n} \sum x \quad \text{equation 2.1}$$

$$\text{Variance } S^2 = \frac{1}{n-1} \sum (\bar{x} - x)^2 \quad \text{" 2.2}$$

$$\text{Standard deviation} = S \quad \text{" 2.3}$$

$$\text{Standard error S.E.} = \frac{S}{\sqrt{n}} \quad \text{" 2.4}$$

$$\text{'t' - value } t = \frac{x_1 - x_2}{S \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}} \quad \text{" 2.5}$$

$$\text{Degrees of freedom D.F.} = (n_1 + n_2 - 2) \quad \text{" 2.6}$$

The 't' value was used to estimate the 'P' value from the t-tables in statistical reference books. If the 'P' estimate was greater than the chosen 'P' value, in this case 0.05, then there was insufficient evidence for the Null hypothesis to be false.

Comparison of marketable yield 't' = 0.67

Comparison of total yield 't' = 1.04

With 26 degrees of freedom, ' P ' > 0.05 in both cases. The probability was greater than the 5% level, therefore there was insufficient evidence for rejecting the Null hypothesis, i.e. there was no significant difference between total, or marketable yield between tubers treated with Fazor and those without.

2.15.2 Sprouting assessment

After five months storage the potatoes from the plots with the highest and lowest residue levels and a control plot, were assessed for sprouting. The criterion for sprouting was if a sprout was greater than 1 cm long then the potato was unacceptable. The results are tabulated in Table 2.11.

2.15.3 Residue analysis of field trials

The method of analysis is described in section 3a.1. The full residue results are detailed in section 4.4. The results are tabulated in Table 2.12.

2.16 Discussion : The use of MH as a sprout suppressant and an assessment of its growth regulant activity

The two seasons of trials at Glasgow University both demonstrated that MH as Fazor does suppress sprouting. Greater sprout suppression was observed in the larger sized potatoes and in plots with the highest residue levels.

The PMB field trials showed more suppression with slow water loss and slower sprout growth than the controls. However the CIPC and the TCNB treated potatoes had less water loss and almost complete sprout suppression.

The PMB trials showed MH to give a similar total yield compared with the controls. However, at grading the treated marketable ware grade was reduced. The Glasgow University field trials assessed yield and found decreased yields in all grades, although the differences were not statistically significant.

The results indicate that MH does suppress sprouting in store. This suppression is characterised by all eyes being active and producing spikelets that develop into small rosettes, similar to small cauliflower heads.

The sprout suppression does not seem to be as effective as with existing sprout suppressants, especially when CIPC and TCNB are used effectively under good store management. However, because it is applied in the field with no disturbance to the stored crop and without some of the problems of CIPC and TCNB outlined in section 1.7, some growers may prefer to use Fazor with the possible benefits of ground keeper control and increased yields. MH may show erratic and short-term control. If MH has only short term control CIPC will still have to be applied with the associated extra expense of contract fogging.

Groundkeepers may be reduced but total control is unlikely, therefore there is still the expense of rogues and selective herbicides if clean crops are to be obtained in successive years.

The promise of extra yield and a better quality product is not always fulfilled. MH can reduce yields, and can reduce the quality due to deformities such as skin cracks and greater numbers of small tubers.

The problems of erratic sprout suppression, inadequate groundkeeper control, reduced yields, and misshapes can all be caused by incorrect application times causing insufficient or excessive MH concentration in the potatoes. The application is dependent on a lot of criteria such as the climate, the state of the haulm, the ability of the soil to withstand machine spraying and the stage of growth of the tubers.

At the time of application the farmer must decide whether he can sell the crops as seed or as ware. If the farmer sprays Fazor then he cannot sell his crop as seed. The farmer will also not be able to keep some of his own crop for seed.

At the application stage in the Glasgow University field trials there were periods of wet weather. There were very few periods that could be forecast to give 24 hours without rain, even at short notice. It is recommended that Fazor needs 24 hours without rain for maximum uptake and effect. The prediction of rainfall in the UK, especially in Scotland, is difficult because of local terrain and peculiarities due to the geography. Agricultural practice does demand rapid uptake from foliar applied growth regulants.

When Fazor is applied in the field it does not necessarily mean another spraying. At the time of application Fazor can be mixed with blight control and insecticide sprays.

The crops today are of various varieties. Individual varieties have different husbandry situation. There is an infinite number of factors inter-relating that can alter a crop's characteristics and properties, and change the optimum time of application of Fazor to get sufficient concentrations for maximum benefit, without excessive residues. Because of the number of criteria that have to be satisfied, and the number of factors to be considered, the use and application of Fazor depends on careful management and experience.

There is still very little known about translocation of assimilates and xenobiotics in the potato crop. With more understanding of the processes and transport systems, foliar applied translocatable agrochemicals may be used more efficiently and effectively without excessive concentrations.

TABLE 2.1

Crop husbandry of 1985-86 Glasgow University field trial

Site :	Garscube Estate, Bearsden, Glasgow OS 1:50000. 548706
Seed :	Maris Piper SE.2
Fertiliser :	Norsk Hydro Maincrop (1400 kg/ha)
Nematicide :	Temik (10 kg a.i./ha)
Block arrangement :	Eight blocks, each containing five drills, four metres long, thirteen seed per drill
Weed control :	Gramonol (5.6 kg/ha) <1% shoot showing
Blight control :	Fortnightly sprays of Patafol fungicide (2 kg/ha) and Rogor insecticide (0.5 kg a.i./ha)
Sprayer :	Knapsack sprayer, Lurmark Ltd
Harvesting :	One drill per block was lifted per week, except for the first week in which two drills were lifted
Storage :	Crop from each drill was stored in 10 kg cardboard boxes at 8+/- 1°C after conditioning at room temperature for one week

(1) Refers to section 2.13

TABLE 2.2

Residue analysis on Glasgow University 1985-86 field trial

Weeks after Fazor spraying	Total MH (ppm)
1	19.5
2	20.7
3	17.8
4	19.1

(1) Full results are to be found in section 3c.2.2.

TABLE 2.3

The PMB field trial crops husbandry

Site	Variety	Fazor* applied	Haulm destruction chemical used	Harvest
1	Record	21 Aug	16 Aug, H SO _{2 4}	30 Oct
2a	Record	21,30 Aug**	11 Sep, H SO _{2 4}	15 Oct
2b	M. Piper			
3	M. Piper	7 Sep	30 Sep, H SO _{2 4}	17 Oct
4	P. Dell	30 Aug	4 Oct, DNBP	22 Oct

* Fazor applied at 5 kg/ha in 300 to 500 litres of water

** Site 2 required a second application because of rain within 24 hours of application

Refer to section 2.14

TABLE 2.4

Residue analysis of the PMB field trials

Site	Cultivar	Mean residue MH level (ppm)*
1	Record	23.1 (18.8, 27.3, 23.2)
2a	Record	7.5 (7.5, 7.5)
2b	M. Piper	17.0 (17.5, 16.5)
3	M. Piper	5.7 (7.3, 3.9)
4	P. Dell	21.7 (22.8, 20.5)

* Each result is the mean of two replicates apart from site 1 which is the mean of three replicates. The replicates are in the brackets.

Refer to section 3a.3.4 for full analytical details

TABLE 2.5

Weight loss of 10 kg samples 1985-86 PMB field trials

Site	Cultivar	Weight loss %			
		MH	TCNB	CIPC	Control
1	Record	4.6	4.3	3.4	5.8
2a	Record	4.8	4.5	3.9	4.7
2b	M. Piper	3.1	3.7	3.9	5.1
3	M. Piper	4.6	4.5	3.9	4.7
4	P. Dell	4.3	3.5	3.9	3.7
Mean		4.2	3.8	3.6	4.4

(1) 13°C storage for two weeks

(2) 5½ months storage at 8 +/-1°C

(3) Refer to section 2.14

TABLE 2.6

Sprouting of 10 kg samples 1985-86 PMB field trials

Site	Cultivar	Sprouting % wt.			
		MH	TCNB	CIPC	Control
1	Record	0	0	0	0.1
2a	Record	0.3	0	0	1.6
2b	M. Piper	0.4	0	0.1	1.0
3	M. Piper	0.1	0	0	0.4
4	P. Dell	0.1	0	0	0.7
Mean		0.18	0	0.02	0.76

- (1) 13⁰C storage for two weeks
- (2) 5½ months storage at 8 ± 1⁰C
- (3) Refer to section 2.14

TABLE 2.7

Total yield 1985-86 PMB field trials

Site	Cultivar	Yields t/ha	
		MH	Control
1	Record	62.1	58.2
2a	Record	56.5	54.0
2b	M. Piper	62.8	63.0
3	M. Piper	54.0	58.6
4	P. Dell	36.7	34.2
Mean		54.4	53.6

- (1) 13°C storage for two week
- (2) 5½ months storage at 8 ± 1°C
- (3) Refer to section 2.14

TABLE 2.8

Grading out-turn 1985-86 PMB field trials

	Washed % Weight	
	MH	Control
Ware size (40-80mm)	95.0	98.0
actual mean weight (t/ha)	51.7	52.4
Sub-ware size (<40 mm)	4.6	2.0
actual mean weight (t/ha)	2.5	1.1
	Percentage of ware size	
Misshapes	1.9	1.1
Growth cracks	3.0	2.7

- (1) 13⁰C storage for two week
- (2) 5½ month storage at 8 ± 1⁰C
- (3) Refer to section 2.14

TABLE 2.9

Crop husbandry of 1986-87 Glasgow University field trial

Site	: Coaldarach Farm, Drymen, Glasgow OS 1:50000 . 469901
Seed	: Maris Piper SE.2
Fertiliser	: Norsk Hydro Maincrop (1400 kg/ha)
Seed dressing	: gamma HCH
Block arrangement	: Fourteen blocks, each containing two drills, five metres long, sixteen seed potatoes per drill
Weed control	: Gramonol (5.6 t/ha) with less than 1% shoot showing
Blight control	: Fortnightly sprays of Patafol fungicide (2 kg/ha) and Rogor insecticide (05 kg a.i./ha)
Fazor spraying	: Seven blocks were sprayed with Fazor (5 kg/ha) at the end of August
Haulm destruction	: Three weeks after Fazor spraying with Reglone (4 t/ha)
Sprayer	: Knapsack sprayer, Lurmark Ltd
Harvesting	: One week after haulm destruction
Storage	: Plots were stored in 10 kg cardboard boxes at 8+/- 1°C after conditioning at room temperature for two weeks

Refer to section 2.15

TABLE 2.10

Mean weight per drill (kg/drill)
1986-87 Glasgow University field trial

Grade	Number of plots*	MH treated	Untreated
Bull	14	0.21 (0.05)	0.30 (0.06)
Seed	14	1.80 (0.12)	1.95 (0.12)
Ware	14	9.10 (0.39)	9.13 (0.42)

* Each plot was 5 metres long (16 seed)

- (1) The standard errors, equation 2.4, are printed in brackets alongside each result
- (2) Refer to section 2.15.1

TABLE 2.11

Percent of sprouted tubers
1986-87 Glasgow University field trials

Plot	MH mean of grade (ppm)	Percent sprouted tubers		
		Bull	Seed	Ware
1	4.7 (least MH)	46.5	41.8	17.4
7	<1.0 (control) -	100.0	100.0	100.0
8	10.7 (most MH)	45.5	27.3	7.4

- (1) 5 month storage at $8 \pm 1^{\circ}\text{C}$
- (2) Typically 30-40 individual tubers per grade at plot
- (3) Refer to section 2.15.2

TABLE 2.12

Total MH residue levels
1986-87 Glasgow University field trials

Plot	MH ware residue levels (>4.5 cm grill)		
1	11.5	(12.3	10.8)
3	15.1	(14.0	16.2)
6	13.7	(11.7	15.7)
8	17.0	(16.3	17.7)
9	15.3	(16.3	14.4)
11	11.5	(10.5	12.5)
13	10.4	(10.1	10.7)

- (1) All treated plots had above the minimum concentration required for successful sprout suppression (quoted by private communication with Uniroyal as 10-15 ppm).
- (2) Refer to section 4.4 for full analytical details

Photograph 2.1

A comparison of Fazor treated and untreated potato tubers.



Husbandry conditions; Table 2.1,
Harvested one week after Fazor application,
Storage for three months at 8 °C,
Left hand box treated,
Right hand box untreated.

Photograph 2.2

A comparison of Fazor treated and untreated potato tubers.



Husbandry conditions; Table 2.1,
Harvested three weeks after Fazor application,
Storage for three months at 8 °C,
Left hand box untreated,
Right hand box treated.

Photograph 2.3
Typical MH treated sprouts.



Husbandry conditions; Table 2.1,
Harvested three weeks after Fazor application,
Storage for three months at 8 °C.

Photograph 2.4

A close-up of Fazor treated and untreated control potato tubers.



Husbandry conditions; Table 2.1,
Harvested three weeks after Fazor application,
Storage for three months at 20 °C.

Photograph 2.5
Typical MH treated sprouts.



Husbandry conditions; Table 2.9,
Storage for six months at 8 °C.

Photograph 2.6
Control untreated sprouts.



Husbandry conditions; Table 2.9,
Storage for six months at 8 °C.

Photograph 2.7
Abnormal sprouting from Fazor treated plots.



Husbandry conditions; Table 2.9,
Storage for six months at 8 °C.

Photograph 2.8
PMB trials, site 1 Fazor treated potatoes.



Husbandry conditions; Table 2.3,
Storage for about six months at 8 °C.

Photograph 2.9
PMB trials, site 2a Fazor treated potatoes



Husbandry conditions; Table 2.3,
Storage for about six months at 8 °C.

Photograph 2.10
PMB trials, site 2b Fazor treated potatoes.



Husbandry conditions; Table 2.3,
Storage for about six months at 8 °C.

Photograph 2.11
PMB trials, site 3 Fazor treated potatoes.



Husbandry conditions; Table 2.3,
Storage for about six months at 8 °C.

Photograph 2.12
PMB trials, site 4 Fazor treated potatoes.



Husbandry conditions; Table 2.3,
Storage for about six months at 8 °C.

ANALYSIS OF MALEIC HYDRAZIDE RESIDUES IN FAZOR TREATED POTATO CROPS3.1 Introduction

The analysis of agrochemical residues is a prerequisite for registration of use. Along with safety and toxicological data there must be a method of analysis, not only of the raw material, but of the residues. These residue methods can be used to correlate concentrations with effects.

Franklin and Loughheed (1964) applied MH and found that 4 ppm was found in tubers 2-4 hours after application. This was insufficient to control sprouting and in fact it stimulated sprouting. 6 ppm was found after 24 hours and this satisfactorily inhibited sprouting for six months at 50°F. 17 ppm was found after 48 hours and this increased the inhibiting effect. Poapst and Genier (1970) related rutted, furrowed appearance on tubers to high concentrations of MH within the tubers.

Different methods of analysis may also be used to measure different pools of a chemical, which may depend on whether the chemical is degraded, conjugated with something else, lost to the environment through gaseous exchange or exudation, or unaffected by the plant.

The initial residue method was that of Wood (1953) described as a reduction of MH to hydrazine by nascent hydrogen from the reaction of zinc granules in boiling concentrated sodium hydroxide. The hydrazine is distilled off and reacts with p-dimethylaminobenzaldehyde in acidic solution to form a yellow coloured chromophore in acid solution which may be measured colorimetrically.

Since then Lane et al. (1958) has added a pre-cook stage and the addition of ferrous chloride to reduce the interferences. The method used in this thesis is explained by Lane (1963) and is described in the Materials and Methods section 3a.1.

Radiolabelled MH has been used in analysis of residues (Callaghan and Grun [1961]), extracted in alcohol from wheat leaves (Tower et al. [1958]), and tea plants (Biswas et al. [1967]), then separated using 2-D paper chromatography or using 2-D tlc extracted from tobacco plants (Frear and Swanson [1978]). Haeberer et al. (1974) developed a GC method for methanol extractable MH using silylation and FID detection. An alumina microcolumn (Haeberer et al. [1974]) or a tlc clean-up (Frear and Swanson [1978]) have been found to be necessary in some situations prior to GC separation.

There are also HPLC methods for MH analysis, including the method of Victor et al. (1984) for detection and measurement of MH in waste water using reversed phase separation and conductivity detection. The method used in this thesis is a methanol extraction, reduction in volume under vacuum, SCX clean-up, and separation by HPLC ion-exchange with UV detection. This method has been developed by Newsome (1980a), and used to measure MH in vegetables and potatoes by Newsome (1980b).

The purpose of this chapter is to investigate and discuss two existing methods already mentioned above. These are Lane's (1963) method for total MH and Newsome's (1980a) method for free MH. Either one may give different correlations with effects, but together they can be used to identify different pools of MH. These different pools are (a) total, (b) methanol soluble MH and (c) free MH in the methanol extract.

If the above pools are measured we can get a rough idea of the adsorbed material, being the difference between total MH and methanol soluble MH, and the metabolised material, being the difference between methanol soluble MH and free MH.

Firstly, the methods are described and their use discussed in association with standardising experiments and inter-lab correlation. Secondly, these methods are used to investigate the different ratios and absolute quantities in a series of field trials explained later.

This chapter is arranged in three sections 3a, 3b and 3c.

Section 3a describes the total MH method of analysis.

Section 3b describes the free MH method of analysis by HPLC.

Section 3c describes the use of the above methods on field-treated potatoes and discusses the results.

3a.1 Total MH : Materials and Methods

The chemical process is described by Wood (1953) as hydrolysis of MH to hydrazine by nascent hydrogen from the reaction of zinc granules in boiling concentrated sodium hydroxide (see equation 3.4 and 3.5, Figure 3a.1). The hydrazine is distilled off and reacts with p-dimethylaminobenzaldehyde in acidic solution to form a yellow coloured chromophore which may be measured colorimetrically (equation 3.6 and 3.7, Figure 3a.1).

3a.1.1 Reagents

- (a) Quick dissolving sodium hydroxide pearls (Formachem Ltd.)
- (b) Zinc granules (May and Baker Ltd. Pronalys AR)
- (c) Ferrous chloride (Hopkin and Williams Ltd.GPR)
- (d) Anti-foaming agent (Bulk glycerol)
- (e) Colour reagent, 2% p-dimethylaminobenzaldehyde (BDH Ltd AR), in 2N sulphuric acid (May and Baker Ltd. Pronalys AR)

3a.1.2 Glassware

Refer to figure 3a.2

- (a) 250 ml RB. three necked reaction flask heated by two bunsens
- (b) 200⁰C quickfit thermometer
- (c) 200 ml quickfit water reservoir
- (d) 6" quickfit condenser with Teflon distillate tube

- (e) Quickfit bubble pipe
- (f) Quickfit T-junction connector for condenser and bubble pipe
- (g) 50 ml measuring cylinder packed in ice

3a.2 Total MH: Procedure

About 2 g of potato sample is added to the 250 ml RB. reaction flask along with 50 g sodium hydroxide granules, 40 ml water, and 10 drops of anti-foaming agent (glycerol). This is then heated to about 160°C then cooled this boils off natural volatile interferences. The mixture is left to cool for about 10 minutes, but may be left for longer periods.

5 g of zinc granules and 0.5 g of ferrous chloride (to stop an unknown natural interference) are added and the glass system is constructed with nitrogen bubbling at about 3 bubbles/second in the 50 ml measuring cylinder containing 4 ml colour reagent.

The mixture is then heated and maintained at temperatures of between 168°C and 173°C with slow additions of deoxygenated water (boiled) until about 40 ml is collected in the 50 ml measuring cylinder. The actual volume is noted .

The resultant solutions are stored in brown tinted bottles and read within three days. Standard solutions are usually reduced in the middle of batch runs because all the coloured solutions decay at room temperature. The decay is linear over a period of two weeks (refer to 3a.3.3).

The solutions were read at 430, 460 and 490 nm on a Pye Unicam SP 1800 UV spectrophotometer and the concentrations calculated from net absorbances.

Calculations of net absorbance 'Abnet'.

$$\text{Abnet} = \text{Ab}_{460} - \frac{(\text{Ab}_{430} + \text{Ab}_{490})}{2} * \frac{\text{Vol.}}{40} \quad \text{equation 3.1}$$

50 µg of MH is used as a standard

$$\mu\text{g of MH in samples} = 50 \mu\text{g} * \frac{\text{Abnet sample}}{\text{Abnet standard}} \quad \text{equation 3.2}$$

The quantity of MH is divided by the weight of potato sample to give the ppm of the fresh weight.

For the standards, a quantity of MH is added to the reaction vessel, usually 40 µg or 50 µg, and treated in the same way as above.

The standard solutions were dilutions of stock solutions. Stock solutions were made up fresh. 0.500g of MH was dissolved in 80 ml of boiling water and after cooling the solution was made up to 100 ml.

Blank controls were measured with 4 ml of colour reagent diluted to 40 ml. Potato material known to have no MH was the untreated control.

Residue levels and untreated controls were corrected for the blank controls.

All standards and samples were duplicated.

The described method was validated below and was found to be linear up to 200 µg of MH with a recovery rate consistently over 90%. The minimum quantification was about 1 µg of MH this corresponded to a residue level of 0.5 ppm in a potato sample. However, lower levels could be detected using larger samples, but there may be a problem of excess frothing.

3a.3 Validation of method for total MH

3a.3.1 Standard curves

The standard curves were calculated from reducing and distilling a number of standard quantities and calculating the Net Absorbance (Abnet). The mean of replicate reductions and distillations are used to draw a standard curve, see Table 3a.1 and 3a.2

3a.3.2 Typical recovery value

A 2g sample of untreated potato mash had 2 ml of 10 ppm MH added. The spiked sample was analysed as a typical potato sample outlined in section 3a.2.

Typical results of a spiked sample are tabulated in Table 3a.3.

Continuous checks throughout analysis confirmed a recovery of over 90% in all analysis using this method.

3a.3.3 Decay of coloured solutions

Four sample solutions of a treated potato were kept in brown tinted bottles for 14 days. The absorbances were read every second day and the net absorbances calculated. Graph 3a.3 shows that the developed colour decays, and that there is a loss of approximately 0.005 net absorbance units per day.

3a.3.4 Inter lab correlation

The 1985-86 PMB field trials, described in section 2.14, were analysed by GC Laboratories in Cheltenham and by the Agricultural Chemistry section at Glasgow University. The results in this section describe the correlation for total MH analysis between Glasgow University Laboratories total MH analysis and GC Laboratories total MH analysis.

Method of sampling and analysis by GC Laboratories Limited

The sampling and analytical method are described below.

On receipt each batch of potatoes was split in half and each designated 'x' and 'y'. For each of the half samples the analysis was duplicated.

The method used by GC Laboratories was described as the colorimetric method of the AOAC (1980 edition, 29.129-29.135, pp. 490-491), and a modified method for the calculation (GC Laboratories own report J 4004/4067 of 11.11.82). This involved the modified calculations.

$$\text{Abnet} = (\text{Ab}_{460} - \text{Ab}_{490}) * \frac{\text{Vol.}}{40} \quad \text{equation 3.3}$$

Their results are tabulated in Table 3a.4.

Glasgow University sampling and analysis method

The samples were received from the PMB at Sutton Bridge, in late November 1985 and stored at 8°C +/- 1 °C until analysis in December 1985.

Twelve potatoes were selected from each batch (batches were about 6 kg). Every twelve potato samples were selectively chosen to represent the same size distribution as the 6 kg batch.

The potatoes were washed, then cut apical to basal twice to give quarters, then cut in halves to give eighths. Opposite eighths from each potato were collected together, weighed, then blended with half the potato sample weight of water.

A 2 g sample was reduced, distilled, and the concentration calculated from the net absorbance.

The results are tabulated in Table 3a.5.

Each result is the average of two replicate 2 g sub-samples from the same blended potato mixture. Except for site 4, it is the average of three replicates.

Each result is corrected for the blank control, no untreated tubers were available for analysis.

Table 3a.6 shows the comparison of results. The Glasgow University results have been recalculated using G C Laboratories amended calculation.

Because of the different samples, methods of sampling batches, methods of reduction and distillation, calculation of results and the variance of any analysis carried out by any two individuals, the results are bound to be different.

However, the results are acceptable replicates of the same batch from two different laboratory analyses.

3a.4 Total MH : Results and discussion

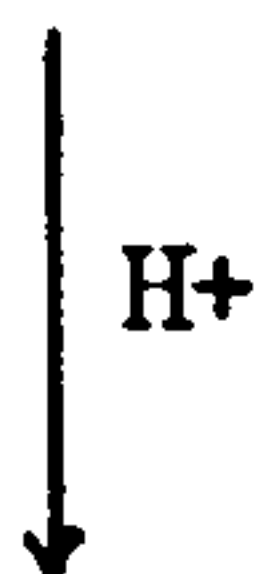
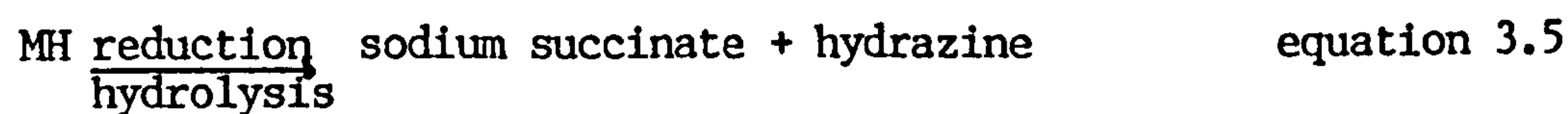
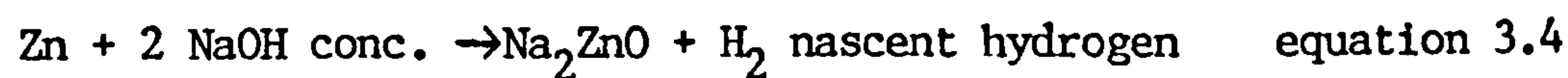
This method is a reliable method for the analysis of potatoes containing MH with a concentration of about 140 ppm down to about 1 ppm. The original method was reliable from about 150 ppm down to about 1 ppm (Lane, 1963).

The main practical problem of this set-up is that the frothing can overflow into the condensing tube. This caustic spillage flows into the measuring cylinder containing the colour reagent. The caustic solution precipitates the chromophore. In most cases concentrated sulphuric acid is added by pipette to dissolve the chromophore and the coloured solution filtered through Watman No. 1 filter paper and the uv absorbance read. In extreme cases the whole process is repeated.

The effects of the frothing can be controlled by regulating the nitrogen flow rate.

The process was tedious but did appear to be effective with replicates being similar. It also correlated very well with the GC Laboratory analysis.

Figure 3a.1 Total MH residue method, equations

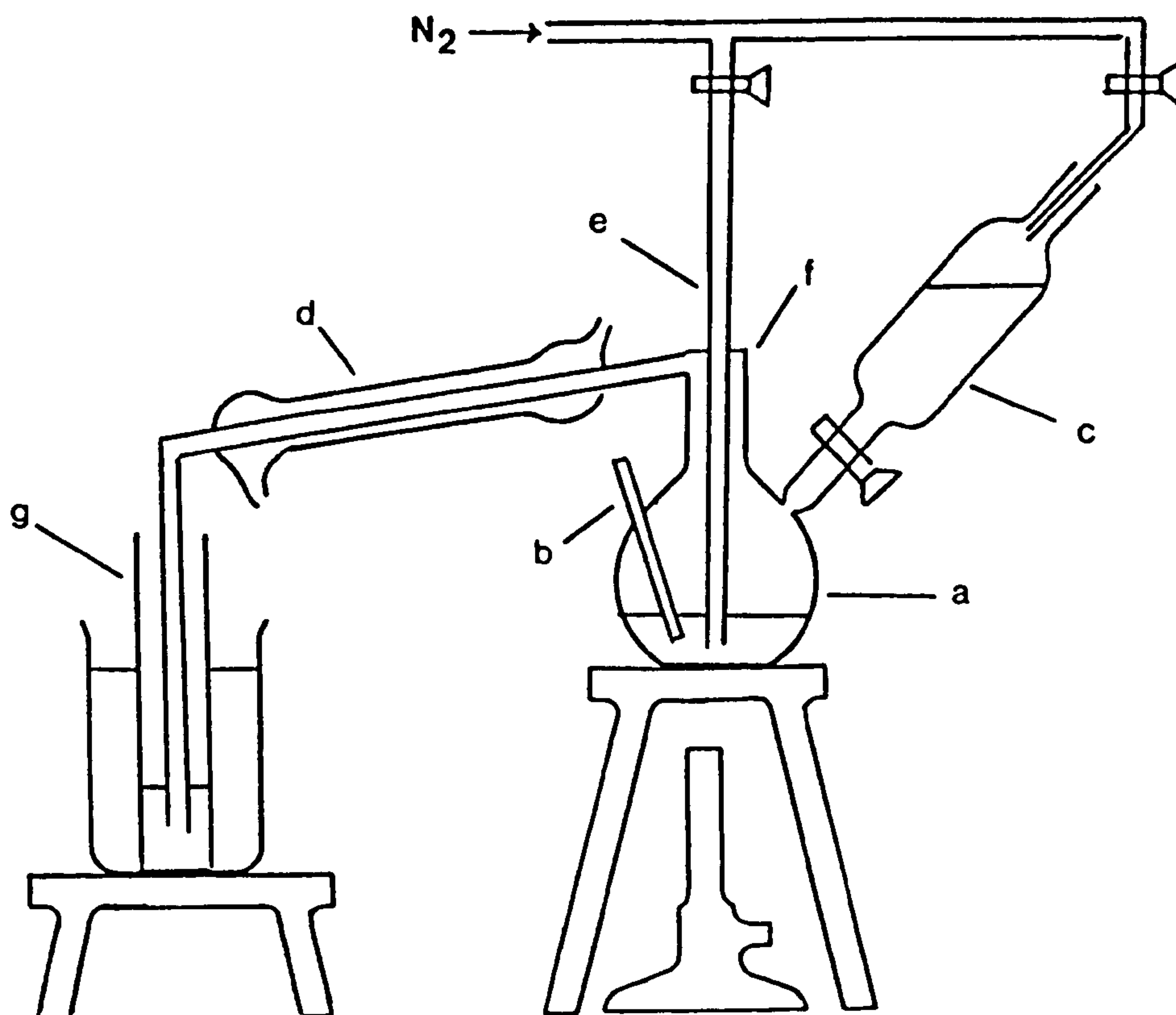


Quinone structure absorbance max. 460 nm

$$\text{Abnet} = \text{Ab460} - \frac{(\text{Ab430} + \text{Ab490})}{2} \quad \text{equation 3.7}$$

The Abnet is found to be linear with the quantity of MH up to 200 ug (Table 3a.2).

Figure 3a.2



- a) 250ml R.B. three necked reaction flask heated by two bunsens.
- b) 200°C Quickfit thermometer.
- c) 200ml Quickfit water reservoir, deoxygenated water with N_2 atmosphere.
- d) 6" Quickfit condenser with teflon distillate tube.
- e) Quickfit bubble pipe regulated at three bubbles per second.
- f) Quickfit T-junction connector for condenser and bubble pipe.
- g) 50ml graduated measuring cylinder, containing the colour reagent, packed in ice.

TABLE 3a.1

Standard curve up to 50 ug

Total MH method

Quantity (ug)	Vol. (ml)	Absorbance			Abnet	Mean
		430	460	490		
0	40.5	0.010	0.000	0.000	-0.005	-0.005
0	40.5	0.010	0.000	0.000	-0.005	
10	38.0	0.116	0.115	0.072	0.020	0.017
10	38.0	0.050	0.050	0.020	0.014	
20	33.5	0.115	0.125	0.030	0.044	0.044
20	36.0	0.105	0.110	0.020	0.043	
30	36.0	0.180	0.200	0.066	0.069	0.066
30	38.0	0.120	0.140	0.030	0.062	
40	39.5	0.130	0.155	0.035	0.072	0.086
40	40.0	0.170	0.205	0.040	0.100	
50	39.0	0.208	0.250	0.070	0.108	0.107
50	40.0	0.180	0.220	0.050	0.105	

See graph 3a.1

Refer to section 3a.3.1

TABLE 3a.2

Standard curve up to 200 ug

Total MH method

Quantity (ug)	Vol. (ml)	Absorbance			Abnet	Mean
		430	460	490		
0	40.0	0.020	0.010	0.000	0.000	0.000
0	40.0	0.020	0.010	0.000	0.000	
50	41.0	0.330	0.420	0.060	0.230	0.24
50	38.0	0.340	0.460	0.060	0.250	
100	39.0	0.680	0.920	0.120	0.510	0.49
100	39.0	0.630	0.860	0.120	0.470	
150	38.0	1.000	1.350	0.180	0.720	0.78
150	39.0	1.120	1.520	0.210	0.830	
200	37.0	1.280	1.730	0.240	0.930	1.03
200	39.0	1.500	2.030	0.260	1.120	

See graph 3a.2

Refer to section 3a.3.1

TABLE 3a.3

Results of a spike sample

Total MH method						
Absorbance						
	Vol.	430	460	490	Abnet	Mean
20 µg std.	33.5	0.125	0.145	0.035	0.054	0.058
	39.5	0.105	0.125	0.020	0.062	
Control	40.5	0.010	0.000	0.000	-0.005	-0.005
	40.0	0.010	0.000	0.000	-0.005	
U.S.	41.0	0.020	0.010	0.010	-0.005	-0.005
	38.5	0.020	0.010	0.010	-0.005	
#	40.0	0.110	0.130	0.035	0.058	0.054
	39.0	0.095	0.115	0.035	0.049	

U.S. = Untreated sample
= 2 g potato spike containing 20 µg MH

Calculated percentage recovery

$$\frac{(0.054 + 0.010)}{(0.058 + 0.005)} * 100 = 101\%$$

Refer to section 3a.3.2

TABLE 3a.4

GC Laboratories Analysis (Total MH)
1985-86 PMB field trials

Results			Total MH mean ppm
Site Ref.		ppm	
4	x	14.2 (16.4 11.9)	14.1
	y	14.0 (14.0 13.9)	
3	x	5.1 (6.3 3.8)	3.9
	y	2.7 (3.0 2.4)	
2b	x	9.7 (11.4 8.0)	14.3
	y	18.9 (15.8 22.0)	
2a	x	4.6 (5.1 4.0)	6.5
	y	8.4 (6.4 10.4)	
1	x	15.1 (13.1 17.0)	16.0
	y	16.9 (16.7 17.0)	

x and y designate the two half samples
Duplicate analysis are in the brackets

The above table is used to correlate GC Laboratories analysis with the
method described in section 3a.1

Refer to section 3a.3.4

TABLE 3a.5

Glasgow University analysis (Total MH)

1985 PMB field trials

PMB ref.	Total	MH	ppm*
4	21.7	(22.8	20.5)
3	5.7	(7.5	3.9)
2b	17.0	(17.5	16.5)
2a	7.5	(7.5	7.5)
1**	23.1	(18.8	27.3 23.2)

* Replicate analysis are shown in brackets

** Site one initial analysis gave poor duplicate analysis results, therefore an extra analysis was carried out

Refer to section 3a.3.4

TABLE 3a.6

Comparison of results

1985-86 PMB field trials

Inter-lab results

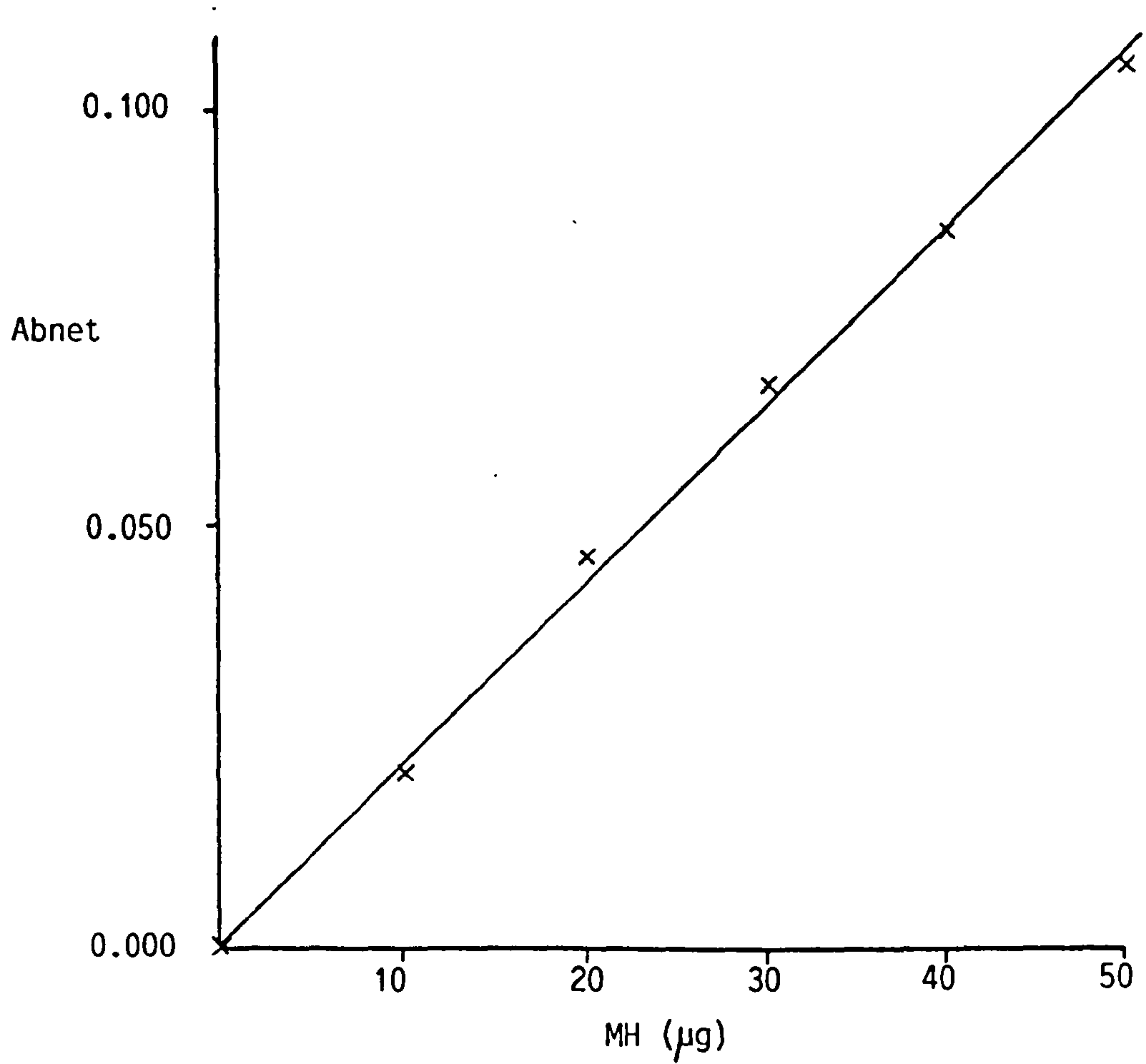
PMB ref.	GC Lab. ppm	GU Lab.* ppm	recalc**
4	14.1	21.7	18.7
3	3.9	5.7	3.5
2b	14.3	17.0	13.1
2a	6.5	7.5	5.6
1	16.0	23.1	20.9

* GU Lab. refers to Glasgow University analytical results

** Recalculated using GC Laboratories amended calculation (equation 3.3)

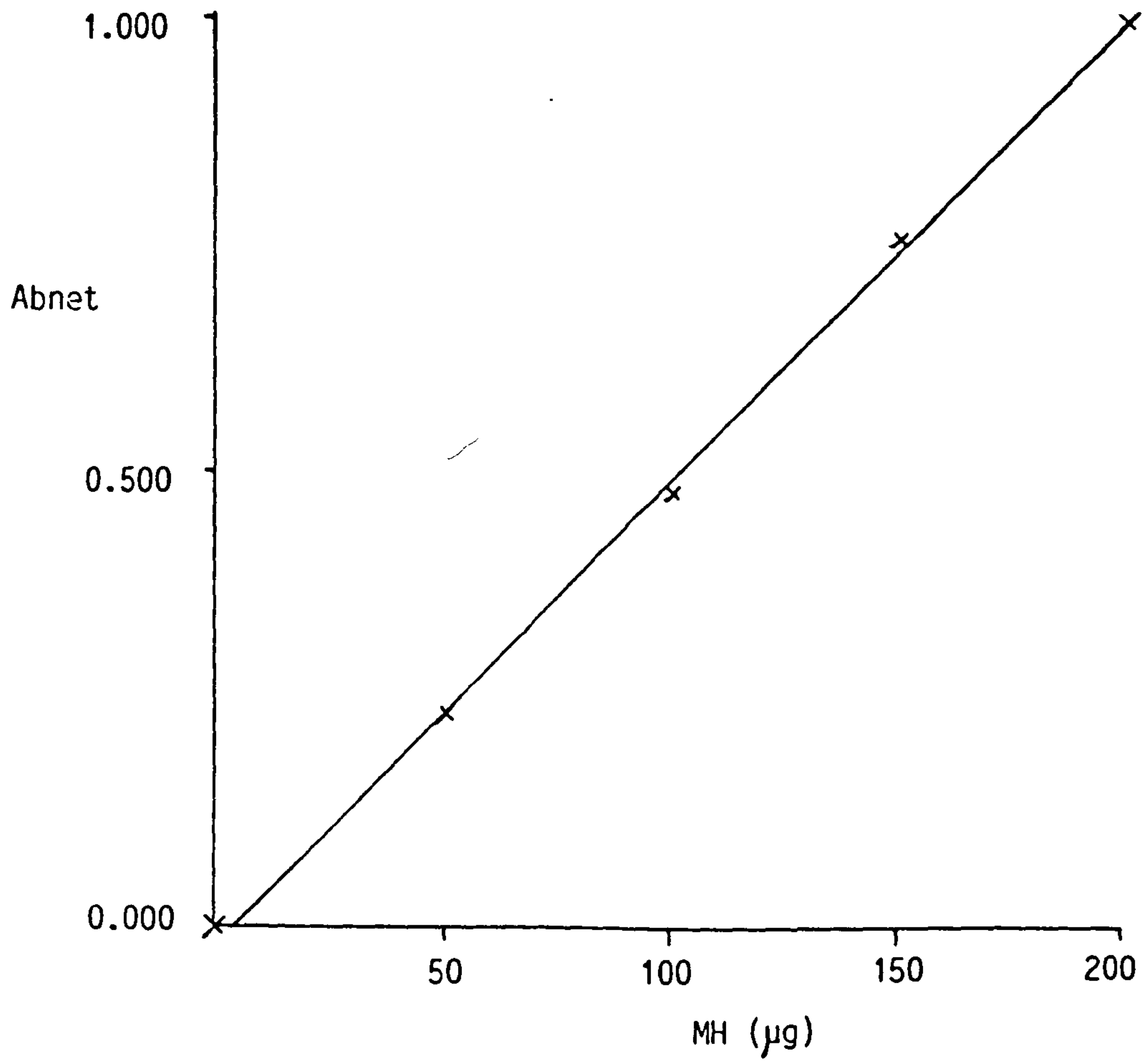
Refer to section 3a.3.4

Graph 3a.1, Total MH standard calibration curve
zero to 50 μ g MH



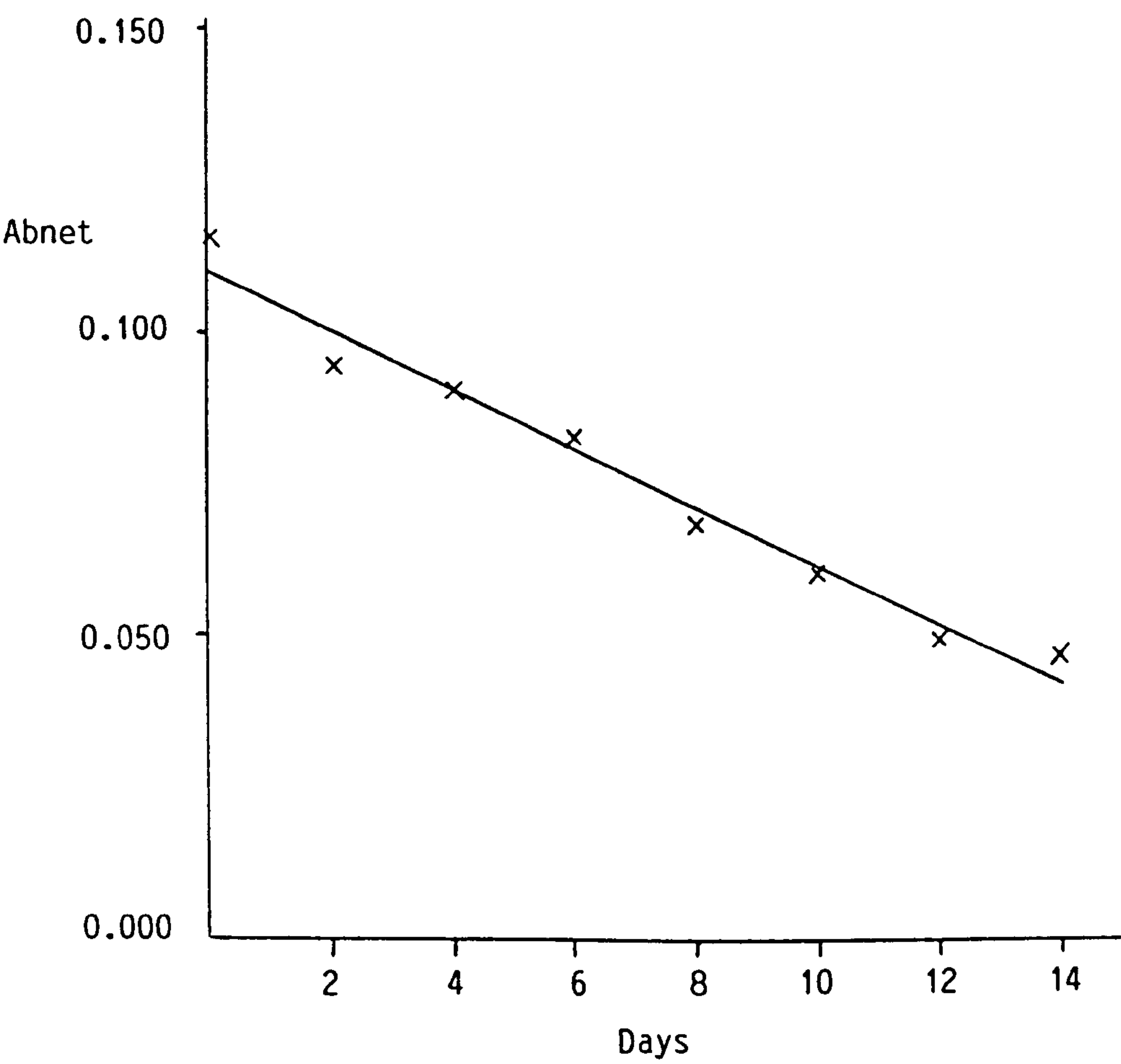
Results taken from Table 3a.1

Graph 3a.2, Total MH standard calibration curve
zero to 200 μ g MH



Results taken from Table 3a.2

Graph 3a.3, Deterioration of Abnet with respect to time.



Refer to section 3a.3.3 for details

3b.1 HPLC residue analysis : materials and Method

3b.1.1 Extraction :

50 g of minced potato material was homogenised with 200 ml of bulk methanol for about two minutes. The mix was then filtered under suction on a Buchner apparatus with Whatman No.1 filter paper. The insoluble material was washed twice with the 100 ml methanol washings of the homogeniser, then made up to 500 ml of methanol.

3b.1.2 Concentration :

A 50 ml aliquot was transferred to a 100 ml round bottom flask, then reduced to about 3 ml on a rotary evaporator (Buchi Rotavapor 'R') water bath during evaporation. The 3 ml fraction was transferred to a 5 ml volumetric flask. Two washings of about 1 ml distilled water washed the round bottomed flask and was added to the volumetric flask.

3b.1.3 Clean-up :

A clean-up column was prepared by a method prescribed by Newsome (1974).

2 ml of the concentrated extract was eluted through a cation exchange column (120 mm x 8 mm i.d.). The cation exchange resin was 'Amberlite 120' or 'Dowex 50w x 200' as hydrogen form activated with 1 M HCl and washed with deionised water. After about 1.8 ml was eluted off the column (void volume was about 2ml), 10 ml was collected in a 10 ml volumetric flask. After filtration of the samples through Millipore filters, (0.45 μ m pores size), the samples were ready for HPLC analysis or stored in a fridge until analysis.

3b.1.4 HPLC Analysis :

The HPLC systems used were 1. : a Perkin Elmer package with; a series 400 pump, ISS-100 automatic injector, LC-90 UV detector set at 313 nm, and a LCI-100 integrator, 2 : a Waters Associates package with a series 6000 pump, U6k manual injector, model 450 variable wave-length detector, and Servoscribe chart recorder.

The HPLC anion exchange columns used were : a Ionosphere tma packed column (250 mm x 4.6 mm), and a column self-packed on a Shandon packer, with Partisil SAX 10 μ m (250 mm x 4.6 mm).

The mobile phase used was 0.1 M acetic acid. The pH of 4.8 was set with concentrated NaOH. The buffer was then filtered under suction on Millipore filter 0.45 μ m pore size. The solvent was eluted at 1.0 ml/min, with continuous helium purge.

3b.2 Validation of the method for HPLC MH

3b.2.1 Elution profile of the clean-up column :

The clean-up column was assessed by applying 2 ml of 100 ppm potassium salt of MH.

A series of 3 ml fractions were collected diluted to 10 ml. The MH was measured on a SP 1800 Pye Unicam UV spectrophotometer at 303 nm. The potassium was measured on a flame photometer. See graph 3b.1 for the profile.

3b.2.2 Linear response of detector :

Standards of MH were made up by dilution of the same stock solution. The stock solution was made up by dissolving 0.500 g of MH in 80 ml of boiling distilled water and made up to 100 ml after cooling. The standards were made up fresh when required.

Each point on the graph is a replicate of two injections.

The area is expressed in 'area units', an empirical unit used by the integrator system.

The height was measured manually.

See graph 3b.2 for the response curve.

3b.2.3 Partisil SAX compared with the Ionosphere tma

The two figures 3b.1, and 2 are injections of a spiked 10 ppm potato extract and a normal extract separated on two different columns a Partisil SAX and an Ionosphere tma.

3b.3 HPLC residue analysis : results and discussion

The cation exchange clean-up column was assessed with : different column sizes, (height and diameter), different sample volumes, and different packings. The column size chosen was 120 mm x 8mm, stoppered with a plug of glass wool, packed with either Amberlite 120 or Dowex 50 w x 200. A large bead size was preferred to avoid blockage, although the flow usually did become unacceptable after 10 to 12 samples had been applied. The column was washed with 1 M HCl after every third sample to keep the column active, and washed with deionised water.

Prior to the final HPLC analysis method being chosen and detailed in previous sections other HPLC conditions were investigated.

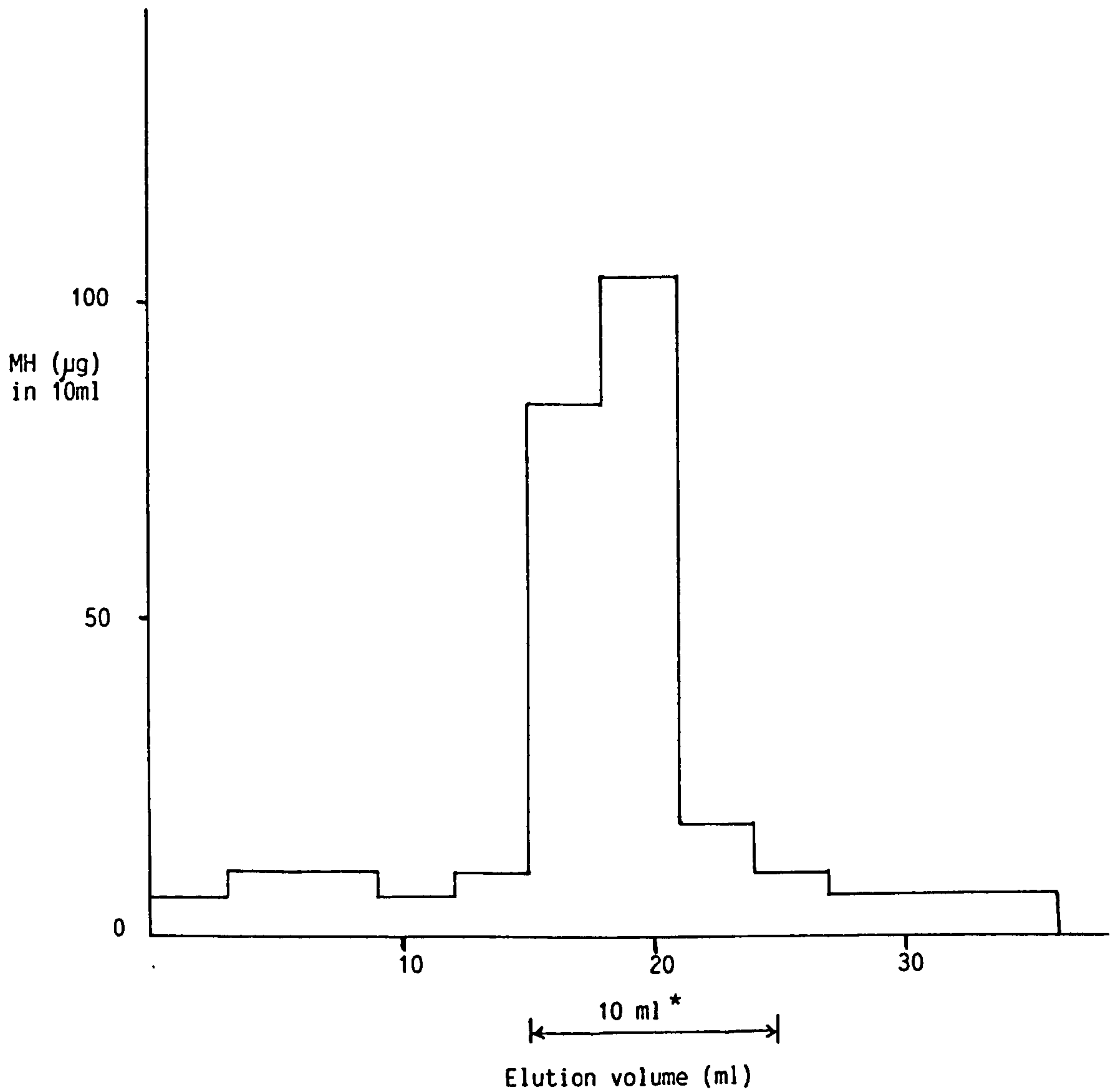
Various concentrations of disodium hydrogen phosphate at various pH conditions and ammonium acetate also of different concentrations and at various pH conditions were used as mobile phases for anion exchange columns (25 cm x 4.5 mm i.d.) packed with Vydac anion material, Ionosphere tma, or Partisil SAX. The final HPLC analysis method was similar to the method used by Newsome (1980a). It was found to give the best retention time for rapid analysis, also demonstrating the best peak shape and insignificant coextracted interference. The Partisil SAX column was packed within Glasgow University on a Shandon HPLC column packer. Thereafter any deterioration of performance was rectified by repacking. The Vydac and Ionosphere were prepacked. The HPLC packings routinely used were Ionosphere tma and Partisil SAX, both columns sufficiently retained MH with 1 ml/min flow of 0.1 M acetic acid pH 4.8. However, the Ionosphere required a longer elution time of 40 min, and had a slight interference with similar retention to MH. The Partisil could be repacked if, and when, the efficiency of the column decreased. The Ionosphere column was used for most of the development work, the Partisil column was used for most of the routine analysis. The integrator had a linear detection with area and height from 100ng to 1000 ng with the same volume injection, 100 μ l.

The system could detect down to about 1 ng, however, the limit of extracted potatoes was about 20 ng this corresponded to about 1 ppm of fresh weight.

The recoveries of MH from spiked potatoes were consistently over 80%.

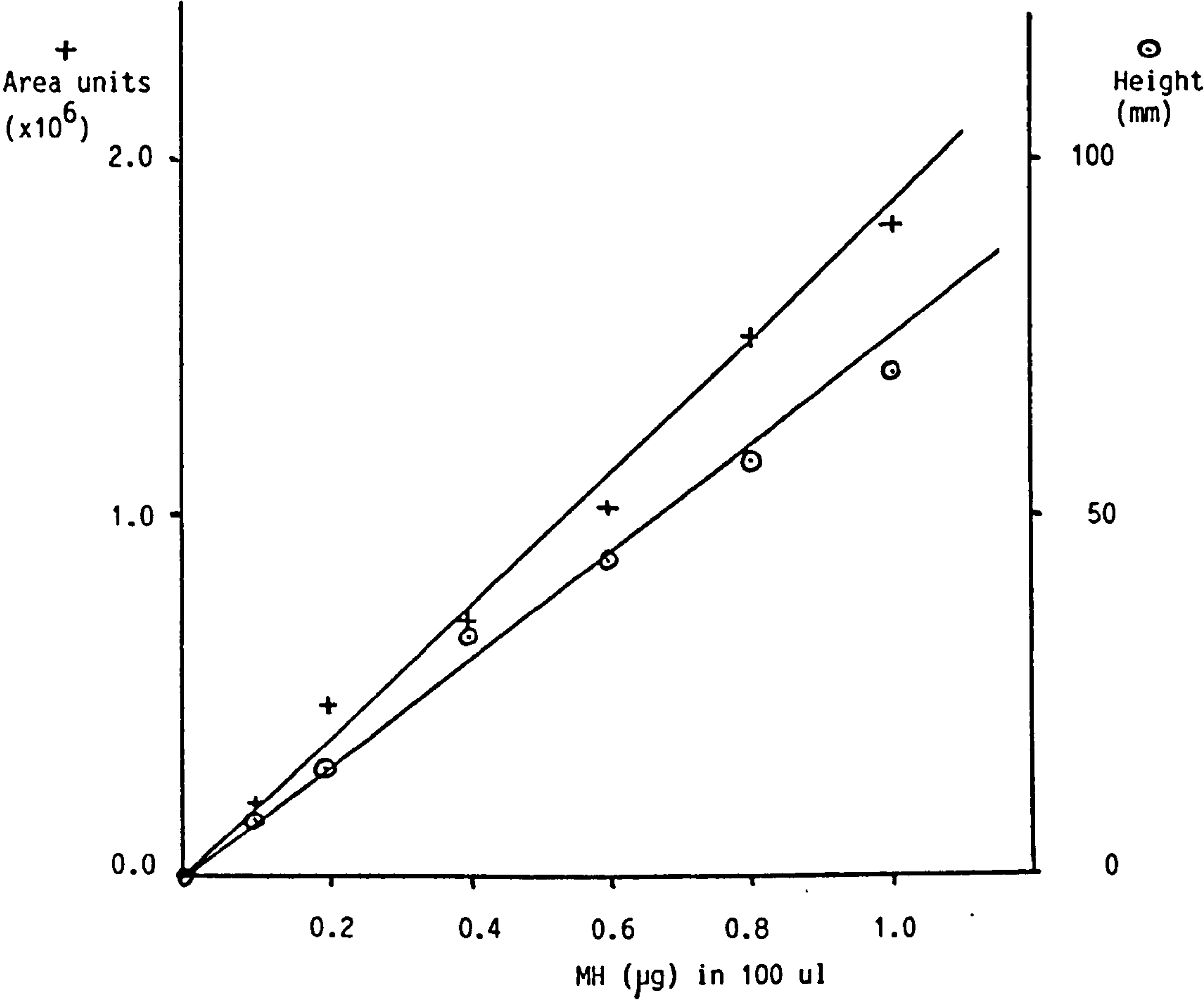
The HPLC method was specific for MH and was well suited for rapid routine analysis. Efficient analysis however also depended on efficient recovery of the concentrate from the round bottomed flask after rotary evaporation, on careful elution through the cation exchange clean-up column, and optimising the integrator counting technique by choice of counting methods.

Graph 3b.1, Typical elution profile of the cation exchange column clean-up used in the HPLC analysis of MH



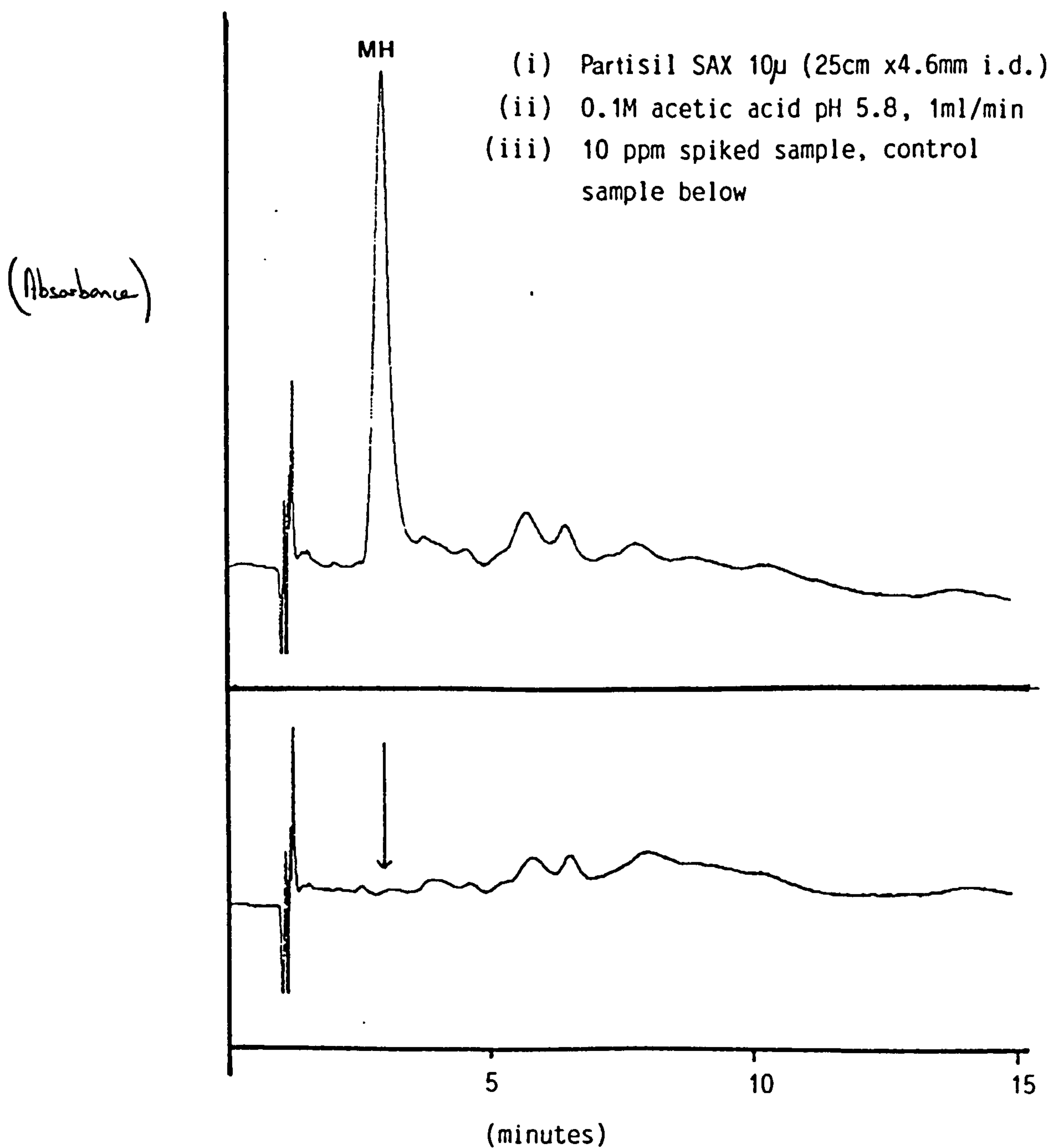
* The three samples between 15ml and 24ml have 90% of the MH applied
The mean quantity of potassium was 1.6ug per 10ml flask
Refer to section 3b.2.1 for details

Graph 3b.2, The linear response of the HPLC U V detector



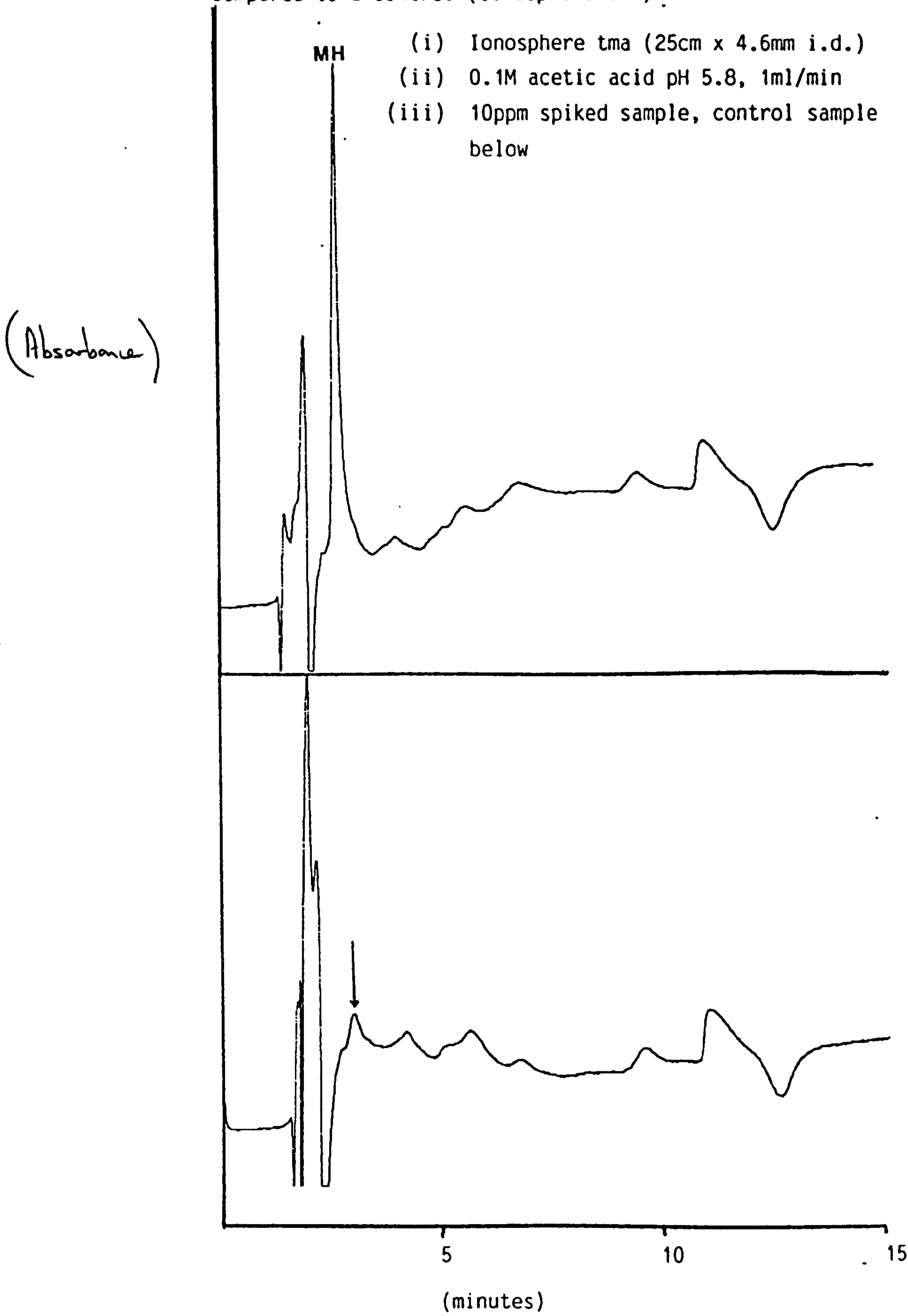
Refer to section 3b.2.2 for details

Figure 3b.1 A comparison of spiked potato extract compared to a control (Partisil SAX 10 μ).



Refer to section 3b.2.3 for details

Figure 3b.2 A comparison of spiked potato extract compared to a control (Ionosphere tma).



Refer to section 3b.2.3 for details

3c.1 Fractionation of MH in field treated potato crop

In this section the two methods were used to measure the residues, the total MH and the methanol soluble MH fraction. Another series of residues were also measured. For the HPLC method the MH was extracted with methanol then concentrated. This concentrate was reduced and distilled by the Lane (1963) method outlined in section 3a.1 to measure the total MH within the methanol fraction.

Firstly it was necessary to assess the fractionation method.

3c.1.1 Assessment of fractionation :

Three batches of potatoes were washed and dried, each batch containing six potatoes.

For each batch the potatoes were halved. One half of each potato was homogenised together with half its weight of water.

About 2 g of the potato mix was reduced and distilled by the method outlined in section 3a.1. This gave the total value.

The other halves of potatoes were minced. About 50 g of the minced potato was extracted and concentrated by the method outlined in sections 3b.1.1 and 3b.1.2. The 5 ml of concentrate represented about 5 g of fresh weight potato.

The residue in the filter paper, after the methanol extraction, was dried overnight in a fume cupboard and weighed. This material was ground by mortar and pestle. 1 g was reduced and distilled in a similar fashion as outlined in section 3a.2.

The results are tabulated in Table 3c.1.

This fractionation method does give close to 100% recovery of the MH. The total MH, the HPLC MH, and the total MH in the methanol extract, can split the MH into different pools after analysis.

3c.2 Fractionation of MH into pools in a field treated potato crop : materials, methods and results

3c.2.1 Harvesting and storage of the field treated potato crop :

Eight plots had been planted, each with five drills, four metres long. See Table 2.1, for full description of the growth of the plots.

After spraying the plots with the field concentration of Fazor, two drills per plot were lifted after one week, one drill per plot lifted after two weeks, one drill per plot lifted after three weeks, and the last drill of each plot lifted after four weeks.

The potatoes from each four metre drill were kept at room temperature in 10 kg nylon net bags for four to five days, then stored in 10 kg cardboard boxes at a constant temperature of 8.0 ± 1 °C.

3c2.2 Total MH analysis :

Twelve potatoes per drill were chosen from each plot. These potatoes were washed and dried, cut from the apical to the basal end twice to give quarters, then cut in half to provide eighths. Two opposite eighths from each potato were collected together and homogenised for three minutes (Alto mix MSE Co. Ltd.) with half their weight of water to give a soup consistency.

Two sub-samples of 2 g were each analysed using the adapted reduction distillation method, outlined in section 3.a.2 to give the total MH content, (see Table 3c.2).

The rest of the potato eighths were immediately frozen and kept for later analysis using HPLC.

3c.2.3 HPLC analysis of frozen potato samples

About 300 g of the frozen potato chunks were allowed to slightly defrost in a warm water bath then crushed and minced in a mincer (Bauknecht mincer). Two 50 g replicate samples of the same minced material were extracted in methanol, concentrated, cleaned, and analysed by the method outlined in section 3b.1, see Table 3c.3.

3c.2.4 Total MH in the methanol fraction :

About 200 g of the frozen potato chunks were slightly defrosted in a hot water bath then crushed and minced in a mincer.

Two 50 g replicate samples of the same minced material were extracted and concentrated by the method outlined in sections 3b.1.1 and 3b.1.2.

About 2 g from each flask was weighed into a reduction vessel, reduced and distilled by the method described in section 3a.2, without the pre-cook stage, or any additions of anti-foaming agent or ferrous chloride, see Table 3c.4.

3c.3 Discussion of fractionation results

The definitions of the results and analysis are explained below.

Total MH (Table 3c.2) is the total MH measured by the reduction, distillation and colorimetric method of Lane (1963) outlined in section 3a.2.

Free MH (Table 3c.3) is the MH measured by the HPLC method adapted from the method of Newsome (1980a) outlined in section 3b.1.

Free MH (Table 3c.3) is the MH measured by the HPLC method adapted from the method of Newsome (1980a) outlined in section 3b.1.

The total MH was found to quickly accumulate (within one week after application) and remained at a plateau up to four weeks after application (refer to graph 3c.1). Free MH had the greatest concentration after one week and thereafter this tended to decrease in concentration and as a percentage of the total MH with time (refer to graph 3c.1).

Total methanol extractable is the total MH in the methanol extract analysed and calculated by an adaption of the method of Lane (1963), (refer to graph 3c.1). Table 3c.4 tabulates the results, Table 3c.5 demonstrates that this fraction increases as a percentage of total MH.

Methanol extracted MH (Table 3c.6) not free MH is a measure of possible conjugates or metabolites, extracted by methanol, that still have the hydrazyl group that would give a positive reaction in the total MH residue method. The metabolites that have been detected and could give a positive response to the detection method are MH and beta-D-glucoside conjugates and hydrazine molecules. Towers et al. (1958) and Frear and Swanson (1978) have detected MH and beta-D-glucoside conjugates in wheat and tobacco respectively. Biswas et al. (1967) suggested hydrazine as a degradation product of MH in tea plants.

The percentage of methanol extracted MH not free MH was found to increase compared to total MH.

Non-methanol extracted MH (Table 3c.7) is a measure of possible conjugates, metabolites or methanol insoluble fractions of MH. Nooden (1970) recognised this fraction in corn and pea seedlings soaked with ^{14}C -MH. The methanol soluble ^{14}C -MH was associated with large particles which was considered to be cell wall fragments. The binding required metabolic energy which suggests a covalent binding and not physisorption. Frear and Swanson (1978) also recognised a methanol insoluble fraction within tobacco plants treated with ^{14}C -MH. Free unchanged ^{14}C -MH was released after acid or base hydrolysis.

The heading 'week n' is the week of harvesting after Fazor application.

Each method detects and measures a different fraction. The reduction and distillation method measures the total MH that is bound chemically or bound physically to the methanol insoluble fraction and the soluble MH in the methanol extract, free and conjugated. The HPLC method measures the free unbound MH extracted by methanol.

They have similar recovery factors and limits of detection in routine use.

The total methanol extracted fraction increases. This is due to the increase in the methanol extracted fraction which is not free MH. This fraction concentration increases with respect to the total MH.

It should also be noted that the total residues may be slightly higher in absolute amounts because most of the untreated residues are less than zero.

Conjugates of MH have been found in other plant systems. They had been extracted by alcohols. Towers et al. (1958) identified a beta-glycoside of MH produced by young wheat leaves. Nooden (1970) also found small quantities of a metabolite compared to the overall amount in the corn roots. These were recognised to correspond to those described by Towers et al. (1958). The major methanol soluble metabolite in tobacco leaves was identified as the beta-D-glucoside by Frear and Swanson (1978).

However Newsome (1980b) using the specific detection method of HPLC, unlike the others described above who used radiolabelled MH could not detect any increase in MH after MH treated potato extract had been treated with beta-D-glucosidase.

Biswas et al. (1967) did not find any glycoside in tea leaves after treatment with radiolabelled MH but did find degradative products of ring cleavage.

There are other possibilities, Nooden (1970) suggested that the polar metabolite may be small molecular weight fractions desorbed from the cell wall fraction with MH attached.

Hughes and Spragg (1958) found MH to bind with sulphydryl groups present in pea protein, including some enzymes. Some of the binding was irreversible.

In Chapter 5 and 6 the metabolite of MH is investigated using radiolabelled studies, and enzymic cleavage and conjugation studies.

Table 3c.1

Trial fractionation results (ppm MH)

	Batch A	Batch B	Batch C
Total MH	8.5 (8.6 8.4)	9.8 (9.8 9.8)	24.0 (25.3 22.7)
MeOH ext. MH	8.3 (9.0 7.5)	8.8 (8.2 9.4)	20.0 (19.6 20.4)
Non MeOH ext. see note (ii)	0.1 (0.5 0.5)	1.0 (5.4 4.8)	1.9 (10.0 9.8)

- (i) The replicates are in brackets.
- (ii) The Non MeOH ext results are average results divided by 5, because the methanol extracted residue is the solid material from the tuber that is assumed 20% of the fresh weight. Therefore, the material is five times concentrated.

Refer to section 3c.1.1.

Table 3c.2

Total MH (ppm)

1985-86 Glasgow University field trials

Week after Fazor application

Plot	Week 1	Week 2	Week 3	Week 4
1*	12.5 (11.7 13.3)	18.3 (22.8 13.7)	12.5 (13.3 11.8)	15.7 (15.8 15.7)
2	-1.1 (-1.1 -1.1)	-0.5 (0.0 -1.0)	-0.2 (-0.4 0.0)	-0.8 (-0.5 -1.0)
3*	21.9 (24.4 19.4)	21.5 (22.8 20.2)	17.7 (18.2 17.2)	19.5 (19.6 19.6)
4	-0.8 (-0.5 -1.1)	-0.5 (-0.5 -0.5)	-0.6 (-0.8 -0.4)	0.0 (0.0 0.0)
5	-1.3 (-1.2 -1.4)	-0.5 (-0.5 -0.5)	-0.9 (-0.9 -0.9)	n.a.
6	-0.3 (0.0 -0.6)	-1.0 (-1.0 -1.0)	-0.7 (-0.9 -0.4)	n.a.
7*	19.6 (19.7 19.5)	13.5 (13.5 13.4)	18.7 (19.4 18.0)	15.7 (15.4 16.1)
8*	24.2 (25.0 23.3)	29.6 (29.7 29.6)	22.4 (20.9 23.8)	25.4 (25.4 25.4)
Mean*	19.5	20.7	17.8	19.1
Mean	-0.9	-0.6	-0.6	-0.4

n.a. = Not available

Replicates are in the brackets below each mean result

* = Treated plots

Refer to section 3c.2.2

Table 3c.3

HPLC MH analysis (ppm)

1985-86 Glasgow University field trials

Week after Fazor application

Plot	Week 1	Week 2	Week 3	Week 4
1	9.2 (8.3 10.0)	6.7 (6.4 7.0)	6.2 (7.1 5.2)	6.1 (6.3 5.8)
3	12.0 (10.3 13.6)	11.0 (9.9 12.0)	5.7 (5.3 6.1)	6.2 (6.6 5.7)
7	8.5 (9.5 7.4)	7.8 (7.8 7.8)	5.0 (4.3 5.7)	7.6 (7.7 7.5)
8	11.0 (10.8 11.1)	11.5 (10.6 12.4)	10.7 (8.4 12.9)	11.0 (12.0 9.9)
Mean	10.1	9.2	6.9	7.7

Replicates are in the brackets below each mean result

Refer to section 3c.2.3.

Table 3c.4

Total methanol extractable MH (ppm)				
1985-86 Glasgow University field trials				
Week after Fazor application				
Plot	Week 1	Week 2	Week 3	Week 4
1	13.3 (13.7 12.9)	11.6 (11.9 11.4)	11.0 (10.9 11.1)	13.2 (13.7 12.6)
3	12.9 (10.3 13.6)	19.7 (20.0 19.5)	8.8 (8.8 8.9)	16.4 (16.4 16.4)
7	7.6 (7.6 7.8)	14.2 (13.0 15.4)	15.9 (14.6 17.1)	12.4 (10.8 14.0)
8	19.6 (19.9 19.9)	21.9 (21.9 21.9)	20.1 (24.0 16.2)	24.3 (23.6 25.1)
Mean	13.5	16.9	14.0	16.6

- (i) Replicates are in the brackets below each mean result
- (ii) The weeks refer to the harvesting period after Fazor application
- (iii) Refer to section 3c.2.4.

Table 3c.5

Analysis of the mean of the residue results
1985-86 Glasgow University field trials

Week after Fazor application	Total MeOH ext. MH (% of total MH)	Free MH (% of total MH)
1	69.2	51.8
2	81.6	44.4
3	78.7	38.8
4	86.9	40.3

Refer to section 3c.3.

Table 3c.6

Methanol extracted MH not free MH

1985-86 Glasgow University field trials

Week after Fazor application	Total MeOH ext. minus free MH (ppm)	% of total MH
1	3.4	17.4
2	7.7	37.2
3	7.1	39.9
4	8.9	46.6

Refer to section 3c.3.

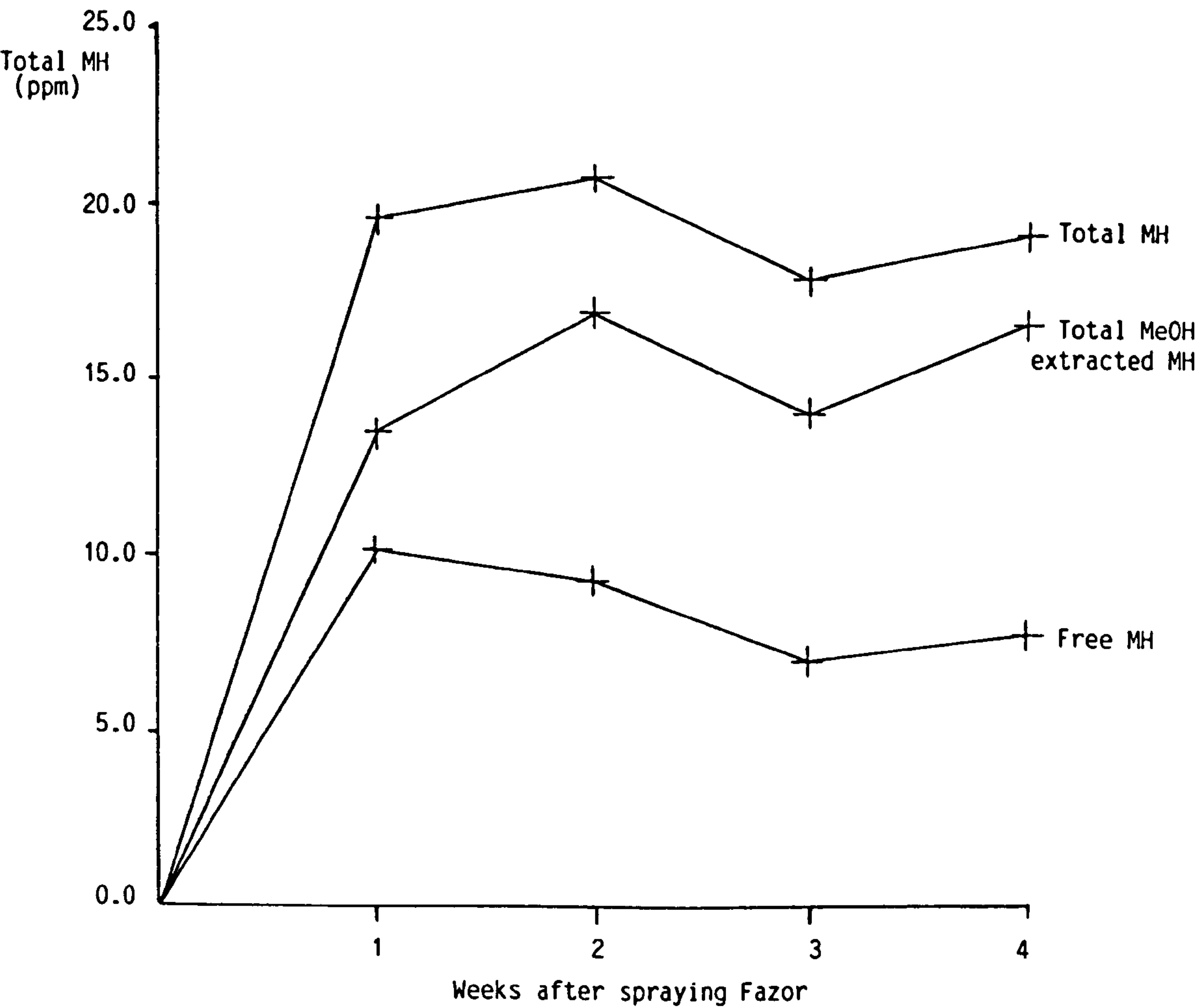
Table 3c.7

Non-methanol extracted MH

Week after Fazor application	Total MH minus total MeOH ext MH (ppm)	% of total MH
1	6.0	30.8
2	3.8	18.4
3	3.8	21.4
4	2.5	13.1

Refer to section 3c.3.

Graph 3c.1, Residue analysis of field treated potato crop



Refer to section 3c.3for the description of the above

THE DISTRIBUTION OF MALEIC HYDRAZIDE WITHIN A POTATO CROP4.1 Introduction

Most of the work on maleic hydrazide has been based on quantity applied, related to effects. Variations in quantity applied, time of application and their effects on water loss, yield and sprouting characteristics have been looked at (Denison [1950], Kennedy and Smith [1951], Paterson et al. [1951], Wittwer and Paterson [1951], Franklin and Thompson [1953], Isenberg [1954], Payne and Fults [1955], Timm et al. [1959], Hansen [1960], Baijal and Kumar [1978], Matlob [1979], Weiss et al. [1980] Davis and Groskopp [1981]).

There has also been some work on relating actual concentrations within the treated tubers to effects. Smith et al. (1959) used the colorimetric method developed by Wood (1953) to investigate the quantities within the tuber related to the performance. Bishop and Schweers (1961) investigated the concentrations of MH within individual tubers and found that it varied greatly. Franklin and Loughheed (1964) found that low concentrations of 4 ppm within tubers stimulated sprouting, and that 6 ppm and higher concentrations had satisfactory inhibition for six months at 50°F. Newsome (1980b) investigated the variation of the methanol soluble free MH fraction during storage.

The effects within the potato store are best related to concentrations of active ingredient within a crop, and not to the quantity applied for the following reasons. The quantity applied in a number of similar field trials may be the same, but other factors may alter the quantity absorbed through the cuticle, translocated, and concentrated in the tuber. For instance, climatic conditions such as rainfall after application, variety, general condition of the plant including water stress, insect and viral attack decreasing the active uptake area. Effects are best correlated with concentration within the tuber. However the question still arises as to which method of analysis should be used.

GC analysis has been used for tobacco residues, (Haeberer et al. [1978], and Frear and Swanson [1978]). HPLC analysis in vegetables including potatoes have been used by Newsome (1980a). All of the above methods extract MH with methanol, and measure the free unbound MH.

In the previous chapters the total residue method, adapted from Wood (1953) and detailed by Lane (1963), was used to investigate the fractionation of pools of MH and the residues in crops. This method was found to be accurate, reasonably sensitive, and reproducible. It had also been used in other residue assessments, was easily accessible in Glasgow University Agricultural Chemistry Laboratories, and is used by Uniroyal Ltd., for routine residue analysis. The method was primarily used in this chapter to assess MH distribution within the potato crop, however, the HPLC method of Newsome (1980a) was also used as an assessment and compared with the total MH method.

This chapter is concerned with the distribution of MH within potatoes and the consequences.

4.2 The concentration of MH in individual tubers

- 4.2.1 It has been reported that sprout control was proportional to the MH applied (Franklin and Thompson [1953]), and Matlob [1979]). Lower levels of MH applied resulted in decreased sprout growth.

In the field trials carried out at Glasgow Univeristy there were some erratic responses. There were a significant number of sprouted tubers (sprouts greater than 1 cm after five months, see section 2.15.2), with more sprouting in the smaller grades of potato tubers, i.e. those tubers that fell through a 3 cm grid.

Bishop and Schweers (1961) measured the concentration of MH in individual potatoes over a period of two seasons with a normal application rate and a double dose (3lb/acre and 6lb/acre respectively). The results are shown below.

	1958 season	1959 season
3 lb/acre	34 (6 - 53)	15 (11 - 18)
6 lb/acre	60 (34 - 88)	38 (12 - 65)

The results are expressed as ppm. The range for individual potatoes are in the brackets.

The results show a large variation between individual potatoes. Double the application rate roughly doubles the residues. Also the 1959 crop, for no reason mentioned, had half the residue of the 1958 crop when 3lb/acre was applied and two thirds the residue when 6lb/acre was applied.

4.2.2 Materials and methods

Eight tubers of equal size, from one treated plot of the 85-86 season, were chosen from their sprouting characteristics after four months in store (see Table 2.1). Each tuber was washed and dried before homogenisation with half its weight of water. Two replicates of 2 g of potato mixture was reduced, distilled, and the released hydrazine was reacted colorimetrically. The full method of analysis is described in section 3a.2.

4.2.3 Results and discussion

Refer to Table 4.1 for sprouting characteristics of individual potatoes.

There is a large variation within a plot of treated tubers, ranging from less than 1 ppm to 141.7 ppm. The highest concentration found is just below three times the WHO limit of 50 ppm. Another two values are just over the limit, however, the average concentration for the whole eight tubers is 42.6 ppm.

Individual tubers show a correlation with total MH residues and sprouting effects. A high residue level decreases sprouting, while a low residue level may not inhibit sprouting sufficiently but the break in apical dominance is still exhibited.

Table 4.1

Sprouting characteristics of individual tubers

Potato	Sprouting characteristics
A —	Normal sprouting pattern from one eye only
B	Normal sprouts from more than one eye
C	
D —	Abnormal sprouts, loss in apical dominance
E	Characteristic MH spikelets from all eyes
F	
G	
H	

Refer to section 4.2.2.

Table 4.2

Residue results of individual potatoes

Potato	Total MH (ppm)	replicates
A	1.0	(<1.0 1.0)
B	4.9	(3.2 6.7)
C	9.6	(10.6 8.6)
D	15.6	(10.8 20.3)
E	141.7	(132.9 150.5)
F	35.5	(29.6 41.5)
G	68.9	(66.0 71.9)
H	64.9	(57.4 72.4)
Mean	42.6	

Refer to section 4.2.2.

4.3 The internal distribution of MH within tubers

4.3.1 The residue levels of existing sprout suppressants :

CIPC and TCNB levels diminish in store. TCNB residues have been shown to be reduced with ventilation and airing (Dalziel [1978]). Increased temperatures during storage and greater length of time of airing both increase the reduction in residues. CIPC residues are also significantly lost with ventilation and can be reduced to acceptable levels with airing (Boyd [1984], Coxon and Filmer [1985]).

Many of the food products using potatoes are firstly washed then peeled. Washing and peeling both reduce TCNB (Dalziel and Duncan [1974]) and CIPC (Ritchie et al. [1983]).

Because MH is applied to the foliage, translocated, and accumulated in the tubers, there is concern that there is no practical method to reduce the residues that will be ingested.

This section investigates the distribution of MH within individual tubers.

4.3.2 Materials and methods :

Six potatoes were chosen from a treated plot of the 86-87 season, with diameters of about 4 to 5 cm. The potatoes were washed, dried, and cut perpendicular to the rose and heel axis to give a potato disc about 4 to 5 cm in diameter and 2 cm thick.

A 2 mm thick strip was peeled off the disc and cut into fine strips. This fraction was called the 'skin'.

A 10 mm thick strip was cut off and cut into fine strips. This fraction was called the 'outer flesh'.

The central core, which was about 20 to 30 mm in diameter, was also cut into fine strips. This fraction was called the 'core'.

The potato flesh was analysed by the adapted method of Lane (1963), and is described in section 3a.2.

4.3.3 Results and discussion :

Maleic hydrazide was present throughout the whole tuber. Refer to Table 4.3.

In every individual potato the core concentration was lower than the skin concentration. In five out of six potatoes the inner concentration was lower than the flesh.

In four out of six potatoes the outer flesh concentration was lower than the skin concentration.

The maximum concentrations would seem to be in the outer flesh including the skin. Lower concentrations tend to be in the core of the tuber.

Table 4.3

MH distribution within potatoes

Potato	Skin	Outer flesh	Core
1	12.5 (11.9 13.2)	9.7 (10.6 8.7)	12.0 (12.2 11.8)
2	26.8 (26.7 26.9)	28.2 (29.1 27.3)	15.0 (14.8 15.2)
3	33.0 (35.7 30.4)	34.0 (32.3 35.6)	23.7 (23.3 24.2)
4	56.4 (57.7 55.1)	54.6 (55.9 53.4)	44.5 (46.1 42.8)
5	22.6 (22.0 23.2)	20.1 (18.2 22.1)	18.7 (18.7 18.6)
6	30.4 (29.6 31.1)	25.2 (23.9 26.5)	22.3 (21.0 23.7)
Mean	30.1	28.6	22.7

The results are expressed as ppm the replicates are in the brackets.

Each replicate has been corrected for the blank control.

4.4 The concentration compared with tuber size grades and correlation between free MH and total MH

4.4.1 At the time of application there is a gradation in tuber sizes. The marketing literature describes the best time to apply MH for an increase in marketable yield, as when the maximum number of tubers required to reach marketable yields are greater than 25 mm.

At the time of application the tubers will be expanding due to a combination of cell division and cell expansion (Artschwager [1927], Reeve et al., [1963a], [1963b]). The larger tubers may slow down their growth and stop, letting other tubers expand (Moorby [1978]).

If MH distribution depends on the leaf assimilates pathway, that is MH accumulates itself in the areas of growth and expansion, then some grades of tuber may accumulate more MH than required and some grades may accumulate less than required.

This section deals with the distribution of MH between grades.

4.4.2 Materials and methods :

The harvested tubers (see Table 2.9 for details of the crops treatments) were graded into bull, seed and ware separated by grids of 3.0 cm and 4.5 cm. It should be remembered that MH is not to be used on crops intended for seed production, the term seed in this context describes a size of tuber.

Twelve bull, eight seed and six ware tubers were taken from each treated plot, and two of the untreated plots. These tubers were washed, dried and halved. Half of the tubers were frozen for analysis by HPLC, and the other half homogenised with a known volume of water then analysed by the adapted method of Lane (1963) and described in full in section 3a.2.

The frozen tuber samples intended for HPLC analysis were allowed to defrost slightly before being crushed and minced. The samples were analysed by the method outlined in section 3b.1.

4.4.3 Analysis of results :

Refer to Table 4.4 and Table 4.5 for results of analysis.

This section assesses the correlation between the two analytical methods of Total MH and Free MH. There are a number of methods to assess correlations. In this section the correlation is assessed using the estimate of the true correlation coefficient 'r' and simple linear regression (Bailey [1981]).

- 4.4.3.1 The correlation coefficient, is used to assess two sets of data if, the two sets of data both resemble a normal distribution pattern, and if the data are plotted there is a concentration in the centre and an unequal fall off along an axis.

The 'r' estimate has a value between -1 and 1. If the value is -1 then there is a perfect negative correlation, and the two sets are independent. If 'r' is between either -1 or 1 and zero, then the relationship is somewhere between a fixed relationship and an independence.

The correlation coefficient is given by

$$r = \frac{\sum (x - \bar{x}) (y - \bar{y})}{\sqrt{\sum (x - \bar{x})^2 \sum (y - \bar{y})^2}} \quad \text{equation 4.1}$$

$$\text{Degrees of freedom} = n - 2 \quad \text{equation 4.2}$$

The 'r' value is used to estimate the 'P' value from correlation coefficient tables found in statistical books. The 'r' value must exceed the value in the table for significance at various levels according to the number of degrees of freedom.

In this section the significant level is the 5% level (0.05). If the estimated 'P' value is greater at the 5% level, then the two values are independent. If the 'P' value is lower than the 5% then there is a relationship. The higher the 'r' value the lower the 'P' value the closer the relationship.

4.4.3.2 Simple linear regression is the method used to predict results from one set of data.

For the equation $y = bx + a$, the estimated linear regression equation is

$$b = \frac{\sum (x - \bar{x}) (y - \bar{y})}{\sum (x - \bar{x})^2} \quad \text{equation 4.3}$$

$$a = \bar{y} - b\bar{x} \quad \text{equation 4.4}$$

4.4.3.3 Initial analysis : The data for free MH and total MH were not kept to their grade classes, the data is assessed as a random number of potatoes representing the spread of potatoes that had been grown, harvested and stored under similar conditions.

The correlation coefficient and the regression calculations were processed by 'Microtab' software program on a 'BBC Minicomputer'.

The data for plot 11 bull grade was discarded because of an indefinite free MH value.

The estimated correlation coefficient :-

$$'r' = 0.701$$

$$r^2 * 100 = 49.1\%$$

The percentage figure means that 49.1% of the results could have been predicted with only one set of figures.

The 0.701 'r' value corresponds to a 'P' value, with 15 degrees of freedom, of less than 0.05, therefore there is a relationship between the free MH and the total MH.

The linear regression equation was calculated as,

$$\text{total MH} = 0.491 (\text{free MH}) + 6.9 \text{ (refer to graph 4.1)}$$

The 'Microtab' program also assessed point 'A' highlighted on graph 4.1 (21.2, 12.4) as being unlikely and very unusual compared to the other data.

The 21.2 ppm value is greater than 1.96 standard deviations from the free MH mean (Higginbotham, [1985]).

4.4.3.4 Final analysis, the estimated correlation coefficient, omitting point A (21.2, 12.4) refer to graph 4.1.

$$'r' = 0.885$$

$$r^2 * 100 = 78.3\%$$

Therefore 78.3% of the results could have been predicted with only one set of data.

The 0.885 'r' value corresponds to a 'P' value, with 15 degrees of freedom, of less than 0.05. This represents a stronger relationship between free MH.

The linear regression equation was calculated as,

$$\text{total MH} = 0.88 (\text{free MH}) + 4.17 (\text{refer to graph 4.1})$$

4.4.4 Results and discussion :

In six out of seven plots the ware grade had more total MH than the seed grade and the bull grade. Plot 13 was an exception. In six out of seven plots the seed grade had more total MH than the bull grade. Plot 13 was the exception.

In six out of seven plots the ware grade had more free MH than the seed grade. Plot 8 was an exception. In six out of seven plots the ware grade had more free MH than the bull grade. Plot 13 was an exception. In six out of seven plots the seed grade had more free MH than the bull grade. Plot 13 was an exception.

In this field trial the smaller sized tubers had less total MH and free MH compared with the larger sized tubers.

The correlation coefficient 'P' values mean that there is a close relationship between free MH and total MH. This allows predictions to be made using the linear regression equation.

If we assume that point marked 'A' (21.2, 12.4) is an extraordinary value and omit it from the correlation coefficient and linear regression equation, we see that there is a stronger correlation between the prediction of total MH result and free MH and vice versa with an ability to predict correctly by 78.3%.

Table 4.4

1986-87 Glasgow University field trial residue results

Total MH (ppm)				
Plot	Bull	Seed	Ware	Average
1	3.5 (3.5 3.5)	6.3 (6.0 6.7)	11.5 (12.3 10.8)	7.1
3	8.2 (7.5 8.9)	9.2 (9.3 9.1)	15.1 (14.0 16.2)	10.8
6	10.5 (9.6 11.3)	12.2 (12.3 12.0)	13.7 (11.7 15.7)	12.1
8	9.7 (8.6 10.7)	14.3 (15.5 13.1)	17.0 (16.3 17.7)	13.7
9	7.1 (6.2 8.0)	11.4 (10.9 11.5)	15.3 (16.3 14.4)	11.3
11	4.8 (4.3 5.3)	9.5 (10.2 8.9)	11.5 (10.5 12.5)	8.6
13	12.4 (12.0 12.9)	9.7 (8.6 10.8)	10.4 (10.1 10.7)	10.8
Mean	8.0	10.4	13.5	

The results are the average of two replicate analyses expressed as ppm

Each replicate has been corrected for the blank control

The untreated plots had less than 1 ppm

Table 4.5

1986-87 Glasgow University field trial residue results

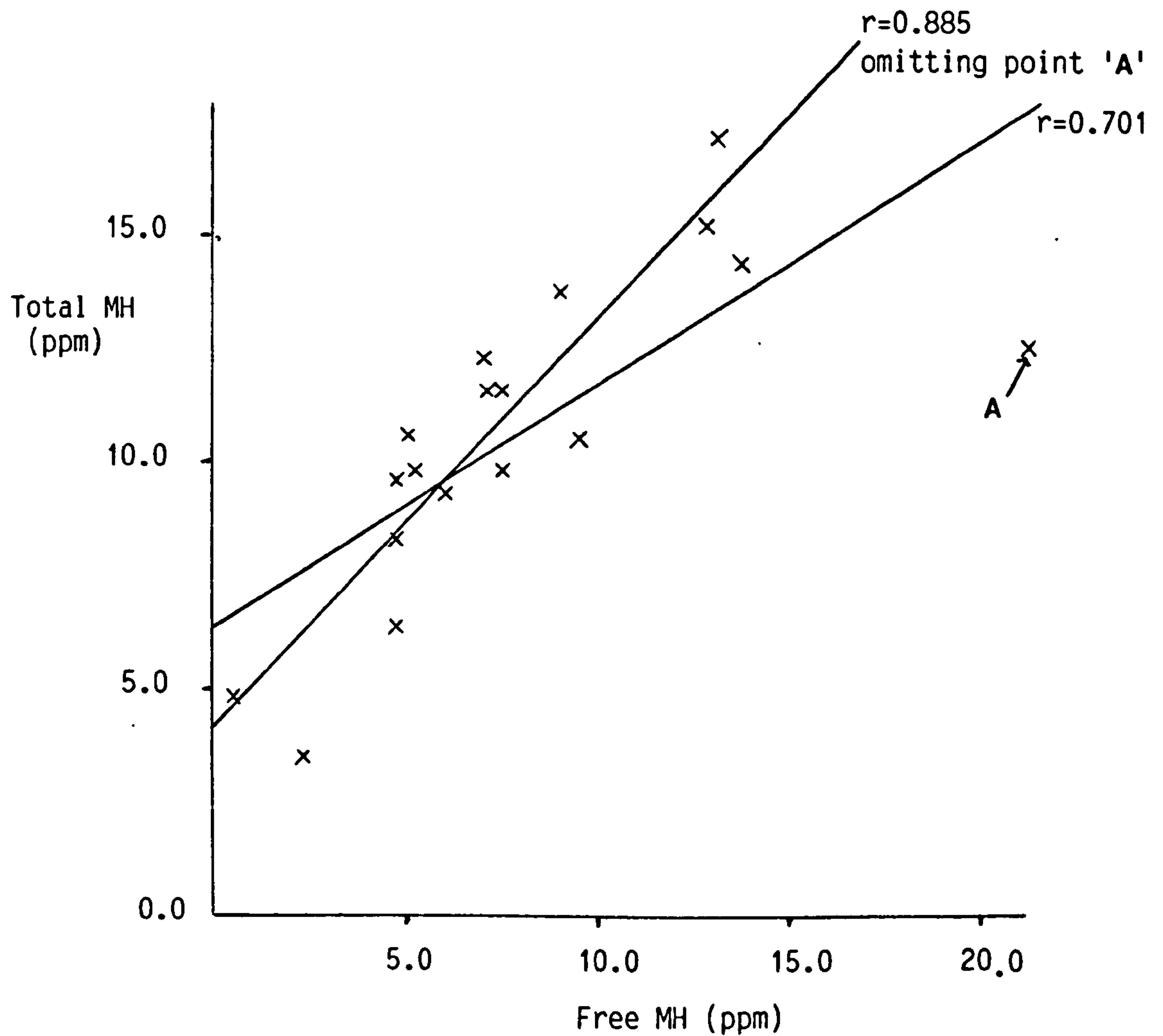
Free MH (ppm)

Plot	Bull	Seed	Ware	Average
1	2.3 (1.6 3.0)	4.7 (4.1 5.4)	7.1 (4.8 9.3)	4.7
3	4.7 (2.5 6.9)	6.0 (6.2 5.8)	12.8 (10.5 15.0)	7.8
6	5.0 (4.9 5.1)	7.0 (6.7 7.3)	9.0 (10.0 7.9)	7.0
8	5.2 (2.5 7.9)	13.7 (13.9 13.6)	13.1 (15.1 11.0)	10.7
11	0.5 (0.4 0.2)	4.7 (4.1 5.3)	7.5 (4.9 10.0)	4.2
13	21.2 (19.1 23.3)	7.5 (7.3 7.9)	9.5 (7.9 11.0)	12.7
Mean	6.5	7.5	9.8	

The results for plot 9 are not available

The replicates are in the brackets

Graph 4.1, The correlation of free MH residue analysis with total MH residue analysis



'r' = correlation coefficient

Refer to section 4.4.3 for the details of the above

4.5 MH residue levels after processing

MH has been shown to be present throughout the whole tuber (section 4.3). CIPC and TCNB have been shown to be reduced prior to processing due to airing (Dalziel [1978], Boyd [1984], and Coxon and Filmer [1985]) and by peeling (Dalziel and Duncan [1974], and Ritchie et al. [1983]).

This section is aimed at investigating whether MH residues are reduced after typical processes such as frying as crisps and boiling.

The specific method of HPLC analysis is used to detect any change of the free unchanged MH in the oil and the non oil residue of fried crisps, and in boiled potatoes.

- 4.5.1. Frying crisps : Potatoes from three treated plots and one untreated plot from week 4 of the 1985-86 season (section 2.15) were peeled and thinly sliced by knife.

A known weight of potato slices were fried for about 90 seconds at 200°C, cooled for 30 minutes and weighed again (see Table 4.6 for weights).

- 4.5.2 Oil extraction (Ritchie et al. [1983]) : Approximately 20 g of fried crisps were homogenised with 40 ml of hexane for one minute, filtered under suction through Whatman No. 1 filter papers. The residue was further extracted with 30 ml of hexane with 2 ml of diethyl ether added. The residue was again extracted with 30 ml of hexane with 2 ml of diethyl ether added.

The filtrates were combined, reduced under suction by rotary film evaporation in pre-weighed 200 ml round bottom flasks. The flasks were weighed again and the percentage of oil extracted was calculated by the difference in weight (see Table 4.6).

The oil fraction and the non-oil residue was kept for analysis.

- 4.5.3 MH in oil fraction : The oil fraction had 2 ml of hexane added to reduce viscosity. MH was extracted with 15 ml of acetonitrile extractant (acetonitrile with 1% of 2.5% sodium chloride aqueous solution) in a 50 ml separating funnel. The solutions were thoroughly mixed then left for two hours.

5 ml of the acetonitrile extractant was reduced to dry on a heating block under a nitrogen stream and redissolved in 1 ml of methanol. The residue was eluted through a Bond Elut C₁₈ clean-up column before injection onto the HPLC system detailed in section 4.5.8.

The results are tabulated in Table 4.7

- 4.5.4 MH in solid non-oil residue : The residue was dried on a watch glass and left overnight in a fumehood.

1 g was shaken for two hours with 2 ml of methanol then left to settle overnight.

1 ml of the methanol extractant was eluted through a Bond Elut C₁₈ column before injection onto the HPLC system detailed in section 4.5.8. The results are tabulated in Table 4.8.

4.5.5 The results are analysed and clarified in Table 4.9.

The total MH in the 20 g fried sample is expressed as a percentage of the total MH in the 20 g fresh weight.

4.5.6 Boiling potatoes : Two 500 g batches of treated potatoes were halved. One half of each batch was cut into two inch cubes and boiled for 15 minutes until soft, then mashed. The other half of each batch was minced fresh.

4.5.7 MH analysis of boiled and fresh potatoes : 50 g of minced, or mashed, potato material was homogenised for two minutes with 200 ml of methanol. The mixture was filtered through Whatman No. 1 filter papers under suction. The homogeniser was washed twice with two 100 ml aliquots of methanol and washed through the residue and added to the filtrate. The filtrate was made up to 500 ml with methanol. A 50 ml aliquot was reduced to about 3 ml (by rotary evaporator at 40°C), made up to 5 ml with distilled water washings of the round bottomed flask.

A 1 ml aliquot from the 5 ml extract was eluted through a Bond Elut SCX column. The SCX column was previously wetted with methanol, conditioned with 0.1 M acetic acid, and washed with distilled water. MH was analysed from the HPLC analysis detailed in section 4.5.8.

The results are tabulated in Table 4.10.

4.5.8 Column : Partisil SAX, 25 cm x 4.5 mm (i.d.)

Mobile phase : 0.1 M acetic acid, pH 4.8 with
conc. sodium hydroxide

Flow rate : 1 ml/min

Injection volume : 100 μ l

Standard : 10 μ g/ml MH

4.5.2 Results and discussion

MH was detected in the potato products after frying and boiling MH treated potatoes.

The majority of the MH was extracted from the non-oil residue of crisps, very little was found in the oil fraction of crisps.

Boiled potatoes had slightly reduced MH levels compared to the fresh weight levels.

Table 4.6

Weight and percentage oil in crisps

Plots	1	4*	7	8
Fresh weight of slices (g)	93.80	66.98	92.60	108.85
Fried weight of slices (g)	32.75	24.93	29.70	36.66
<u>In 20 g fraction</u>				
Weight of oil (g)	10.75	11.75	10.09	10.88
percentage oil	53.8 %	58.5 %	50.5 %	54.4 %
Weight of residue by difference (g)	9.25	8.25	9.91	9.12
percentage of residue	46.2 %	41.2 %	49.5 %	45.6 %

* = untreated plot

Refer to section 4.5.1 and 4.5.2

Table 4.7

MH residue results of crisps (oil extract samples)

Plot	MH in 100 μ l** HPLC injection (ng)	Total MH in 20 g sample (μ g)
1	11	0.33
4*	n.d.	n.d.
7	9	0.27
8	n.d.	n.d.

* Untreated plot

** The MH detected is at the limits of the HPLC detection

n.d. Not detected

Refer to section 4.5.3

Table 4.8

MH residue results of crisps (non-oil residue samples)

Plot	MH in 100 μ l HPLC injection (ng)	Total MH in 20 g sample (μ g)
1	129	24.1
4*	n.d.	n.d.
7	252	49.9
8	164	29.9

* Untreated plot

n.d. Not detected

Refer to section 4.5.4

Table 4.9

Analysis of residue results of crisp samples

In 20 g sample

Plot	Fresh Weight		Fried sample		The percent of total MH found in fried product (%)
	Total MH (ppm)**	Total MH (µg)	Total MH (µg)		
1	6.1	122	24		19.7
4*	n.d.	n.d.	n.d.		-
7	7.6	152	50		32.9
8	11.0	220	30		13.6

* Untreated plot

** Analytical results from section 3c.2

n.d. Not detected

Refer to section 4.5.5

Table 4.10

MH residue results of boiled potatoes

		Free MH (ppm)
Uncooked	1	14.9
	2	13.3
Cooked	1	11.3
	2	7.4

'1' and '2' refer to two treated batches of field treated potatoes

Refer to section 4.5.7

4.6 The distribution of MH within a potato crop

Section 4.3 has demonstrated that individual potatoes, treated with Fazor, have a wide range of concentrations (1.0 ppm to 141.7 ppm) that is reflected by their sprouting characteristics. Potatoes showing inadequate sprout suppression have low MH levels. This was also observed in field treated plots in section 2.12.2. Plots with low mean MH levels had poor inadequate sprout suppression compared to plots with higher mean MH levels with adequate sprout suppression.

Section 4.3. shows MH is distributed throughout the whole tuber with slightly lower levels of MH in the central flesh. In processing the potato skin is usually peeled and discarded. The majority of skin applied chemicals are also discarded with the skin. MH treated tubers after peeling still have the majority of the MH within the flesh.

Section 4.5 shows that MH is still present, as the active ingredient, after boiling and frying. Some of the observed loss in concentration due to boiling may be due to expansion and uptake of water during boiling therefore decreasing the concentration compared to the fresh weight. MH is probably washed out of the potato flesh during boiling although the boiling water was not analysed.

The process of frying does decrease the total MH that may be extracted (see section 4.5.5), however, this may be due to the extraction methods being inefficient in extracting all the MH. The frying process may alter the MH available by excluding MH by chemical binding of the MH to unknown fractions within the potato. However, free MH is still detected after 90 seconds of frying in corn oil at 200°C.

Section 4.4 showed that smaller tubers had less MH than larger tubers. This may be due to the final stages of growth of the crop in the field. The parameters that control growth and expansion of tubers are still not fully understood. If MH readily translocates in the phloem and the expanding tubers act as a sink for the assimilates in the phloem, then MH may be found in higher concentrations under circumstances where the smaller tubers have the greater demand for assimilates than larger tubers. It is unclear at the time of MH application whether larger tubers are still growing and smaller tubers may catch up with accelerated growth. It should also be remembered that MH halts cell division in tubers therefore larger tubers which grow due to cellular expansion will pull more MH into the potato flesh.

Section 4.4 also demonstrates the correlation between the two methods. The correlation factor of 0.885 reflects a close relationship that allows predictions of free MH from total MH data and vice versa.

AN INVESTIGATION OF THE METABOLITE OF MALEIC HYDRAZIDE FOUND IN POTATO
TUBERS USING RADIOTRACER ANALYSIS

5.1 Introduction

This chapter was initiated because of the findings from chapter three. In chapter three two methods of residue analysis were used to detect MH. The difference between the two methods highlighted the possibility of a metabolite.

This chapter describes the use of radiolabelled MH to attempt the identification of any metabolites.

Radiolabelled MH had been used in tracer studies and degradation of ^{14}C -MH in a variety of crops. MH showed good mobility, translocating to the actively growing areas with a radial effect. Most of the recovered ^{14}C -MH, but not all of the radiolabel, was accounted for. Smith et al. (1959) suggested either, a protein complex, a glucoside, or a degradation product.

Towers et al. (1958) fed ^{14}C -MH and ^{14}C -glucose to young wheat leaves. After ethanol extraction, concentration, spotting on chromatography paper, and 2-D elution firstly with water saturated with phenol then secondly with n-butanol/acetic acid/water (4/1/5, v/v). The trials with ^{14}C -MH gave three spots. One was identified as ^{14}C -MH, the two other spots were termed U_1 and U_2 . U_1 after hydrolysis with acid or emulsin gave ^{14}C -MH. Feeding wheat plants with ^{14}C -glucose and unlabelled MH gave a number of spots including U_1 and U_2 . After acid or emulsin hydrolysis U_1 released ^{14}C -glucose. It was concluded that U_1 was a beta-glucoside of MH, a detoxification product.

Biswas et al. (1967) fed ^{14}C -MH to tea plants and suggested ring cleavage producing lactic acid, succinic acid, maleimide, and hydrazine. The tea plants were grown in ^{14}C -MH medium for seven days, extracted in hot ethanol, concentrated, and spotted on chromatography paper. The paper was twice eluted in a 2-D fashion with water saturated with phenol, then n-butanol/acetic acid/water (4/1/5^v/v). Two unknowns were detected termed U_1 and U_2 , not to be confused with Towers et al. (1958) metabolites also termed U_1 and U_2 . Infra-red analysis of the metabolites Biswas et al. suggested that U_2 most likely contains lactic acid and some other product, U_1 most likely contains succinic acid, maleimide and/or hydrazine. However this work was not conclusive. The Avena first internode test revealed that both U_1 and U_2 increased the growth rate, compared to controls. U_1 and U_2 had similar activity to indoleacetic acid IAA.

Nooden (1970) investigated the uptake, metabolic stability, and the binding of MH to some unidentified macro-molecules. He found the MH ring structure to be stable and MH incorporated by an active binding to macro-molecules in the older parts of plants. Most of the ^{14}C -MH was bound to very large particles.

The behaviour and fate ^{14}C -MH was investigated by Frear and Swanson (1978) in tobacco plants. They found ^{14}C -MH was translocated rapidly to actively growing tissue in a source to sink pattern. Ring degradation was not a significant metabolic pathway. The major methanol soluble metabolite was isolated and identified as the beta-D-glucoside. Unchanged MH was released from the methanol insoluble fraction by acid and alkaline treatment.

5.2 The techniques used in ^{14}C radiolabelled analysis

There are numerous methods of detecting radiolabels with different emissions and are well reviewed by L'Annunziata and Legg (1984). This section explains the methods used in this analysis, liquid scintillation and autoradiography, of ^{14}C labelled molecule.

- 5.2.1 Liquid scintillation : The ^{14}C atom will decay to ^{14}N releasing a beta particle (B^-). If liquid scintillation is to be used the beta particle excites the electrons in the solvent. The energy of the Pi electrons are transferred to a primary fluor which will emit photons.

The photons emitted are collected by a photomultiplier and counted. The greater the activity the larger the scintillation count.

- 5.2.2 Autoradiography is the production of an image on a photographic emulsion by nuclear radiation emitted from a particular material or body. The beta particles emitted by ^{14}C atoms cause ionisation of bromide ions within silver bromide crystals. An orbital electron from a bromide ion is ejected. The ejected electrons travel from one ion to another until trapped in defects within the silver bromide crystal. At this point the silver ions become reduced to silver atoms.

The affected crystal becomes visible when the developer reduces all the silver in the affected crystal to metallic silver (black grains). The fixer dissolves the unreduced crystals, leaving a black image caused by nuclear radiation.

5.3 The aims of this chapter

The aim of this chapter was to identify and characterise the metabolites of MH within the potato plant.

To achieve these aims radiolabelled MH was synthesised, applied to potato plant leaves, extracted from the potato tubers, separated and quantified.

5.4 The synthesis of MH

The method used to synthesize MH closely resembles the initial industrial synthesis described by Harris and Schoene (1951) and is described below. The synthesis is fully detailed in section 5.5.

- 5.4.1 Synthesis outline : Equimolar portions of hydrazine sulphate and maleic anhydride were refluxed in an acidic solution for about one hour. After cooling, solid MH is filtered from the initial condensation.

A conical flask (200 ml) was used with a magnetic stirrer and boiling stones to stop boiling. The flask was heated by a heated magnetic stirrer. The mixture was refluxed for one hour.

The synthesised crystals were recrystallised by boiling the crystals, cooling, filtration of the solid MH, washing of the crystals with cold water, and finally drying at 100°C for 20 minutes.

The refinements above yielded 3.3 g (27.5% yield) of solid MH with greater than 88% purity.

Yields and purity were measured from melting point determinations, infra-red spectral data, and HPLC analysis with uv identification and quantification. The methods of yield and purity calculations are detailed in section 5.6.

Further refinements of purifying maleic anhydride by sublimation and better techniques in recrystallisation increased yield to 37.7% with greater than 95% purity.

5.5 The synthesis of (4,5-¹⁴C) MH

All chemicals or solvents were of Analar or HPLC grade

5.5.1 50 mCi of 2,3-¹⁴C maleic anhydride (specific activity 25 mCi/mmol) was purchased from Amersham International plc. The maleic anhydride was received as crystals in a N₂ filled ampule. The seal was broken and the crystals were dissolved in about 2 ml of methanol and transferred by pipette to a ground glass necked 200 ml conical flask. The methanol was evaporated off under reduced pressure.

5.5.2 8.14 g (0.083 moles) of sublimed maleic anhydride and 13.01 g (0.100 moles) of hydrazine sulphate were added to the conical flask with 40 ml of 2.5 M HCl.

The above mixture was refluxed for one hour, with a magnetic stirrer and a few boiling stones, at 110°C on a heated magnetic stirrer. The solution was then left to cool to room temperature.

5.5.3 The precipitated MH crystals were filtered through Whatman No. 1 filter paper and washed with about 60 ml of 2.5 M HCl. The filtrate was again refluxed for about one hour and left to cool overnight. The cooled filtrate was filtered through the same filter paper containing the first precipitated crystals.

5.5.4 The crystals were washed with cold distilled water. The crystals were then scraped into a conical flask (200 ml) and boiled in 100 ml of distilled water, then filtered straight from boiling, through Watman No. 1 filter paper on a Buchner apparatus under suction.

The filtrate was left to cool for two hours, after which the solid MH crystals were filtered through Watman No. 1 filter paper. The filter paper containing the solid MH was dried at 100°C for two hours. The dried crystals were stored in a brown tinted bottle during spectral identification, purity and liquid scintillation calibration.

5.6 The purity of the synthesized ^{14}C -MH

3.73 g of dried crystals (39% yield of theoretical maximum) was collected from the filter paper after synthesis.

The melting point was observed to be 299°C (296 - 298°C, Weast [1979]).

The infra-red spectral analysis was consistent with Analar MH (Hopkin and Williams). Refer to figures 5.1,2 and 3 for spectral drawings of maleic anhydride, Analar MH, and synthesised ^{14}C -MH.

The purity was calculated by HPLC analysis by two different systems.

System 1 (1) Spherisorb-Ph (Phenyl)-3 μ (10 cm x 4.5 mm)
 (2) Acetonitrile/H₂O (80/20 v/v) 1 ml/min
 (3) uv detection of 313 nm

Purity = 95%

System II (1) Partisil SAX -10 μ (25 cm x 4.5 mm i.d.)
 (2) 0.1 M Acetic Acid pH 4.8, 1 ml/min
 (3) uv detection at 313 nm

Purity = 94%

The purity was also calculated by the UV absorption of 10 ppm solutions on a SP 1800 Pye Unicam UV-VIS detector at 330 nm. The ¹⁴C-M.H. was found to be 94% pure.

5.7 The activity of the synthesized ¹⁴C-MH

The activity of the synthesised ¹⁴C-MH was calculated by measuring the activity of different volumes of a 100 ppm standard made from dilutions from a 0.1% standard (100 mg dissolved in 80 ml of boiling water, made up to 100 ml after cooling to room temperature).

100, 200 and 1000 μ l of the 100 ppm standard (¹⁴C-MH) were added to 5 ml of scintillation fluid (Ecoscint, National Diagnostics) in 20 ml plastic counting vials. The vials were counted for 30 minutes each (Phillips liquid Scintillation Counter, PW 4700).

The results are tabulated in Table 5.1.

The linearity of the counting method was assessed by counting the same volume of different concentrations by the method detailed above.

100 μ l of 0.2, 0.4, 0.6, 0.8 and 1.0 ppm standard solutions were diluted from the 10 ppm solution used for activity assessment.

The results are tabulated in Table 5.2.

5.8 The efficiency of the autoradiography

The efficiency of the detection method was assessed by spotting the plates with known concentrations. X-ray photographic plates were exposed to the tlc plates for a time period. The X-ray plates were then developed and fixed.

0.1 ml of 5, 50, 500 and 5000 ppm ^{14}C -MH (in distilled water) was gradually spotted on a tlc plate (Kieselgel H254, 250 μ) allowing the previous drop to dry. The tlc plate was then placed on X-ray photographic film (Agfa-Gavaert Osray M3, 24-30 cm). The tlc plate and X-ray film was then sandwiched between blotting paper and aluminium foil, held together by hardboard fixed with elastic bands. These plates were stored in cardboard boxes for one month.

The X-ray photographic plates were developed (Ilford Phensiol) and fixed (Ilford Hypam).

The 0.5 μg spot could not be detected, the 5 and 50 μg spots were faint and the 500 μg spot was distinct and clear.

5.9 The application of the synthesized ^{14}C -MH to the potato plant

Six seed tubers (cv Maris Piper, SE2) were grown in grow bags (locally bought) in a well aerated greenhouse with white painted windows, and watered every day.

2.5 g of ^{14}C -MH and an equimolar volume of diethanolamine were added to 500 ml of distilled water (0.5% ^{14}C -MH). 0.25 ml of surfactant (Tween 20) was also added to improve uptake.

The solution was painted onto the leaves of three potato plants ten days after blossom fall.

5.10 Residue analysis of the ^{14}C -MH treated potato tubers

The harvested tubers were analysed by the methods outlined in sections 3a.2 (total MH), section 3b.1 (free MH) and 3c.2.4 (total MH in methanol extracted fraction).

The results are tabulated in Table 5.3.

5.11 Radiolabelled trace analysis of the ^{14}C -MH treated potato tubers

5.11.1 The concentrated methanol extract, from the residue analysis of free MH was filtered. Two 500 μl sub-samples from treated tubers and two sub-samples from untreated tubers were mixed with 5 ml of Ecoscint scintillation fluid (National diagnostics) and counted for 80 minutes (Phillips liquid scintillation counter, PW 4700). The concentrated methanol extract from untreated tubers was spiked with ^{14}C -MH similar to 5 ppm and 10 ppm treated tubers. This concentrate was also filtered and counted as described above.

A 100 ppm standard was used as a calibration standard (500 μl into 5 ml Ecoscint scintillation liquid).

The results are tabulated in Table 5.4.

5.11.2 Calculations

Samples :

Mean activity of ^{14}C -MH extract minus mean activity of control equals 913.85 dpm

50 μg ^{14}C -MH = 5633 dpm

$$\frac{913.85 \text{ dpm}}{5633 \text{ dpm}} \times 50 \mu\text{g} = 8.1 \mu\text{g}$$

8.1 μg from 500 μl therefore 16.2 ppm

Recoveries :

$$5 \text{ ppm: } \frac{203.3 \text{ dpm}}{5633 \text{ dpm}} \times 50 \mu\text{g} = 3.6 \text{ ppm (72\% Recovery)}$$

$$10 \text{ ppm: } \frac{31.57 \text{ dpm}}{5633 \text{ dpm}} \times 50 \mu\text{g} = 5.6 \text{ ppm (56\% Recovery)}$$

5.11.3 Approximately 100 μl of the concentrated methanol extract of both treated and untreated potatoes were applied to tlc plates (4 plates treated, 4 plates untreated). The plates had been coated with Kieselgel H, 250 μ (25 cm x 25 cm), dried for 30 minutes at 100°C , equilibrated overnight at ambient room conditions. 100 μl of 1000 ppm ^{14}C -MH was also applied under the above conditions as a reference standard (4 plates).

The plates were eluted twice. The first run was done with water saturated with phenol, dried overnight at room temperature. The second run was eluted perpendicular to the first run with iso-propanol/0.1 M acetic acid (50/50, v/v) again dried overnight at room temperature. Other elution solvents were assessed such as n-butanol/acetic acid/H₂O (4/1/5 v/v) iso-butanol/acetic acid/H₂O (4/1/5 v/v). The above system of iso-propanol/0.1M acetic acid (50/50 v/v) gave reasonable R_f values with distinct MH spots and very little tailing.

Two reference standard plates were detected by charring with 5M H₂SO₄ spray, then heating in a 100°C oven for 20 minutes.

All other plates (4 treated, 4 untreated, 2 reference plates) were individually placed in intimate contact with X-ray plates (Agfa-Gevaert, Osray M3 24 x 30 cm) and sandwiched between blotting paper, then aluminium foil and hardboard held firmly together by elastic bands.

After one, two, three and six months, one treated plate, and one untreated plate were developed and fixed.

After one month the reference standards were developed and fixed.

- 5.11.4 After one month the reference standards were detected (Rf 1st solvent = 0.96, Rf 2nd solvent = 0.71).

No spots were detected up to three months. Traces were noticed with similar Rf values of the reference standards after six months.

- 5.11.5 A two centimetre square of silica, with the centre corresponding to the centre of the developed spot, was scraped off treated and untreated plates. The silica was gently shaken with 1 ml of distilled water for one hour, then filtered through a pasteur pipette plugged with cotton wool. 500 μ l of the filtrate was mixed with 5 ml of Ecoscint (National Diagnostics Ltd) and counted for 30 minutes (Phillips liquid scintillation counter, PW 4700).

100 μ l of the filtrate was injected onto the HPLC system detailed below.

Column - Partisil SAX 10 μ (25 cm x 4.6 mm i.d.)

Mobile phase - 0.1 M acetic acid, pH 4.8, 1 ml/min

Detector - uv detection at 313 nm

The filtrate from the silica extract from the ^{14}C -MH treated plates had higher counts than controls (24.0 dpm compared to 7.0 dpm).

HPLC analysis showed that the ^{14}C -MH treated extract contained MH. The control extract was found to have no detectable MH.

- 5.11.6 A matrix of 2 centimetre sided squares (24 squares, an area sided by 4 and 6 squares) was scraped off one tlc plate that had ^{14}C -MH concentrated potato extract applied and eluted as outlined in section 5.11.3.

Each silica sample was shaken with 1 ml of methanol for one hour, filtered through a pasteur pipette with a cotton wool plug. 500 μl was mixed with 5 ml of Ecoscint and counted as detailed on previous page.

For the results refer to Figure 5.4

The results indicate a second area of radiolabelled activity. This is further investigated in the next section 5.11.7.

- 5.11.7 Eight tlc plates were made up with Kieselgel H (250 μ , 25 x 25 cm). Four plates were spotted with 0.1 ml of a concentrated methanol extract of ^{14}C -MH treated potatoes. Two were spotted with 1% ^{14}C -MH. Two were not spotted.

All eight were eluted firstly with water saturated with phenol, then in a perpendicular direction with iso-propanol/0.1M acetic acid (50/50, v/v).

The two standard MH plates and two ^{14}C -MH treated extract plates were developed by spraying with 5 M H_2SO_4 then charring at 100°C for 20 minutes. These were used to establish the R_f values for ^{14}C -MH.

From the ^{14}C -MH Rf values the position of the other area of activity (not free MH) was calculated. The silica from this area on the remaining tlc plates was scraped off, shaken with 1 ml of methanol, filtered through a pasteur pipette plugged with cotton wool. The filtrate was evaporated under nitrogen on a heated block, redissolved in 0.5 ml of toluene. The toluene extracts were analysed by infra-red spectral analysis.

No structure, apart from those related to methanol, could be distinguished.

5.12 Results and discussion

The synthesis of radiolabelled MH (^{14}C , 3,4 position) was sufficient to provide 3.73 g (39% of yield), with an activity of approximately $3.2 \times 10^{-11} \mu\text{Ci/g}$.

The application and translocation of 0.5% ^{14}C -MH solution, containing 0.1% Tween 20, to potato plant leaves was successful. The total M.H. concentration was 140.5 ppm, the free MH concentration was 80.0 ppm, the total MH extracted by methanol was 112.5 ppm. The scintillation analysis of the methanol extract detected 16.2 ppm.

The autoradiographs, developed after six months exposure to the tlc plates, showed a trace of radioactive material which was found to be MH. No other spot was observed. However, an area of the tlc plates, not corresponding to MH was extracted and radioactivity was detected. The structure of the radioactive trace spot was not elucidated.

One reason that the scintillation counts of the methanol extracted concentrate were low may be due to quenching.

Quenching is (1) a disruption in the energy transfer caused by absorption of the beta particles, (2) disruption in the energy transfer between solvent and fluor and (3) absorption of the photons decreasing the photomultiplier response. Quenching can be caused by molecules that absorb light in the visible region and interact chemically with the solvent fluor.

The methanol extracted concentrate would have other coextracted molecules that obviously had interfered with the efficiency of the scintillation process and the counting method. Clean-up methods such as reversed phase clean-up cartridges could have increased the counting efficiency by adsorbing coextracted interferences. Any clean-up method could have decreased the activity by adsorbing ^{14}C -MH metabolites.

The main problem of the autoradiographs was the low activity. Because of the low activity the X-ray photographic film had to be exposed to the tlc plates for six months, even then the only faint trace of activity was MH. No metabolite was detected by autoradiographs although one area of the tlc plate was extracted with methanol and showed activity. No radiolabelled structure was identified. This may be due to slight differences in the tlc elution solvents sufficiently different from the original elution solvents used on the original tlc plates for autoradiographs. This slight difference in chromatographic conditions may have altered the elution of the metabolite to a different area of the tlc plate. The metabolite may not have dissolved in the toluene required for i.r. spectral analysis, therefore no structure could be detected.

If the initial ^{14}C -maleic anhydride had been of stronger activity then it may have been possible to develop the X-ray plates within a month and may have shown metabolites that could have been extracted from the tlc silica and allowed spectral analysis and identification of possible metabolites. The cost of highly active ^{14}C -MH or ^{14}C -maleic anhydride was prohibitive to repeat the work in this area. The time taken to synthesise ^{14}C -MH, reapply to potato plants, and reanalysis would extend over the time limit of this thesis and would not be completed.

Table 5.1

¹⁴C-MH Specific activity

Volume (ul)	Quantity (ug)	Mean of 3 counts (dpm)	μCi (x10 ⁻³)	μCi/g (x10 ⁻¹¹)
1000	100	7993	3.6	3.6
200	20	1036	0.6	3.0
100	10	642	0.3	3.0

Mean specific activity = $3.2 \times 10^{11} \mu\text{Ci/g}$

Refer to section 5.7

Table 5.2

The linearity of the calibration curve

Concentration (ppm)	Quantity (μg)	Mean of 3 counts (dpm)
1.0	0.5	27.3
0.8	0.4	24.7
0.6	0.3	19.7
0.4	0.2	15.3
0.2	0.1	10.0

- (i) Refer to section 5.7
- (ii) Graph 5.1 demonstrates the linearity

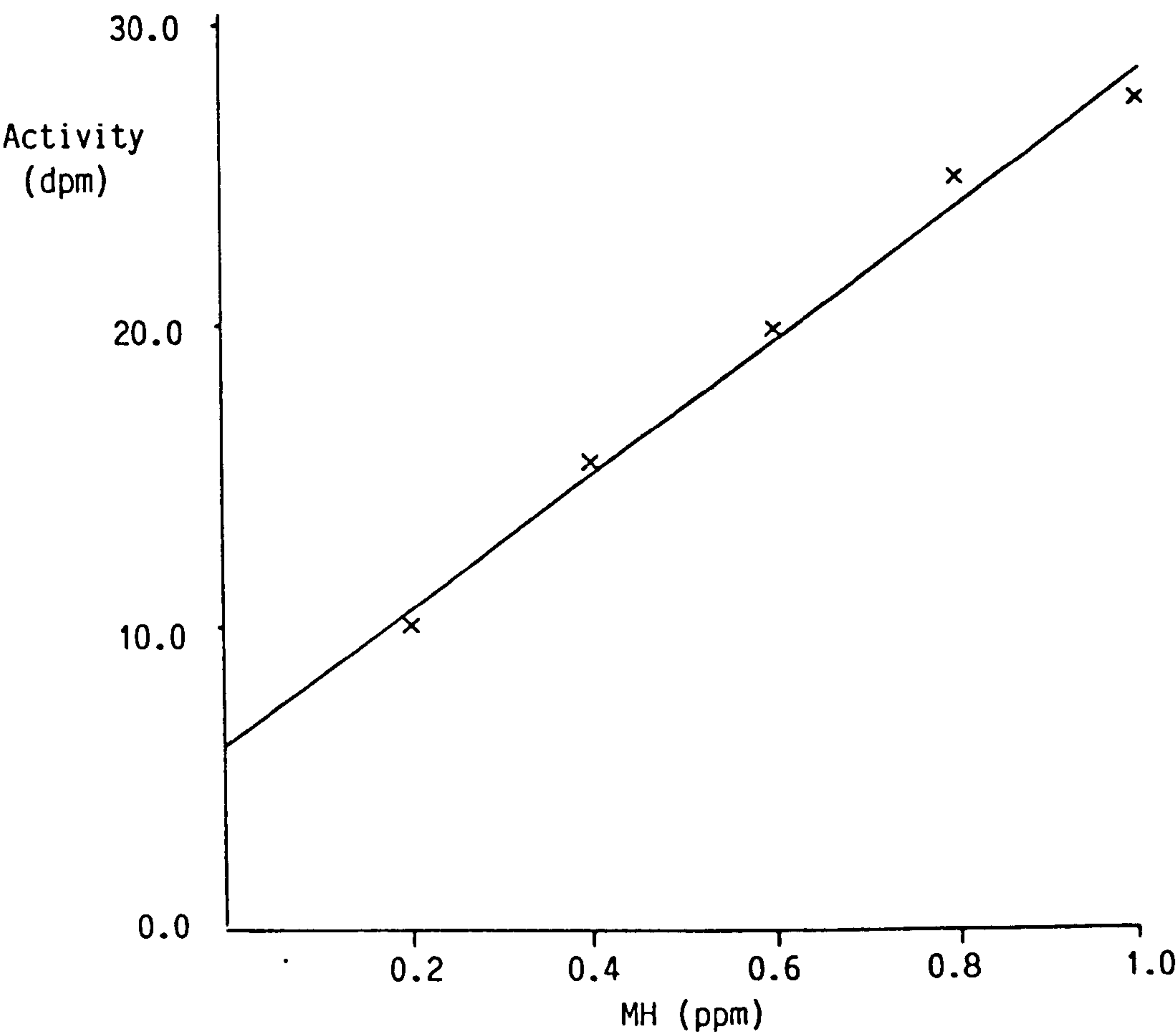
Table 5.3
Residue analysis of tubers from ¹⁴C-MH treated potato plant

	MH (ppm)
Total MH	140.5
Total MH in methanol extracted fraction	112.5
Free MH	80.0

- (i) Untreated potatoes had less than 1 ppm total M.H.
- (ii) Refer to section 5.10

Total 5.4	
Scintillation counts of tuber extract from ¹⁴ C-MH treated potato plant	
Description	Mean of 3 counts corrected for background (dpm)
Control extract I	8.7
II	7.0
¹⁴ C-MH treated extract I	905.3
II	938.0
5 ppm spike	202.3
10 ppm spike	315.7
100 ppm standard	5633.0

Graph 5.1, Linearity of ¹⁴C-MH calibration curve



Refer to section 5.7 for details

Figure 5.1
i.r. spectrum of maleic anhydride
Refer to section 5.6

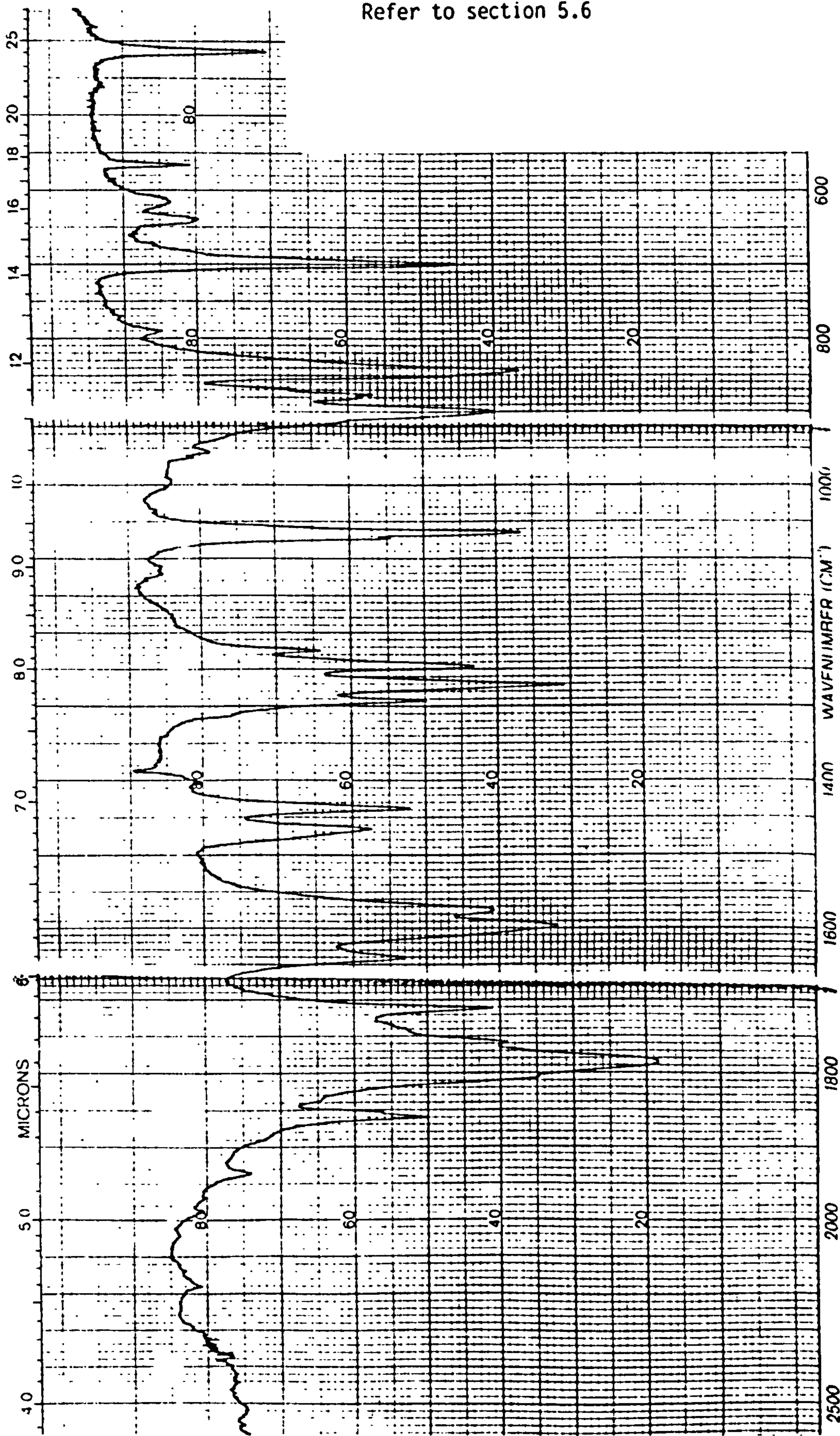


Figure 5.2

i.r. spectrum of MH (analar)

Refer to section 5.6

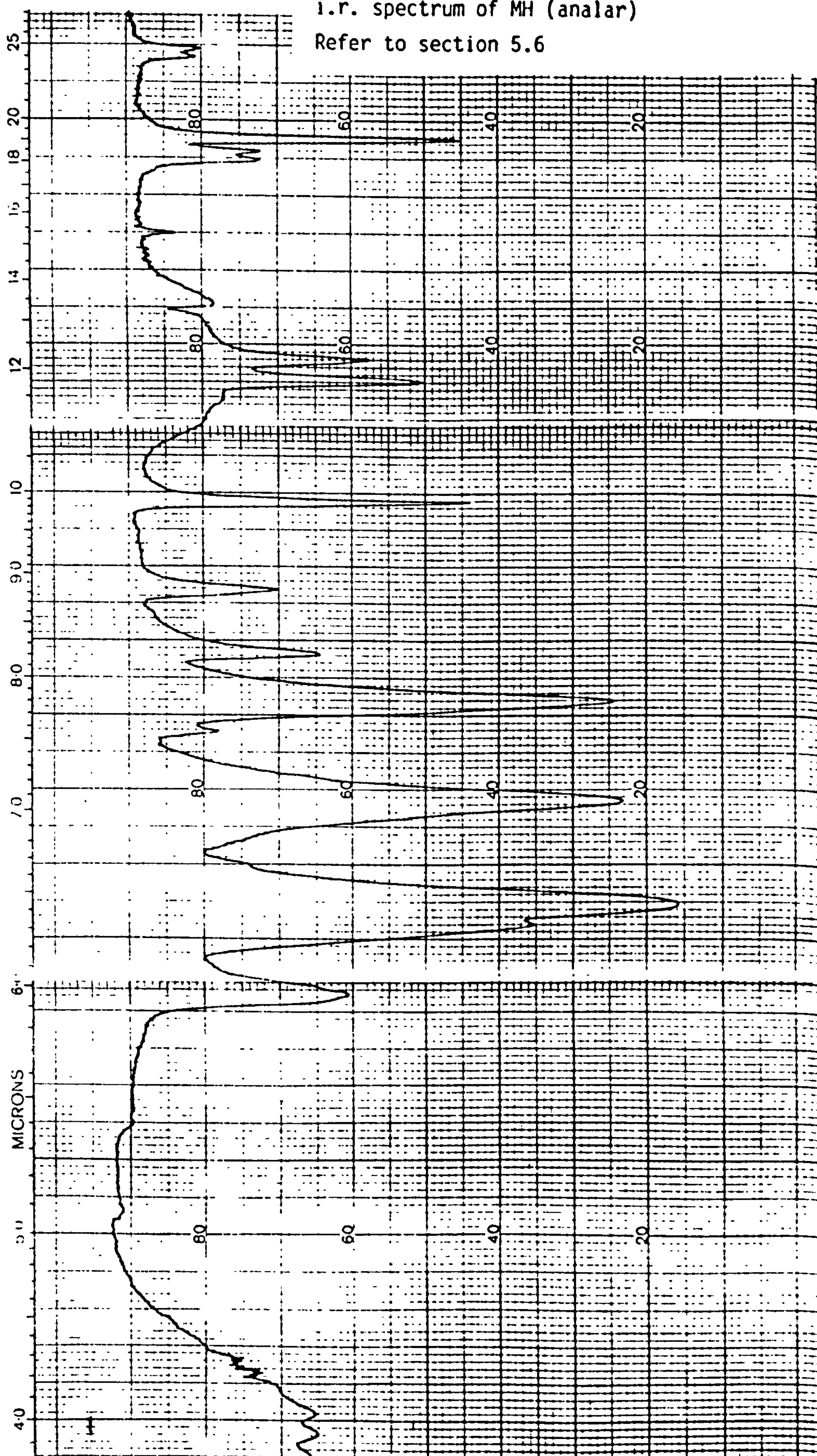


Figure 5.3
i.r. spectrum of synthesized ^{14}C -MH
Refer to section 5.6

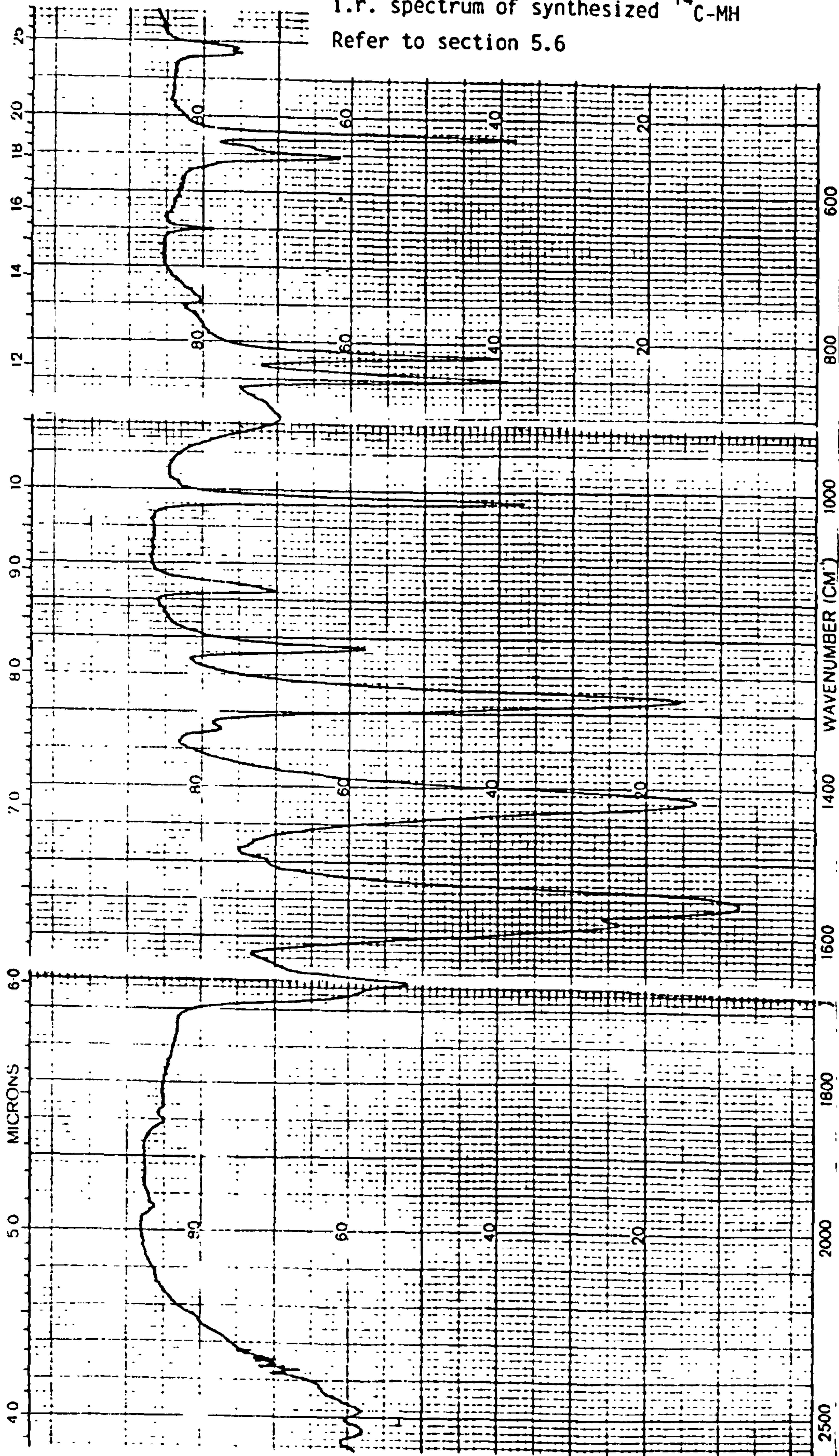
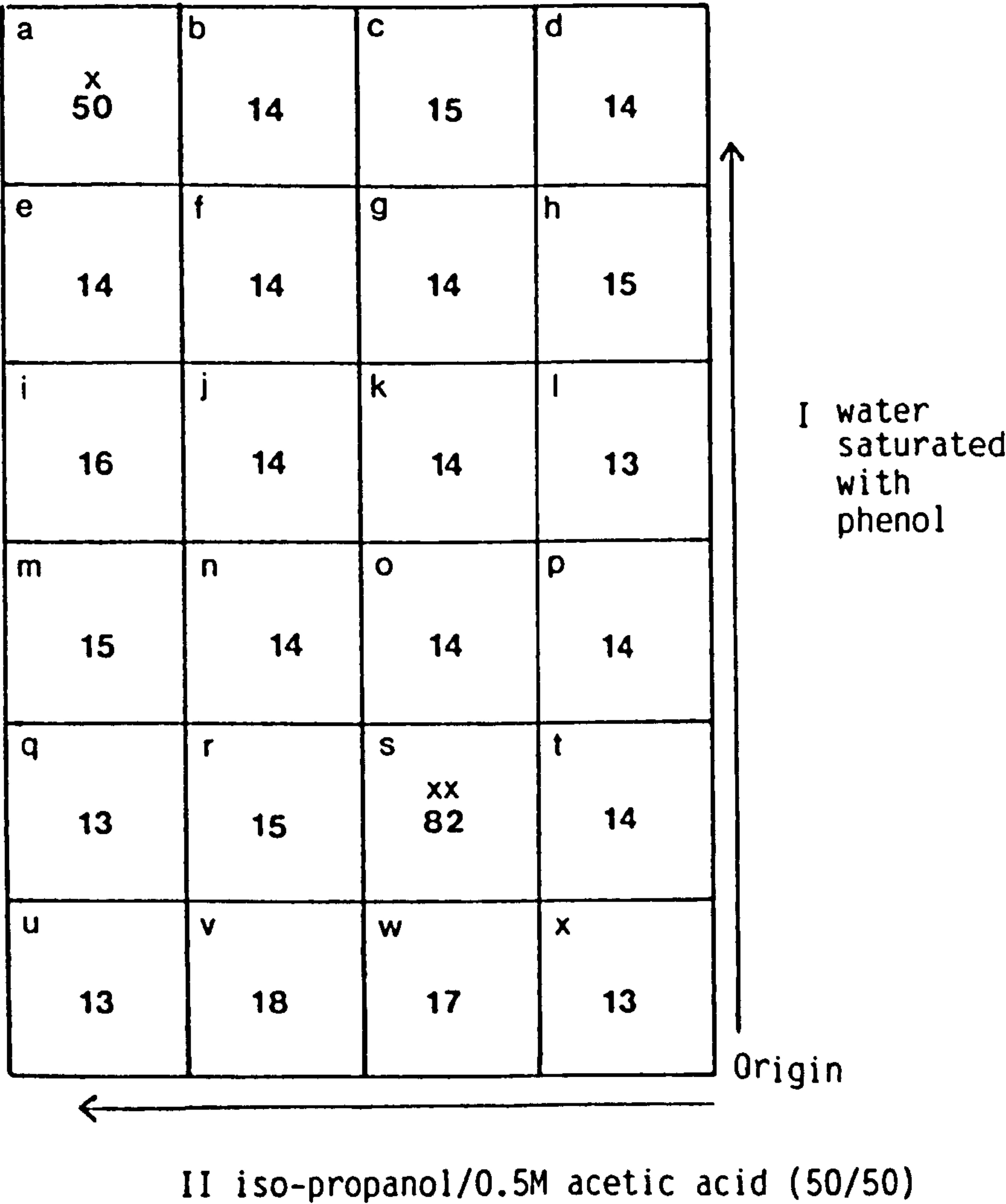


Figure 5.4, Results of activity of 2cm² fractions of the tlc plate spotted with ¹⁴C-MH treated potato extract.



In the centre of each square the figure represents the detected activity (dpm).

^x Square a was extracted with distilled water. The extract was found to contain MH. The other squares were extracted with methanol (HPLC grade).

^{xx} Square s indicates activity, not free MH

A STUDY OF CONJUGATION OF MALEIC HYDRAZIDE WITH GLUCOSE OR GLUTATHIONE6.1 Introduction

The work in this chapter attempts to identify possible metabolites by the effect of chemical reagents and specific enzymes on methanol extracted potato juice from MH treated potatoes.

Towers et al. (1958) applied ^{14}C -MH to wheat leaves and identified the ethanol soluble metabolite as a beta-D glucoside. This metabolite was detected after separation by paper chromatography using 2-D chromatography (1st direction :- phenol saturated with water, 2nd direction :- n-butanol/acetic acid/water, 4 :1:5, v/v). The metabolite was detected by radioautographs and identified as the beta-D glucoside after hydrolysis with (1) Emulsin and (2) 2N HCl for two hours.

Frear and Swanson (1978) applied ^{14}C -MH to tobacco plants. MH and a major metabolite was extracted with methanol. The major methanol extracted metabolite was identified as a beta-D glucoside. The metabolite released glucose after hydrolysis at 100°C for two hours with 1 N HCl. Glucose was released after enzymic hydrolysis with a beta-D glucosidase for 20 hours in 0.05 M acetate buffer at pH 4.5.

Newsome (1980b) could not detect any MH after hydrolysis, of a methanol extracted concentrate of MH treated potatoes, with a beta-D glucosidase (5 mg/ml in 0.1 M acetate pH 5.0) incubated at 37°C for 30 minutes.

Glutathione (GSH) has also been suspected of reacting with MH, Hughes and Spragg (1958) found that glutathione accumulated in cells where division was inhibited by MH. They postulated that MH reacted with protein thiol groups and prevented their reduction by glutathione during mitosis. Coupland and Peel (1971) also found MH to bind to thiol groups.

MH is also known to inhibit enzymes by binding to thiol groups. Baker (1961) found MH to inhibit oxygen uptake by tobacco tissue due to MH reacting with thiol groups in the dehydrogenase enzyme. Hughes and Spragg (1958) also found MH to inhibit pea seedling starch phosphorylase and beta-amylase but not trypsin or aldolase. The action of inhibition was explained as MH binding with thiol groups in the enzymes.

Thiol groups acting as nucleophiles have been found to form additional products with maleic acid and fumaric acid (Morgan and Friedmann [1938], although reaction with fumaric acid was slower.

There have been a number of reports of glutathione (GSH) conjugating with herbicides in plant systems. This metabolism is not an addition reaction but a substitution of a chlorine atom typically. The substitution of the chlorine atom in the triazine group of herbicides is catalysed by a glutathione-S-transferase system. This enzyme system is extracted by 0.1 M phosphate buffer pH 6.8, is insoluble in 50% saturated ammonium sulphate, and is soluble in dilute salt solutions (Frear and Swanson [1970]), Lamoureaux et al. [1970], Lamoureaux et al. [1971], and Lamoureaux et al. [1972]).

The purpose of this chapter is to attempt to characterise the binding of any possible conjugate by hydrolytic methods that would release free MH and be detected by HPLC or by attempting to synthesize conjugates thereby decreasing free MH which again would be detected by HPLC.

Section 6.2 uses non-specific methods to attempt hydrolysis with acid and alkali.

Section 6.3 uses a specific glucosidase to cleave any glycosides and release MH.

Section 6.4 attempts the biosynthesis of conjugates of MH with reduced GSH and UDPG with extracted potato protein as catalyst.

6.2 Acid and alkaline hydrolysis of potato juice extracts

This section attempts to cleave MH from a conjugate metabolite or to extract MH if it is adsorbed onto cellular fractions. A covalent bond may be acid or alkaline labile, physically bound MH may be desorbed and it might be expected that MH would be released. If MH is released there will be an increase in free MH, detectable by HPLC analysis, in the concentrated extract. This assumes that the MH structure is not affected and remains intact.

6.2.1 Experimental : standard solutions and treated tuber extracts were subjected to acid and alkaline hydrolysis.

- (1) Weak hydrolytic conditions : 500 g of treated tubers were minced, 50 g of this mash was homogenised with 200 ml of methanol for two minutes. After filtration (Whatman No. 1) under suction, and two washes with about 100 ml of methanol, the filtrate was made up to 500 ml with methanol. The whole sample was reduced under vacuum to 50 ml on a rotary evaporator. 4 ml sub-samples of the concentrated potato extract was pipetted into eight 10 ml sample vials. 4 ml samples of standard MH (10 ppm in distilled water) was pipetted into eight 10 ml sample vials. Two of the standard vials and two of the sample vials had added to them 0.5 ml of 0.9 M hydrochloric acid (AR grade), or 0.5 ml of 0.9 M sodium hydroxide (AR grade). The other eight standard and sample vials had 0.5 ml of distilled water added.

All the vials, except two standard vials and two sample vials with distilled water added, were heated at 80°C for one half hour.

The acid hydrolysis vials had 0.5 ml of 0.9 M sodium hydroxide added. The alkaline hydrolysis vials had 0.5 ml of 0.9 M hydrochloric acid added. Other vials had 0.5 ml of distilled water added. All the vials were made up to 5 ml. 2 ml aliquots of each vial was eluted through a strong cation exchange column (Dowex 50w x 200, 120 mm x 8 mm i.d.) and collected in a 10 ml volumetric flask.

- (2) Strong hydrolysis : the method was similar to the gentle hydrolysis conditions.

0.5 ml of either 5 M hydrochloric acid or 0.5 ml of 5M sodium hydroxide was used for hydrolysis and neutralising. The vials were heated at 80°C for two hours and kept to approximately 4.5 ml with additions of hot distilled water by pipette.

All the vials were analysed for MH by the method explained in section 3b.1.4 and summarised below.

1. Column : Partisil SAX 10 μ , 25 cm x 4.5 mm i.d.
2. Elutant : 0.1 M acetic acid pH 5.8 1 ml/min.
3. uv detection at 313 nm.

6.2.2 Results : The results in Table 6.1 are expressed as an index where the control vial is the standard. Each result is an average of the two replicate vials.

Weak hydrolysis of the standards did not decrease the MH. Weak hydrolysis of the sample and heating the control samples reduced the MH detected by HPLC. The weak acid hydrolysis was reduced by 24% while the heated control and the weak alkaline hydrolysis reduced the MH by 5%.

Strong hydrolysis of the standards and samples, and heating the control, all reduced the MH detected by HPLC. The standards were reduced by acid and alkaline hydrolysis by 23% and 35% respectively and the control was reduced by 13%. The alkaline hydrolysis sample was reduced by 55% and the control was reduced by 21%. The result for the acid hydrolysis is not available because of interferences of the HPLC chromatographs.

6.3 A study of MH cleaved from a glucose conjugate by a specific enzyme (beta-D-glucosidase)

This section investigates the increase in MH in potato extract by the action of a glucosidase.

Firstly the glucosidase activity is assessed.

- 6.3.1 Almond-beta-D-glucosidase had been purchased from Sigma Ltd., (beta-D-glucosidase glucohydrolase) and stored in the freezer for some time. The specific activity was expressed as being able to release 1 μ mole of glucose from salicin per minute at pH 5.0 and 37°C. The specific activity was calculated by its ability to release o-nitrophenol from o-nitrophenyl-beta-D-glucopyranoside.

2 ml betaD-glucosidase (0.5 mg/ml in 0.1 M sodium acetate pH 5.0) was incubated with 2 ml of 2 mM O-nitrophenyl-beta-D-glucopyranoside made up in 0.1 M sodium acetate pH 5.0.

Four vials were mixed, two were plunged immediately into boiling water for one minute, the other two were incubated for 20 minutes at 37°C in a water bath. After incubation the vials were plunged into boiling water for one minute. All vials were made up to 10 ml with 0.1 M sodium acetate pH 5.0 and filtered through Whatman No. 1 filter papers.

The absorbance of the samples were recorded, at 405 nm on a Pye Unicam SP 1800 UV-VIS spectrophotometer, against o-nitrophenol standards made up in 0.1 M sodium acetate pH 5.0.

6.3.2 Results : The calibration curve is tabulated in Table 6.2

Mean sample absorbance (corrected for controls) = 0.125

0.125 Abs = 0.22 mM o-nitrophenol

0.22 mmol L⁻¹ = 2.2 μ moles of o-nitrophenol in 10 ml

2.2 μ moles of o-nitrophenol released after 20 minutes incubation at 37°C, pH 5.0

Specific activity was calculated as 0.11 μ moles/min
The enzyme activity is approximately one tenth of its stated activity. Its concentration in section 6.3.4.1 will be suitably adjusted.

6.3.3 The release of MH from potato tuber extract by the action of beta-D-glucosidase.

6.3.3.1 Experimental : The potato extract was extracted as in section 3b.1.1.150 ml of the methanol extract was reduced under vacuum to 15 ml on a rotary evaporator. The pH was adjusted to pH 5 with 0.5 M acetic acid. 2 ml of the concentrate was pipetted into six 10 ml vials. 2 ml of beta-D-glucosidase (0.5 mg/ml in 0.1 M sodium acetate, pH 5.0) was added to two of the vials. 2 ml of 0.1 M sodium acetate pH 5.0 was added to the remaining four vials.

The two vials with the enzyme and two of the other control vials were incubated for half an hour at 37°C while the other two controls remained at room temperature.

After the incubation the four vials were plunged into boiling water for one minute then left to cool. All six vials were made up to 5 ml, filtered through pasteur pipettes plugged with cotton wool. 2 ml aliquots were eluted through a strong cation exchange column (Dowex 50 w x 200, 120 mm x 8 mm i.d.) and collected in 10 ml volumetric flasks.

The MH was separated and detected by HPLC analysis as explained in 3b.1.4 and outlined below.

1. Column: Partisil SAX 10 μ , 25 cm x 4.5 mm i.d.
2. Elutant: 0.1 M acetic acid pH 5.8, 1 ml/min
3. Detection : uv absorbance at 313 nm.

6.3.3.2 Results : The results are tabulated in Table 6.3.

1.6 µg/ml was released after incubation.
Therefore 16 µg was released in 10 ml.
Therefore 40 µg was released from the 5 ml reaction flask. The reaction flask had approximately 2 g of potato material extract. Therefore 40 µg of MH was released from 2 g potato material. The enzyme activity released 20 µg of MH per gram of potato material.

6.4 The extraction potato protein as an enzyme source

The potato protein was extracted from the leaf portion and the potato tubers as a source of catalytic enzymes that would induce conjugation of MH to a glucose source or to reduced glutathione.

6.4.1 Potato leaf protein extract : Four seed tubers (cv Maris Piper SE2) were planted in two twelve inch pots filled with peat and kept well watered. The seed was germinated and grown outside for two months. The stems were cut at the base and the leaves were well washed and rinsed in distilled water. The extraction procedure was an adaption of the method of Frear and Swanson (1970) and Lamoreaux et al. (1972).

The potato leaves were extracted by homogenising 50 g of the excised potato leaves with 250 ml of cold 0.1 M sodium dihydrogen phosphate pH 6.8 with 25 g of Polyclar AT and 10 g of ice for three minutes. The homogeniser had been stored overnight in a freezer.

The mixture was filtered under vacuum through a celite pad on Whatman No. 1 filter paper into a Buchner flask surrounded by ice.

The filtrate was further clarified by 15 minutes centrifugation (3000 rpm). The supernatant was decanted and had ammonium sulphate added to make a 50% saturated solution (60 g salt to 170 ml supernatant). The solution was left in ice for forty minutes, the precipitated protein was spun down by centrifugation (3000 rpm), the supernatant was discarded.

The crude protein fraction was dissolved in 140 ml of distilled water and made up to 25% saturated ammonium sulphate (25 g).

After the ammonium sulphate dissolved the solution was left in ice for 25 minutes before centrifugation (3000 rpm) for 15 minutes. The supernatant was collected and the protein was again precipitated by addition of ammonium sulphate to make 50% saturated solution, then left for one hour in ice.

The mixture was centrifuged (3000 rpm) for 15 minutes. The precipitated protein was dissolved in distilled water (50 ml) and used immediately as the potato leaf protein or frozen and defrosted when required.

- 6.4.2 Potato tuber protein : The potato tuber protein was extracted in a similar fashion as the potato leaf protein (section 6.4.1) as detailed by Duncan (1965).

500 g of potato tubers (cv Maris Piper) were washed, thinly sliced mixed with activated charcoal (15 g) then minced in a mincer and stored overnight in a freezer. The minced potato mash was squeezed through muslin and the juice was clarified by centrifugation for 20 minutes (3000 rpm) yielding 130 ml of juice.

The protein was precipitated and partially purified by precipitation, dissolution and clarification, and finally precipitation as described in section 6.4.1.

6.5. A study of a conjugation between MH with activated glucose (UDPG)

This section investigates the possibility of conjugating MH with the activated glucose form uridine diphosphoglucose (UDPG, Sigma Ltd).

6.5.1 Experimental : 2 ml of 0.5 mM MH made up with 0.1 M sodium dihydrogen phosphate pH 6.5 had 2 ml of 0.5 mM UDPG added which was also made up in 0.1 M sodium dihydrogen phosphate pH 6.5. 1 ml of potato leaf protein was added and incubated at 25°C. Vials were also incubated with denaturated potato leaf protein.

Vials were taken from the incubation system and plunged into boiling water for one minute. Vials were denaturated after 0, 15, 30 and 60 minutes.

The solutions were filtered through a pasteur pipette plugged with cotton wool.

The solutions were analysed by HPLC detailed in section 3b.1.4.

6.5.2 Results : The results are tabulated in Table 6.4.

It is obvious that no MH had been conjugated with glucose by the method described.

6.6 A study of conjugation between MH with reduced glutathione

This section investigates the possibility of conjugating MH with reduced glutathione using (1) potato protein and (2) a mammalian glutathione-S-transferase.

6.6.1 Experimental : 16 vials had 2 ml of 0.5 mM glutathione (dissolved in 0.1 M sodium dihydrogen phosphate, pH 6.5) and 2 ml of 0.5 mM MH added. Eight vials had 1 ml of potato leaf protein (section 6.4.1) the other eight had 1 ml of potato tuber protein (section 6.4.2) added.

Two vials with the potato leaf protein and two vials with the potato tuber protein were immediately plunged into boiling water for one minute, made up to 10 ml when cool, then filtered through a pasteur pipette plugged with cotton wool.

The other vials were incubated at 25°C. After 15, 30 and 60 minutes four vials (two with leaf protein, two with tuber protein) were denatured and filtered as above.

The MH was detected and quantified by HPLC analysis described in section 3b.1.4.

6.6.2 Experimental : This section investigates a mammalian enzyme system that may conjugate MH to glutathione.

Horse liver glutathione-S-transferase was purchased from Sigma Ltd. 5 mg of equine liver transferase was dissolved in 2 ml of 0.1 M sodium dihydrogen phosphate, plus 1 mM EDTA at pH 6.5.

The assay was similar to the previous section (section 6.6.1), where 2 ml of 0.5 mM glutathione and 2 ml of 0.5 mM MH (made up in 0.1 M sodium dihydrogen phosphate with 1 mM EDTA, pH 6.5) were incubated at 25°C with 0.1 ml of enzyme. Vials were plunged into boiling water for one minute after incubation, cooled, made up to 10 ml and filtered through a pasteur pipette plugged with cotton wool. Vials were incubated for 0, 15, 30 and 60 minutes.

The MH was analysed by HPLC similar to that in section 3b.1.4.

- 6.6.3 The sample solutions were also analysed for thiol groups with Ellmans reagent which is specific for thiol groups (Orfanos et al. [1980]).

All samples and standards were diluted 1:2 with 0.1 M sodium dihydrogen phosphate pH 6.8. 2 ml of sample and standards had added 1 ml of Ellmans reagent (238 mg 5, 5-dithio bis [2nitrobenzoic acid] in 100 ml of 0.1 M sodium dihydrogen phosphate). The absorbance was read after ten minutes at 415 nm on a Pye Unicam SP 1800 UV-VIS spectrophotometer against a range of standards. Standard curve is detailed in Table 6.5.

- 6.6.4 Results of conjugation assays.

Table 6.5 tabulates the standard calibration curve of glutathione with Ellmans reagent.

Tables 6.6 and 6.7 detail the concentrations of MH and glutathione after attempts to conjugate with potato leaf protein.

Tables 6.8 and 6.9 detail the concentrations of MH and glutathione after attempts to conjugate with potato tuber protein.

Tables 6.10 and 6.11 detail the concentrations of MH and glutathione after attempts to conjugate with a glutathione-S-transferase.

6.7 Results and discussion

Strong acid and alkaline hydrolysis are found to decrease MH standards. Weak and strong acid and alkaline hydrolysis reduced the MH that could be detected.

MH was released from potato juice extract by the action of a beta-D-glucosidase. However, no conjugate could be synthesised with MH and UDPG (Uridine diphospho glucose) by potato leaf protein.

A conjugate could not be synthesised between MH and glutathione by potato leaf or tuber protein nor a glutathione-S-transferase.

If MH was released from some conjugate the increase may be masked by decomposition and degradation. The action of non-specific hydrolysis may alter the MH structure and therefore may not show on the chromatographic trace at the characteristic retention time of MH standards. The hydrolytic conditions may catalyse reactions that would bind MH in the sample extract to other molecules.

MH was cleaved from a potato juice extract by a beta-D-glucosidase although no conjugate could be synthesised. There have been glucoside metabolites of MH found in other plant systems (Towers et al. [1958], Frear and Swanson [1978]). However, Newsome (1980) could not detect any conjugate of MH and glucose residues in potato tubers. The method used by Newsome (1980b) was specific cleavage by a beta-D-glucosidase. There are two possibilities why Newsome's results do not show MH after cleavage. (1) the enzyme used in this thesis was more specific or more active, (2) the detection system was more sensitive and able to detect the slight increase in MH.

The study of MH conjugation with UDPG and reduced glutathione shows negative results. The potato protein may not have catalysed the reaction between MH and glutathione (or MH and UDPG). Frear and Swanson (1970) found some plant tissue had low or no detectable glutathione-S-transferase. The enzyme was found in corn leaves, sorghum leaves, sugarcane, johnson grass and sudan grass. Root tissue of corn and sorghum however contained little enzyme activity. Peas, oats, wheat and barley contained no detectable enzyme activity.

The extraction process would be a heterogeneous mix of enzymes, possibly deactivating the UDPG or reduced glutathione. There is further scope to investigate conjugation reactions once the potato protein enzymes could be clarified. Lamareaux et al. (1972) suggests a method for purification of a glutathione-S-transferase. Any further studies to investigate GSH conjugates should use purified enzyme systems.

Table 6.1

The results of hydrolytic conditions on MH

	<u>Weak hydrolysis</u>		<u>Strong hydrolysis</u>	
	<u>Standard</u>	<u>Sample</u>	<u>Standard</u>	<u>Sample</u>
Control	100	100	100	100
Control heated	101	95	87	79
Acid hydrolysis	101	76	77	NA
Alkaline hydrolysis	102	95	65	45

NA Due to interference in the chromatogram with the same retention as MH, the result is not available

Refer to section 6.2.2.

Table 6.2

Calibration curve of o-nitrophenol absorbance

mM o-nitrophenol	Abs 405 nm
0	0.01
0.4	0.22
0.8	0.44
1.2	0.65
1.6	0.87
2.0	1.17

- (i) Refer to graph 6.1
- (ii) Refer to section 6.3.2.

Table 6.3

The results of beta-D-glucosidase on potato juice extract

Description of treatment	μ g/ml
Control	8.1 (9.1, 7.1)
Incubation, no enzyme	8.3 (9.6, 6.9)
Incubation, enzyme	9.9 (10.6, 9.2)

- (i) Replicate vial results are in brackets
- (ii) Refer to section 6.3.4.2.

Table 6.4

MH levels during attempts of conjugating MH with UDPG
(uridine diphospho glucose)

Time (minutes)	MH (mM)
0	0.20 (0.199, 0.200)
15	0.20 (0.200, 0.200)
30	0.20 (0.198, 0.199)
60	0.20 (0.199, 0.200)

(i) Replicate vial results are in brackets

(ii) Refer to section 6.5

Table 6.5

Glutathione calibration curve

Glutathione (mM)	Abs 415 nm
0.00	0.24
0.05	0.37
0.10	0.52
0.15	0.67
0.20	0.80

- (i) Refer to graph 6.2
- (ii) Refer to section 6.6.4.

Table 6.6

MH levels during attempts at conjugating MH with glutathione
(potato leaf protein)

Time (minutes)	MH (mM)
0	0.20 (0.199, 0.202)
15	0.20 (0.199, 0.204)
30	0.20 (0.203, 0.202)
60	0.20 (0.198, 0.202)

- (i) Replicates are in brackets
- (ii) Refer to section 6.6.

Table 6.7

Glutathione levels during attempts at conjugating MH with glutathione
(potato leaf protein)

Time (minutes)	Abs 415 nm
0	0.55 (0.55, 0.55)
15	0.53 (0.56, 0.49)
30	0.49 (0.48, 0.49)
60	0.53 (0.53, 0.52)
Control 60 minutes (no glutathione)	0.23 (0.23, 0.23)

- (i) Replicates are in brackets
- (ii) Refer to section 6.6.

Table 6.8

MH levels during attempts at conjugating MH with glutathione
(potato tuber protein)

Time (minutes)	MH (mM)
0	0.20 (0.198, 0.201)
15	0.20 (0.199, 0.199)
30	0.20 (0.199, 0.202)
60	0.20 (0.202, 0.201)

- (i) Replicates are in brackets
- (ii) Refer to section 6.6

Table 6.9

Glutathione levels during attempts at conjugating MH with glutathione
(potato tuber protein)

Time (minutes)	Abs 415 nm
0	0.68 (0.70, 0.66)
15	0.72 (0.71, 0.72)
30	0.68 (0.68, 0.68)
60	0.70 (0.68, 0.72)
Control 60 minutes (no glutathione)	0.20 (0.20, 0.20)

- (i) Replicates are in brackets
- (ii) Refer to section 6.6.

Table 6.10

MH levels during attempts of conjugating MH with glutathione
(glutathione-S-transferase)

Time (minutes)	MH (mM)
0	0.20 (0.198, 0.201)
15	0.19 (0.198, 0.199)
30	0.20 (0.200, 0.201)
60	0.20 (0.200, 0.199)

(i) Replicates are in brackets

(ii) Refer to section 6.6.

Table 6.11

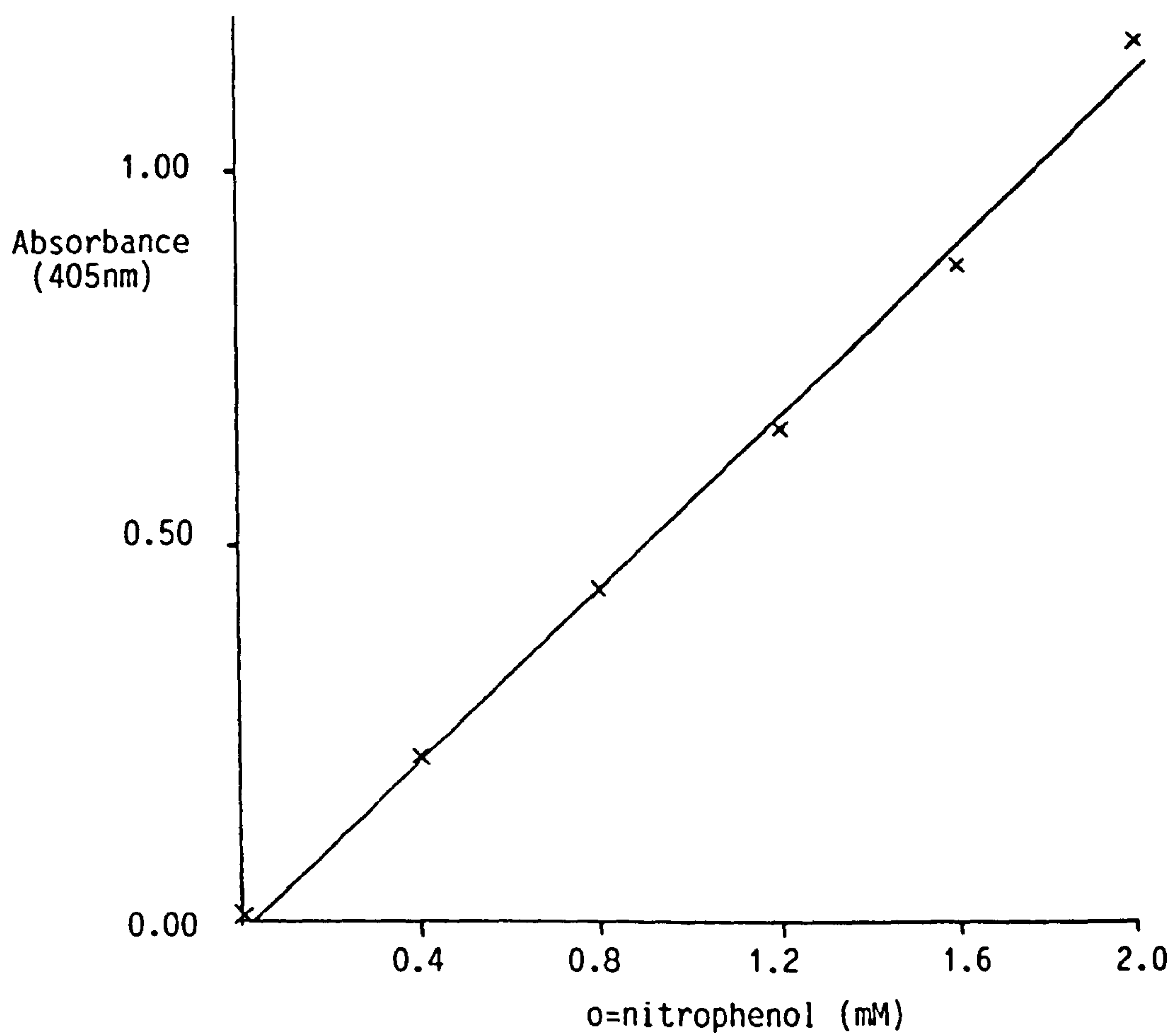
Glutathione levels during attempts at conjugating MH with glutathione
(glutathione-S-transferase)

Time (minutes)	Abs 415 nm
0	0.62 (0.61, 0.62)
15	0.60 (0.60, 0.60)
30	0.61 (0.61, 0.61)
60	0.62 (0.61, 0.62)
Control 60 minutes (no glutathione)	0.20 (0.20, 0.20)

(i) Replicates are in brackets

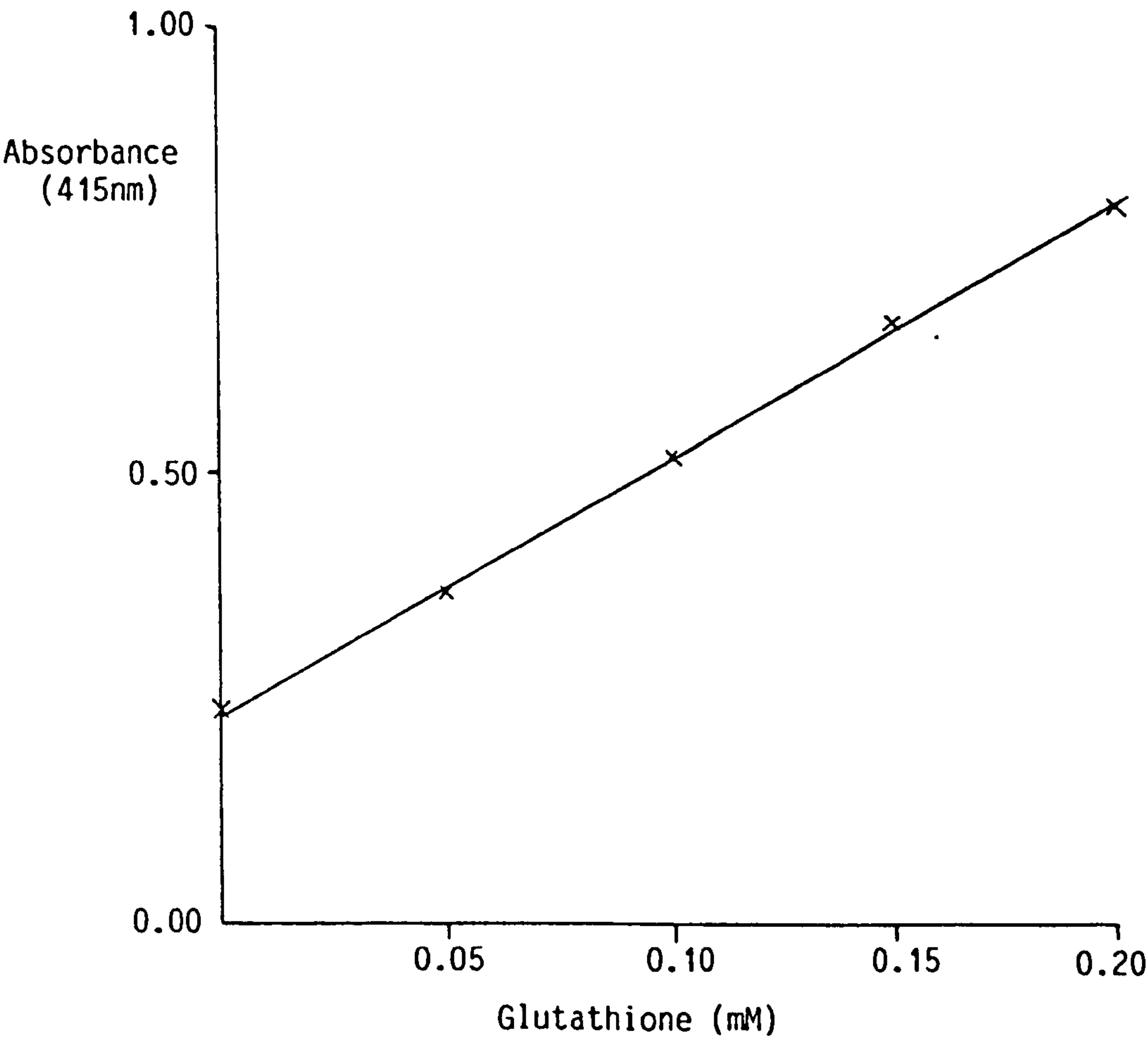
(ii) Refer to section 6.6.

Graph 6.1, Calibration curve of o-nitrophenol



Refer to section 6.3.1.1

Graph 6.2, Glutathione calibration curve
(reacted with Ellmans reagent)



Refer to section 6.6.3 for details

THE EFFECT OF MALEIC HYDRAZIDE ON WOUND HEALING IN TUBERS7.1 Introduction : The process of wound healing

The process of wound healing reduces water loss maintaining an acceptable product and preventing weight loss. The process also prevents the spread of disease organisms into cut tubers (Shapalov and Edson [1919], Fox et al. [1971], Paterson and Gray [1972]). Wound healing is a process which has been studied extensively over the years (Priestley and Woffenden [1923], Thomas [1982]).

Priestley and Woffenden (1923) described how a wounded tuber cut surface was blocked within a period of 12-36 hours at room temperature by suberin deposition occurring on or just below the wounded surface.

After a few more days the cells immediately below start to divide to form a periderm and the cells of this layer are suberised to complete the wound healing process. This simplistic description is still accepted today and is pictorially represented in Figure 7.1. The processes have been investigated by subsequent workers and the factors known to affect wound healing are temperature, humidity, oxygen and carbon dioxide levels, cultivar, and agrochemicals. These factors and their effects are described below :

- (1) Temperature : it has been known for some time that the optimum temperatures are between 20°C and 25°C (Artshwager [1927], Thomas [1982], Priestley and Woffenden [1923]). In commercial stores it is normal practice to allow a curing period of two to three weeks at 12°C to 13°C when potatoes are first taken into store to allow wound healing.
- (2) Relative humidity : Artshwager (1927) recommends that the optimum relative humidity for wound healing is greater than 80% R H

- (3) Oxygen and carbon dioxide levels : lowering oxygen concentration retards or prevents periderm formation and suberin development (Lipton [1967]). Increasing the carbon dioxide concentration also retards or prevents periderm formation and suberin development (Wiggington [1974]).
- (4) Cultivar : the effect of cultivar has been noted by Preistley and Woffenden (1923) and Artshwager (1927), although Wiggington (1974) was unsure in that these effects could be due to maturity and not cultivar. McGee et al. (1985b) pinpointed cultivars with good wound healing ability. These were Desiree, Bintje and Pentland Hawk. Poor wound healing cultivars were Doon Star, Pentland Crown and Majestic.
- (5) Chemical effects : Cunningham (1953) found MENA (the methyl ester of naphthalene acetic acid) delayed surface suberisation and that the periderm formation was either delayed or completely absent. He found that TCNB (1,2,4,5-tetrachloro-3-nitrobenzene) did not delay suberin although deposition was irregular. Furthermore periderm formation did occur but was delayed.

Reeve et al. (1963a, 1963b) shows that CIPC (1-methylethyl [3-chloropheny] carbamate) inhibits wound periderm formation at rates of 10 ppm and 100 ppm vapour based on tuber weight and emulsion dips of 0.5% and greater. McGee (1984) and Leonard et al. (1986) confirm that CIPC is detrimental to wound healing. However TCNB did not show any adverse effects and may also improve wound healing.

This chapter investigates the effects of MH on the wound healing process. The process is investigated by the traditional histological examination of surface tissue and by a more recent method of measuring the rate of water loss from cut potato discs. The comparison should give an indication of the effect MH has on wound healing.

7.2 Histological examination of cut tubers treated with MH

The traditional method of assessing wound healing has been by histological examination of cut potato tissue using stains and light microscopy. The areas of activity have been primarily suberin development and cell division.

This section uses two stains, Sudan III to identify suberin and Toluidine blue to differentiate cells dividing from other cells.

7.2.1 Potato treatment and storage

44 kilograms of Cyprus grown ware (c.v. Cara) were purchased locally. The potatoes were thoroughly cleaned then soaked in tubs of sodium hypochlorite (1%) for ten minutes and rinsed well in distilled water.

All the tubers were cut with a razor blade to reveal an area between 2-3 cm diameter. The potatoes were split into five batches.

Two batches had 100 µl of boiled distilled water applied to the cut surface and allowed to dry at ambient room conditions. One of these batches was stored in a 10 kg cardboard box with 100 g of CIPC crystals (20 mg/kg) in a petri dish with wire gauze cover. The other batch was stored in a 10 kg cardboard box as a control.

The other three batches of cut potatoes had solutions of MH added. Solutions of MH were made up in boiled distilled water to concentrations of 10, 100 and 1000 ppm, applied in 100 µl volumes deposited by a microsyringe and left to dry. The MH treated batches were also stored in 10 kg cardboard boxes.

All potatoes were stored with the cut surface facing upwards, with no cut surfaces leaning against any other surface.

7.2.2 Staining solutions

All chemicals and solvents were Analar, HPLC grade or similar.

- (1) FAA solution : 500 ml of 95% ethanol, 50 ml glacial acetic acid, 100 ml formaldehyde, and 350 ml of distilled water were mixed and stored as a preservative (Nielsen [1973]).
- (2) Sudan III : 1 g of Sudan III was dissolved in 100 ml of ethanol and filtered. This stock solution was diluted with FAA at a 1:20 ratio (Nielsen [1973]).

Normal cell walls were unaffected by the stain, Suberin stains red.

- (3) Toluidine blue : 0.5 gm in 1000 ml of distilled water. Suberised cell walls are stained blue. Normal cells are stained purple. It also helps to differentiate dividing cells (McGee [1984]).

7.2.3 Assessment of the extent of suberisation and cell division

The suberisation and cellular division was assessed by rank. Each section (16-20 sections per treatment per day) was assessed for :

(1) Intensity of suberisation :

- 0 - No suberisation
- 1 - Suberisation just visible
- 2 - Suberisation clearly visible
- 3 - Strong suberisation

(2) Thickness of suberised layer in cell layers to the nearest 0.5 cell units.

(3) The depth of suberisation below surface. The number of cell layers above the suberised cells measured to the nearest layer.

On each day of tissue examination, five potatoes were taken from the boxes. From each potato two blocks of about 1 cm^2 was cut with a razor from each cut surface.

These blocks were stored in FAA solution until stained. This was usually between two and four hours. Four sections less than 0.5 mm thick were hand cut with a razor from each block. Two of these sections were stained with Sudan III. The other two sections were stained with Toluidine blue. After at least an hour the sections were placed on microscope slides with cover slips to reduce dessication. The slides were studied with a Nikon binocular (x30) magnifier. The sections were illuminated from above.

7.2.4 Results : The suberin development and cellular activity measurements are tabulated in Table 7.1 and 7.2 respectively.

The results are tabulated as units of cellular thickness with the standard errors (SE) included.

$$SE = \frac{S}{\sqrt{n}} \quad \text{equation 7.1}$$

$$S^2 = \frac{\sum x^2 - \frac{(\sum x)^2}{n}}{n-1} \quad \text{equation 7.2}$$

x = individual values

n = number of values

S^2 = variance

S = standard deviation

The suberin layer developed over the nine days on all treated tubers and the controls. On day three the three MH treated sections had a greater suberised layer than the control. The CIPC treated layer had a lower thickness of suberin. On day six the low and high MH concentrations, and the CIPC had a greater suberin layer than the control, however, the middle concentration had a shallower suberin layer. On day nine the low and high MH levels were still greater than the control, the CIPC had the same thickness. The middle MH concentration had the shallower suberin layer.

The Toluidine blue shows that all treatments have a thicker area of cellular activity than the control after three days. After six days the low and high MH treatments still had a thicker area of cellular activity than the control. After nine days the high treatment still had a thicker area of activity than the control whereas the low and middle concentration had drastically decreased areas of activity.

The method and stains were found to be very subjective and would require a good deal of experience and histological practice. The differences in thickness show that the suberin development does increase for all treatments. However this method does not seem to give a clear objective picture of suberin development.

The Toluidine blue staining illustrates a very confusing picture and once again is very subjective and not useful in practice for the non-experienced, lacking histology practice.

7.3 An assessment of wound healing by the rate of water loss from cut potato discs treated with MH

Because of this subjectivity in the histological assessment of wound healing a method was developed by Jarvis and Duncan (1979) adapted from Kalattukudy and Dean (1974). This method assesses wound healing by calculating the rate of water loss from cut potato discs. It has been shown to correlate with histological examination (McGee [1984]) and has been shown to assess wound healing and has been used to demonstrate inhibition of wound healing by CIPC and no detrimental effects by TCNB [McGee et al. [1985a], [1985b], Leonard et al. [1986]].

The method detailed by Jarvis and Duncan (1979) was used in this section to assess the rate of water loss in artificially treated cut potato discs treated with MH and in field treated tubers.

The CIPC was not included as a positive control in this series of assessments because of it has well-documented detrimental effects reported in the work of Leonard et al. (1986). The above work by Leonard et al. (1986) had been carried out at Glasgow University during this thesis time span. No duplication was considered necessary.

7.3.1 Method of assessment : Potatoes were taken from store from the 1986-87 season (see Table 2.9) one month after harvesting and four months after harvesting. The potatoes used for assessment by applying MH solutions were bought locally (cv Maris Piper). All potatoes for assessment were hand washed and dried. Each potato was dipped in methanol and flamed twice. 11 mm diameter cores were cut through the waist, 4 mm thick discs were cut from the cores just below the surface of the potato skin. Each potato yielded between eight and twelve discs.

The discs were placed in inverted petri dishes, the bottom portion which had previously had agar (1.2% agar) poured and set. This maintained a constant high humidity during the wound healing process. Each petri dish contained six cut potato discs.

Fresh cut potato discs were cut every day of water loss assessment.

On the day of measuring the water loss a petri dish with lid was weighed. All weights were measured to 4 decimal places. The potato discs were transferred to this petri dish and weighed again. The petri dish containing the cut potato discs were placed under a jet of unheated air from a hair dryer suspended 30 cm above the open petri dish for 90 seconds then re-weighed. The petri dish was again placed under the jet of unheated air for three 20 second periods. Each time the petri dish was weighed.

The rate of water loss was calculated from the weights of potato discs and the loss in water over the three separate 20 second periods.

The relative humidity and temperature were also noted under the hair dryer.

The resistance to water loss is a combination of internal and external resistance. The external resistance to water loss is primarily increased by high relative humidity and decreased by high temperature.

The internal resistance is due to the properties of the potato discs. A well developed suberin, and cell division producing a periderm, will increase the resistance to water loss.

The rates of water loss are calculated and expressed by the equations 7.3 and 7.4 below (McGee [1984]).

$$r \text{ ext.} = d (1-RH)/E_o \quad \text{equation 7.3}$$

$$r \text{ int.} = d (1-RH)/(E-r \text{ ext}) \quad \text{equation 7.4}$$

$r \text{ ext.}$ - total external resistance to water loss

$r \text{ int.}$ - total internal resistance to water loss

E - rate of water loss per unit area of aged discs

E_o - rate of water loss per unit area of fresh discs
at the airstream temperature

RH - relative humidity

7.3.2 This section assesses the rate of water loss from cut potato discs with MH applied in solution.

Discs from locally bought ware tubers (cv Maris Piper) were cut and prepared with either 100 μ l of sterilised water (control) or 100 μ l of 10, 100 and 1000 μ g/ml MH (prepared in distilled water) deposited by a microsyringe.

The discs were assessed over three, six and nine days. The results are expressed as total resistance and are tabulated in Table 7.3.

- 7.3.3 This section assesses the rate of water loss from potato discs cut from field treated potatoes stored over a storage season. Treated ware tubers of the 1986-87 season were compared with untreated ware tubers at the beginning of the storage season (one month after harvest) and after four months storage at $8 \pm 1^{\circ}\text{C}$.

The results are expressed as internal resistance and are a direct comparison of the tissues ability to wound heal, and are tabulated in Table 7.4.

The discs were assessed over three, six and nine days.

- 7.3.4 Results : The results are calculated as the mean value of the number of similar treated petri dishes with three 20 second duration measurements per plate. The standard errors (SE) are calculated by equation 7.6 (McGee [1984]).

$$\bar{x} = \frac{\sum x}{n} \quad \text{equation 7.5}$$

$$SE = S \sqrt{\frac{S^2}{n-1}} \quad \text{equation 7.6}$$

$$S^2 = \frac{\sum x^2 - (\sum x)^2}{n-1} \quad \text{equation 7.7}$$

x - individual values

x - mean

SE - standard error

S^2 - variance

Section 7.3 has assessed the effect of MH on wound healing by the rate of water loss from cut potato discs. Section 7.3.2 suggests that cut potato discs with MH applied in solution do not have a reduced ability to wound heal, over nine days ageing, compared with the controls.

Section 7.3.3 suggests that MH field treated tubers do not differ from the controls over nine days ageing at the initial stages of storage.

After four months storage the MH field treated tubers have a better wound healing ability compared to the controls. However, it should be noted that the controls had broken dormancy and the tubers were in the early stages of sprouting.

7.4 Discussion : The effect of MH on wound healing in tubers

The sections in this chapter suggest that MH does not reduce or increase the wound healing process when potatoes are first harvested. As potatoes come out of store after a few months MH does not hinder the wound healing process compared to the controls.

There is no previous work on the effect of MH on wound healing. MH is known to inhibit cell division (Greulach and Haesloop [1954], Nooden [1969]). Cell division has been known to be an intrinsic part of the wound healing process since 1923 (Preistley and Woffenden [1923]). It might have been expected that MH would reduce the cell division and therefore reduce the wound healing ability. This section has shown that MH does not adversely affect the wound healing process.

The work of Leonard et al. (1986) is graphically displayed in graph 7.5 and shows the effects of CIPC on wound healing. Comparing graph 7.4 with graph 7.5 demonstrates that MH has no detrimental effects compared to control tubers.

However, the wound healing process was only assessed over nine days because it would be the time period immediately after the initial cut or graze that would allow infection into the tuber. MH may affect the wound healing process after nine days when the cell division is the major factor in wound healing.

The rate of water loss was only assessed on one cultivar (Maris Piper) and the histological examination only assessed on imported Cara from Cyprus. Other cultivars may show different results.

The wound healing process is also affected by the maturity (McGee et al [1985a]). MH in potatoes of different maturities may also show different results.

MH has phenolic properties (Miller [1955]). Phenolic compounds have stimulated the formation of a thicker suberin layer in potatoes (Simonds et al. (1953) and have been known to be associated with wound barrier formation in vegetables (Craft and Andia [1962])).

Table 7.1

Development of suberin layer (Sudan III)

	3 days	6 days	9 days
Control	0.88 (± 0.06)	1.29 (± 0.07)	1.58 (± 0.05)
CIPC	0.82 (± 0.06)	1.52 (± 0.05)	1.53 (± 0.09)
10 ppm MH	0.90 (± 0.05)	1.42 (± 0.08)	1.67 (± 0.05)
100 ppm MH	0.95 (± 0.03)	1.25 (± 0.06)	1.53 (± 0.06)
1000 ppm MH	0.93 (± 0.04)	1.46 (± 0.05)	1.75 (± 0.06)

- (i) Refer to graph 7.1
- (ii) Refer to section 7.2
- (iii) The standard errors (equation 7.1) are represented in the brackets.

Table 7.2

Thickness of cellular activity (Toluidine blue)

	3 days	6 days	9 days
Control	1.27 (\pm 0.20)	1.29 (\pm 0.06)	1.32 (\pm 0.10)
CIPC	1.46 (\pm 0.13)	1.27 (\pm 0.11)	1.28 (\pm 0.12)
10 ppm MH	1.35 (\pm 0.10)	1.34 (\pm 0.06)	1.00 (\pm 0.09)
100 ppm MH	1.45 (\pm 0.08)	1.28 (\pm 0.06)	1.05 (\pm 0.10)
1000 ppm MH	1.34 (\pm 0.09)	1.31 (\pm 0.08)	1.39 (\pm 0.11)

- (i) Refer to graph 7.2
- (ii) Refer to section 7.2
- (iii) The standard errors (equation 7.1) are represented in the brackets.

Table 7.3

The total resistance of cut potato discs aged with
MH applied in solution

	Day zero	Day 3
Control	0.22162 ± 0.03019	0.23681 ± 0.01052
10 ppm MH	-	0.22763 ± 0.00578
100 ppm MH	-	0.24024 ± 0.00700
1000 ppm MH	-	0.24542 ± 0.00494

	Day 6	Day 9
Control	0.26098 ± 0.00382	0.62880 ± 0.04601
10 ppm MH	0.25968 ± 0.00497	0.58961 ± 0.04376
100 ppm MH	0.26637 ± 0.00385	0.66163 ± 0.03512
1000 ppm MH	0.26340 ± 0.00492	0.66880 ± 0.02435

- (i) The results are expressed as the total resistance
 (dm⁻¹ S) ± SE (equation 7.6)
- (ii) Refer to section 7.3.2
- (iii) Refer to graph 7.3

Table 7.4

The internal resistance of cut field treated potato discs

Beginning of storage season November 1986

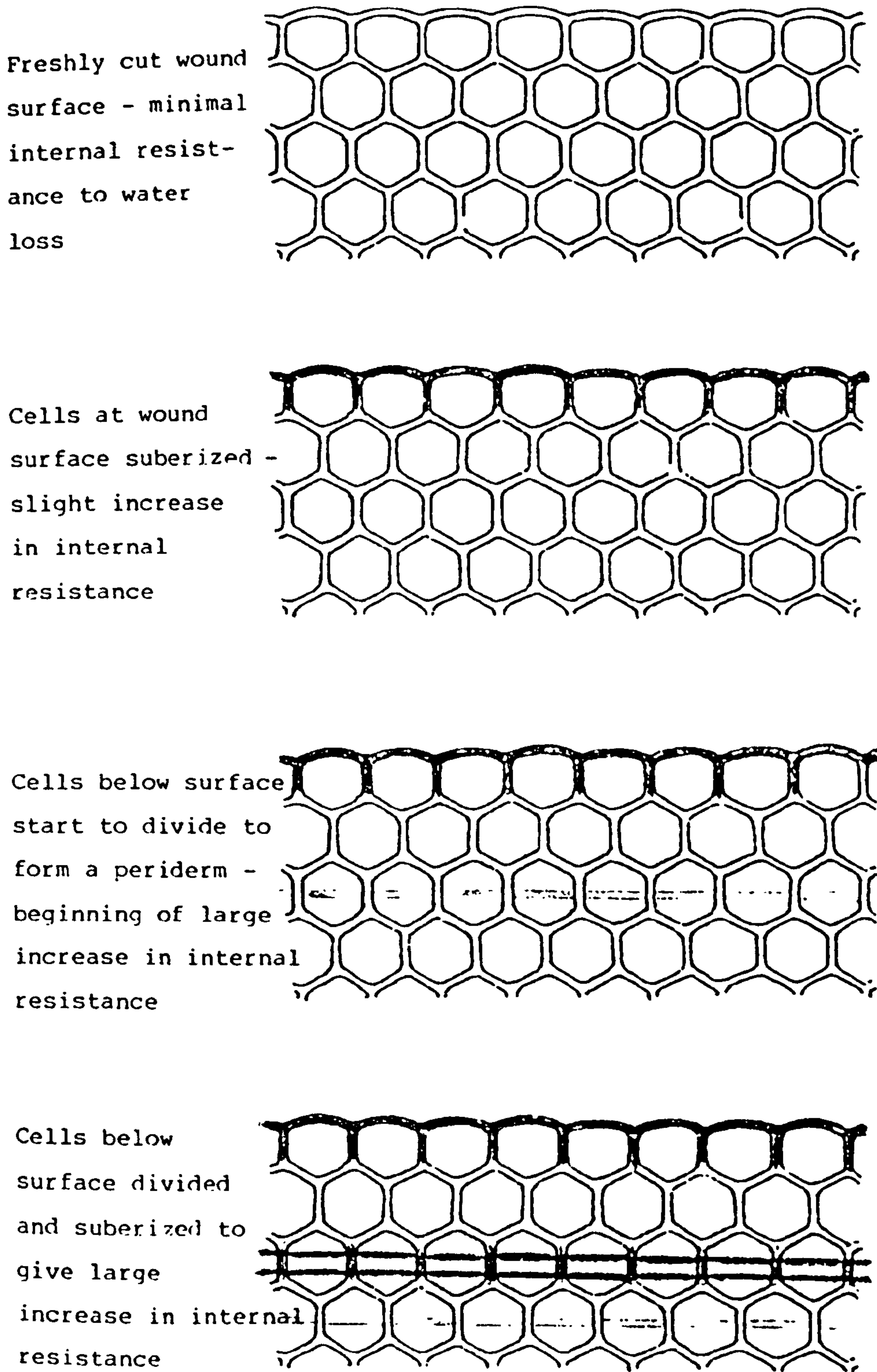
	Day 3	Day 6	Day 9
Untreated	0.02788 ± 0.00503	0.24727 ± 0.01734	0.42415 ± 0.03080
Treated	0.02505 ± 0.09464	0.26253 ± 0.02045	0.41643 ± 0.03154

End of storage season February 1987

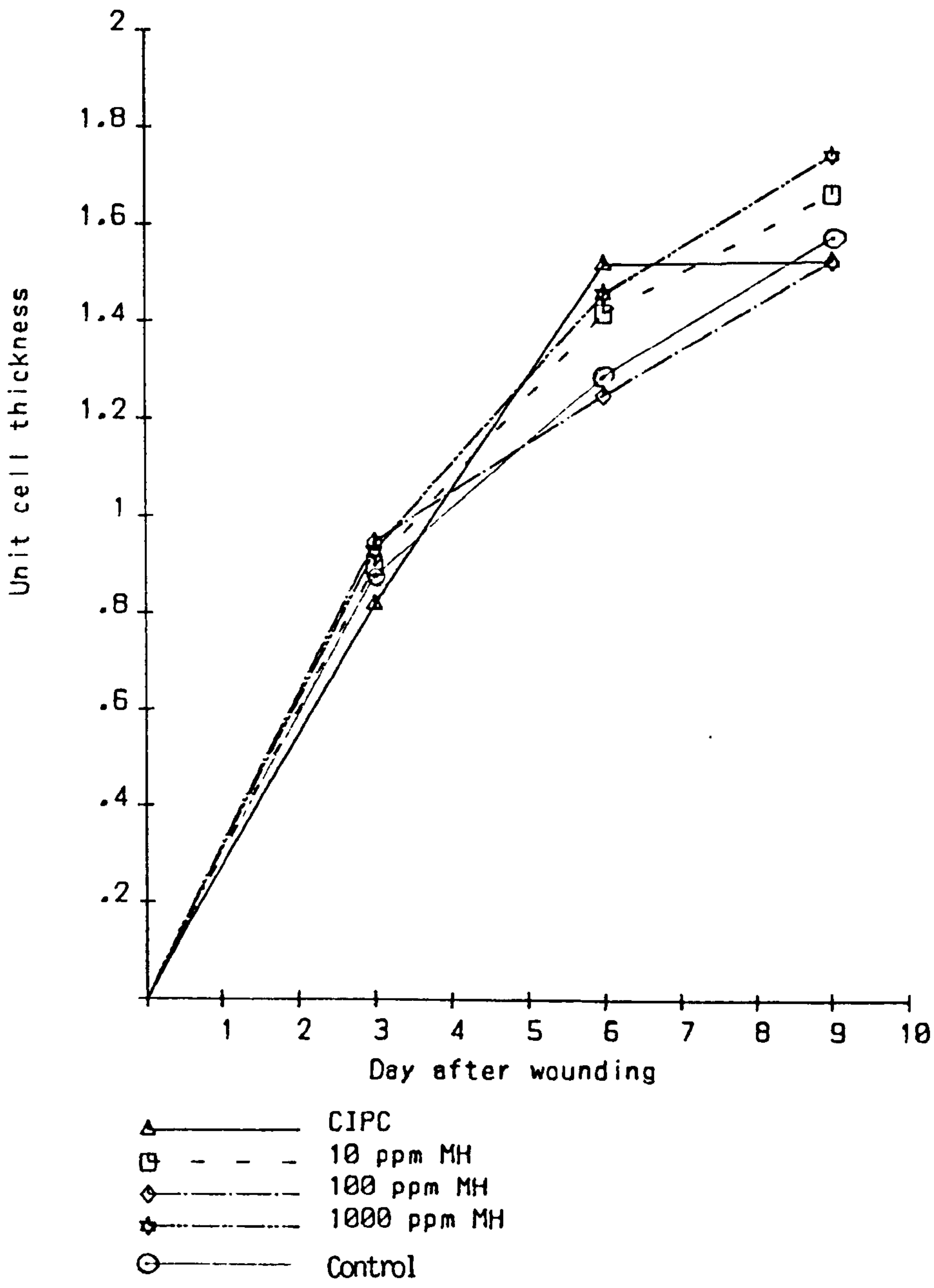
	Day 3	Day 6	Day 9
Untreated	0.04615 ± 0.00592	0.13069 ± 0.01191	0.24336 ± 0.01977
Treated	0.05755 ± 0.0106	0.13470 ± 0.01196	0.28931 ± 0.02493

- (i) The results are expressed as the internal resistance
 (dm⁻¹ S) ± SE (equation 7.6)
- (ii) Refer to section 7.3.3
- (iii) Refer to graph 7.4

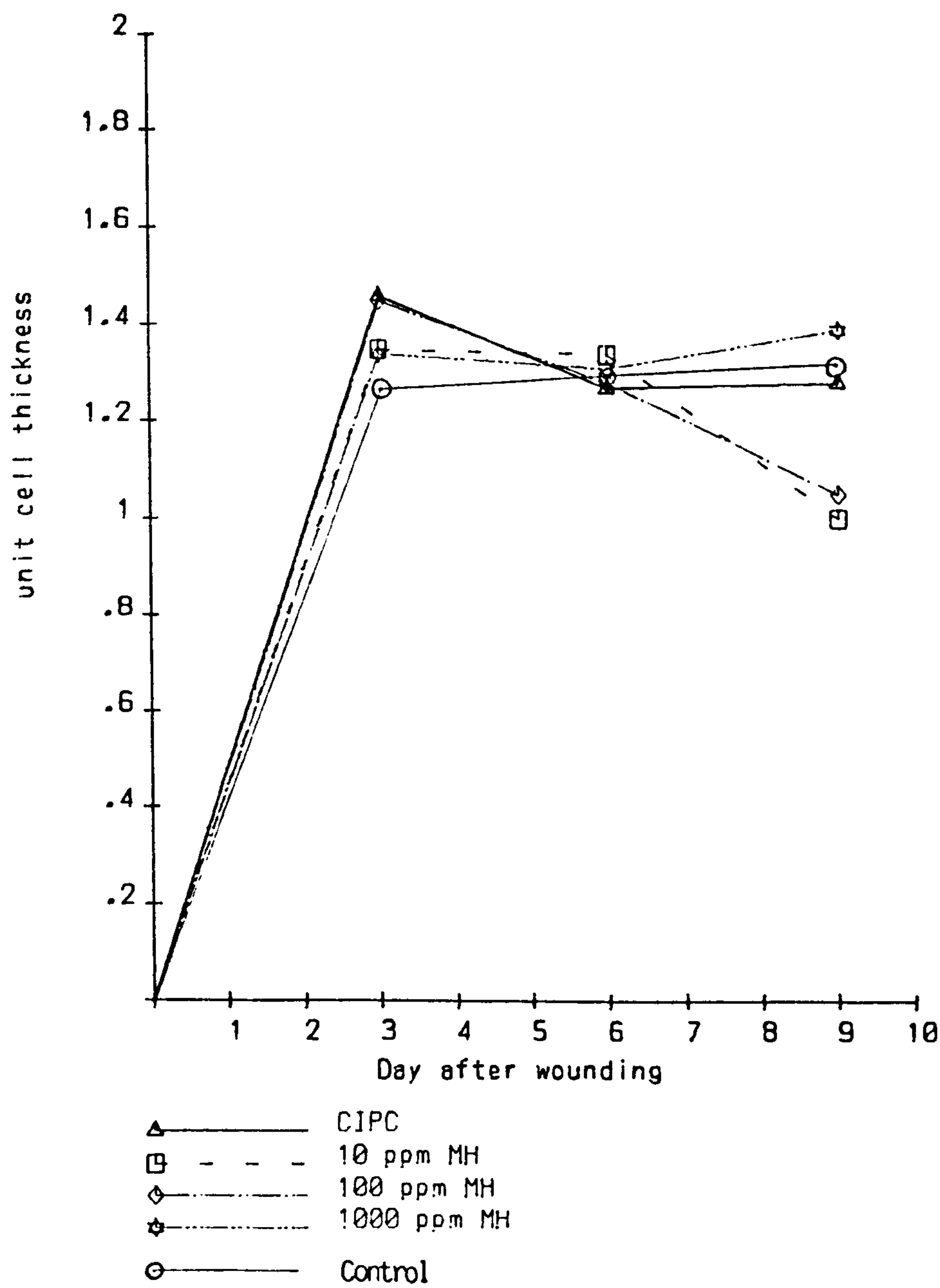
Figure 7.1 Schematic representation of the wound healing process
in potatoes.



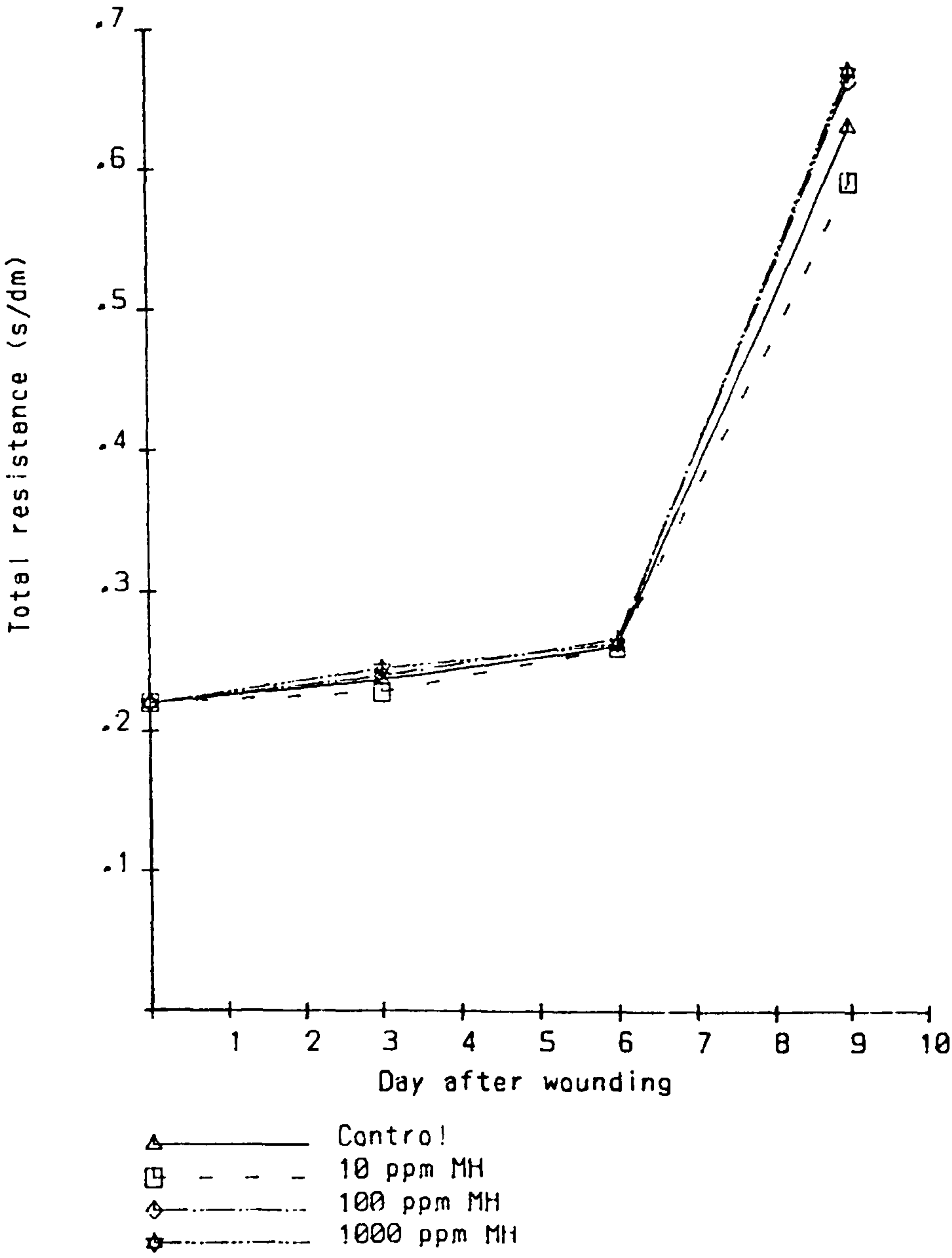
Graph 7.1
The development of the suberin layer
refer to Table 7.1



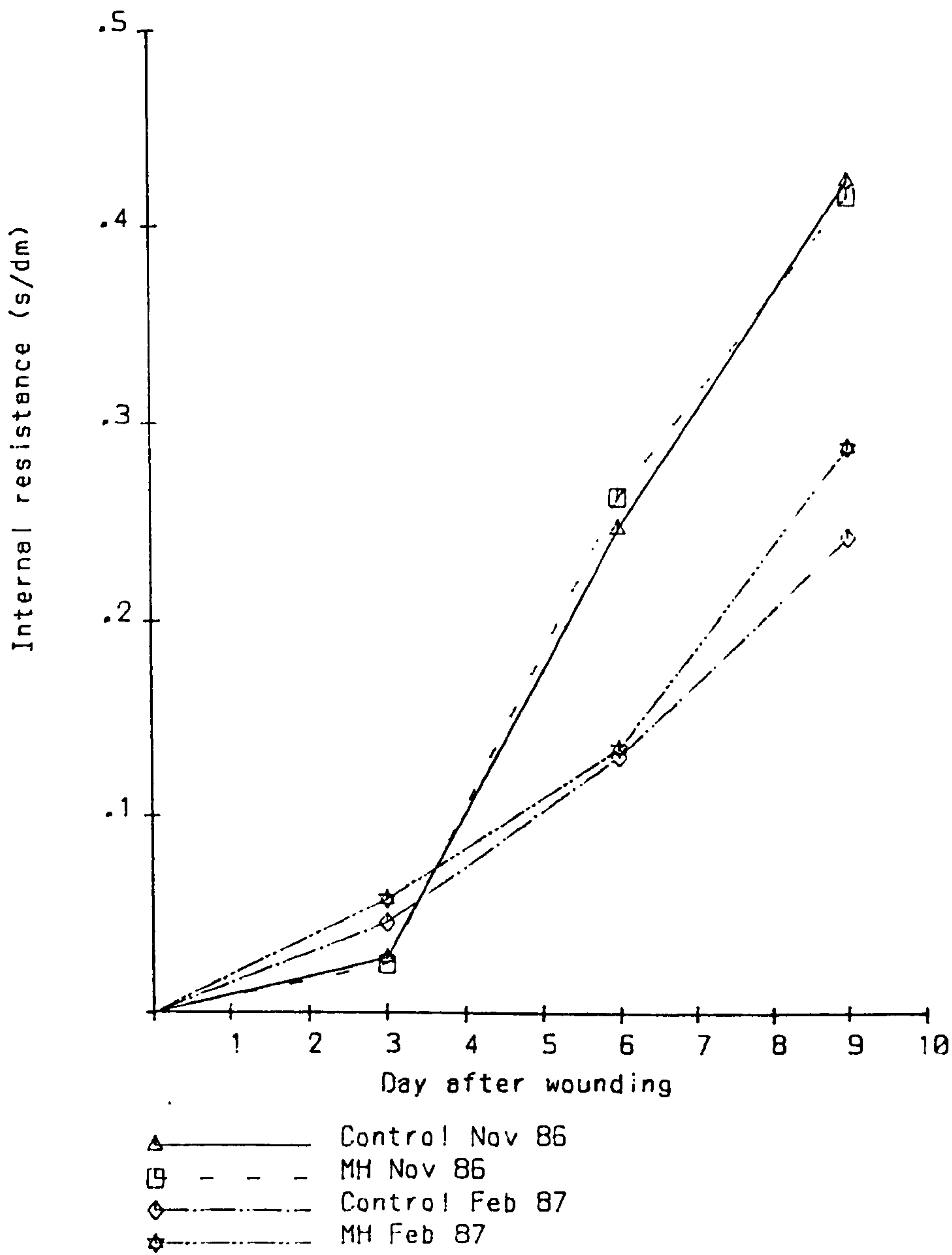
Graph 7.2
The increase in cellular activity
at the wound surface
refer to Table 7.2



Graph 7.3
Assessment of wound healing
MH applied in solution
refer to Table 7.3



Graph 7.4
Assessment of wound healing
Field treated potatoes
refer to Table 7.4



Graph 7.5 The effect of CIPC on the internal resistance of wounded tubers.

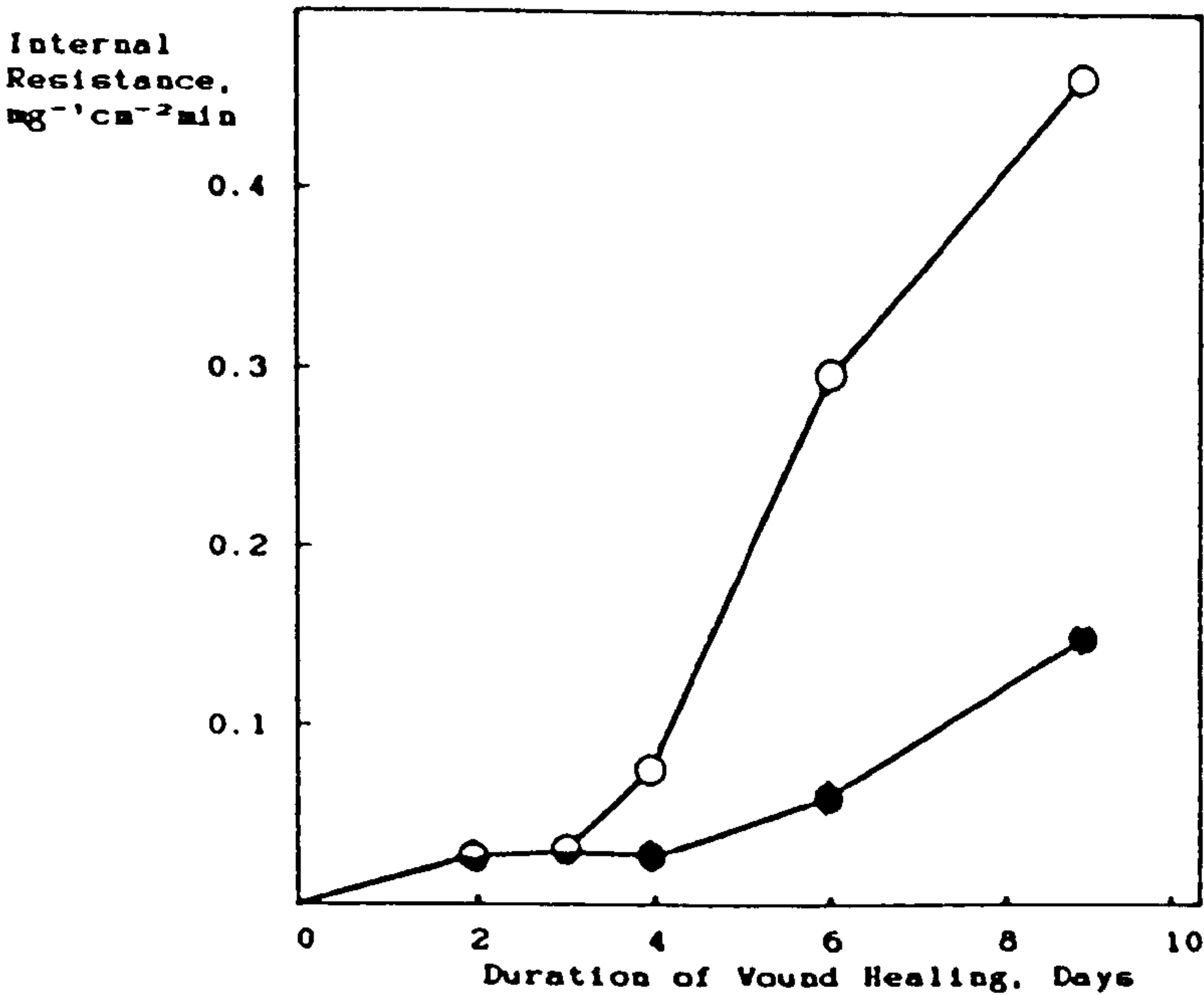


Figure 1. Effect of chlorpropham (50µg ml⁻¹) on rate of water loss from tuber discs, cv. Vilja, at 19°C. Control, o; chlorpropham, ●.

Reproduced by kind permission Leonard et. al. (1986).

CONCLUSIONS AND DISCUSSION OF THE EFFECTS AND RESIDUES
OF MALEIC HYDRAZIDE WITHIN THE POTATO CROP

This thesis has shown maleic hydrazide to be an effective sprout suppressant stored at 8°C. However there was not complete sprout suppression. Effective suppression by MH, as Fazor, relies on successful translocation of foliar applied MH to all tubers at the correct stage of growth of the tubers. In field trials, mainly in North America, reviewed in chapter two, MH is described as a sprout suppressant with reservations. Complete sprout suppression is not always the case. Increased concentrations of foliar applied MH increase sprout inhibition (Paterson et al. [1951], Franklin and Thompson [1953], Kennedy and Smith [1951]). Storage at lower temperatures also increased sprout inhibition (Matlob [1979], Franklin and Thompson [1953]). Late harvested tubers also had greater sprout inhibition (Franklin and Thompson [1951]). Some weak sprouts were noted while others had normal sprouts. It was shown in section 4.2 that inadequate sprout suppression correlates with total MH residue levels.

Uniroyal Ltd. marketing literature claims that the use of Fazor controls groundkeepers. It is unlikely that Fazor will give complete suppression and control of ground keepers. Because of incomplete control potato growers will still have to employ rogues and possibly still use selective herbicides. Thomas and Smith (1983) also consider groundkeeper control as inconsistent, Lutman (1986) doubts whether the inhibiting effect of MH can sustain groundkeeper control until May and June after Fazor application in August.

The marketing literature also claims Fazor to have growth regulant properties that would give a greater marketable yield. In field trials conducted at Glasgow University (section 2.15.1) MH was found not to give any statistically significant increase in yield of marketable yield (potatoes greater than 3 cm). The PMB field trials (section 2.14.2) resulted in MH field treated potatoes yielding higher than controls. However the marketable yield had a lower yield after being graded for misshapes and growth cracks. Previous work on yield has found conflicting results. Some workers have found no difference (Bishop and Schweers [1961], Chung [1983], Bevis and Jewell [1986], Peddie et al [1986]), others have found reductions in yield (Cibes et al. [1955], Hansen [1960]) while some have found increases in marketable grades (Weiss et al. [1980], Davis and Groskopp [1981]).

MH was distributed throughout the whole potato tuber, unlike other sprout suppressants. CIPC and TCNB can be reduced by ventilation (Dalziel [1978], Boyd [1984], Coxon and Filmer [1985]). CIPC and TCNB residues can be reduced by washing and peeling (Dalziel and Duncan [1974], Ritchie et al. [1983]). There is doubt about the environmental and health risks of MH (summarised in section 1.11). There should be concern that MH is present throughout potatoes (section 4.3) and the processes of crisping and boiling show intact MH (section 4.5) within the processed foods.

Chapter three used two residue methods to investigate residue levels in field treated potato crops. The initial method to determine MH residues was developed by Wood (1953). Adapted methods have been used by Lane et al. (1958), Smith et al. (1959), Bishop and Schweers (1961), Lane (1963), Franklin and Loughheed (1964), Poapst and Genier (1970). The method used in this thesis is an adaption of the method of Lane (1963) and was found to give good correlation with the residues detected by GC Laboratories (section 3a.3.4). The results were found to be consistent and reasonably sensitive (limit of detection 1 ppm). The HPLC method by Newsome (1980a) was found to give a fraction of the total residue. This is the free unbound MH extracted by methanol. The total residue could be fractioned into methanol soluble MH (including metabolites) and free unbound MH (section 3c.1.1). Using this fractionation procedure there was a fraction of the total MH that increased over a period of four weeks after spraying Fazor (Table 3c.6). This fraction was extracted in methanol and was not free unbound MH. The HPLC method was found to correlate well with the total MH method to the crop (section 4.4.4).

Chapter five describes the work involved in synthesising ^{14}C -MH (4,5) its application and radiotracer analysis of the residues. The synthesis was successful in providing 3.73 g (39% yield) with an activity of $3.2 \times 10^{-11} \mu\text{Ci/g}$. After application to the potato plants the harvested tubers had a total MH residue level of 140.5 ppm, and the free MH concentration was 80.0 ppm. The radiotracer analysis with tlc separation of a potato juice extract and detection by radioautographs revealed a trace of activity that was identified as ^{14}C -MH. Another area of the tlc plate also showed activity however this component was not identified.

The metabolites of MH were not resolved in this thesis. However future work should use similar methods as those used in chapter five. The limiting factor in the determination of the metabolites was the low activity of the synthesized MH. Future work with higher specific activity should help to identify the structure of any metabolites.

Weak acid and alkaline hydrolysis of concentrated field treated potato juice decreased the MH concentration detected by HPLC. Strong acid and alkaline hydrolysis reduced the MH in the standards and samples (section 6.2). Frear and Swanson (1978) used acid and alkaline hydrolysis to release non-methanol extractable MH, however the detection method was radiotracer analysis and not a specific detection method. The acid and alkaline hydrolysis may release a structure derived from MH possibly a degradation product. Nooden (1970) identified a non-methanol extractable fraction also by radiotracer analysis. He found that the MH was bound to large particles in corn and pea seedlings. It was suggested that these large particles were cell wall fractions that required metabolic energy for binding. This would suggest covalent bond and not physisorption.

The action of a beta-D-glucosidase on field treated potato juice released free MH (section 6.3). This suggested a metabolite of MH is produced in potatoes. This metabolite is probably the beta-D-glucoside. This metabolite has been recognised by other workers (Towers et al. [1958], Frear and Swanson [1978]). A metabolite could not be synthesised between MH and uridine diphosphoglucose (UDPG) with potato leaf and tuber protein as catalysts (section 6.5). A metabolite could not be synthesised between MH and glutathione with potato leaf and tuber protein (section 6.6).

The effect of MH on wound healing has never been assessed previously. The effect of MH was assessed by histological examination of cut potato tissue stained with dyes to identify suberin development and cell division (section 7.2). The histological studies were found to be very subjective and not precise enough to observe slight changes. The assessment method of Jarvis and Duncan (1979) has enabled a good comparison of the potato flesh ability to wound heal. There was no difference between MH treated potato discs and controls including CIPC treated tubers discs (section 7.3.2).

There were no differences in calculating the rate of water loss from field treated potatoes and controls on recently harvested potatoes (section 7.3.3). There was a reduced ability within controls compared to field treated potatoes. Untreated tubers by this time were showing one inch long sprouts. If the controls had been kept at 4°C instead of 8 °C then similar rates of water loss to the MH treated tubers would probably have taken place.

The future of MH as Fazor depends on a number of factors. Firstly, it must be able to compete with existing chemical sprout suppressants (CIPC and TCNB). The existing chemicals have proved themselves through the years as efficient and consistent with good store management. However there have been doubts expressed as to their safety; CIPC is a carbamate, and TCNB is an organochlorine, both should be handled with caution. MH has not sufficiently proved itself as a sprout suppressant as yet. The use of MH as a translocated sprout suppressant, and that it is known as a cell division inhibitor, should be carefully assessed before its use on food products. There is not enough evidence to clear MH from its health hazards label, therefore its use should be noted and controlled. It is known that MH is present within the tuber, therefore future work should concern itself not with its translocation and effects, but with the form it is in the potato as free MH or as a metabolite and to investigate the function of any metabolite found.

- Anon. (1984) 1, 2-Dihydropyridazine - 3, 6-dione : in Dangerous properties of industrial materials report, Von Nostrand Reinhold, New York, p 1061.
- Anon. (1985a) Historical data summary from 1955. Potato Marketing Board, London.
- Anon. (1985b) Potato statistics bulletin. Potato Marketing Board, London.
- Anon. (1985c) Potato storage. Potato Marketing Board, London.
- Anon. (1985d) Potato storage chemicals. Potato Marketing Board, London.
- Appleton, M.D. Haab, W. Eisenstadt, M.L. Rodgers, R. and Thomas, C.J. (1981) Incorporation of maleic hydrazide into ribonucleic acid of *Saccharomyces cerevisiae*. J. Agric. Food Chem., 29, 986-989.
- Artschwager, E. (1927) Wound periderm formation in the potato as affected by temperature and humidity. J. of Agric. Res., 35 995-1000.
- Audus, L.J. (1963) Chapter 15 : Natural growth substances, in Plant growth substances, Leonard Hill, London, pp 384-404.
- Baigal, B.D. and Kumar, P. 1978) The effect of MH on plant growth and tuber yield of potato. Comp. Physiol. Ecol., 3⁽¹⁾, 43-44.
- Bailey, N.T.J. (1981). Chapter 6 : The use of t-tests for small samples, in statistical methods in biology, Hodder and Stoughton, London, 2nd edition, pp 43-51.

Baker, J.E. (1961) A study of the action of maleic hydrazide on processes of tobacco and other plants. *Physiol. Plant.*, 14, 76-87.

Barnard, E.E. and Warden, R.L. (1950) The effects of various herbicides on weed control stands and yields of Netted Gem Potatoes. Research Report 7th Ann. North Central Weed Control Conf. p181.

Bevis, A.J. and Jewell, S.N. (1986) Preliminary results from the use of chemicals or cultivations to control potato groundkeepers. *Asp. App. Biol.* 13 201-208.

Beveridge, J.L. Dalziel, J. and Duncan, H.J. (1981a) The assessment of some volatile organic compounds as sprout suppressants for ware and seed potatoes. *Pot. Res.*, 24, 61-76.

Beveridge, J.L. Dalziel, J. and Duncan, H.J. (1981b) Dimethyl naphthalene as a sprout suppressant for seed and ware potatoes. *Pot. Res.* 24 77-88.

Bishop, J.C. and Schweers, V.H. (1961) Sprout inhibition of fall-grown potatoes by airplane applications of MH. *Amer. Pot. J.*, 38, 377-381.

Biswas, P.K. Hall, O. and Mayberry, B.D. (1967) Metabolism of maleic hydrazide in tea, *Camellia Sinensis*. *Physiol. Plant.*, 20, 819-824.

Boyd, W.D. (1984) The headspace analysis of potato volatiles. PH. D. Thesis, University of Glasgow.

Brian, P.W. and Hemming, H.G. (1957) A relation between the effects of gibberellic acid and indolylacetic acid on plant cell extension. *Nature*, 179, 417.

Brown, W. (1947) Effect of chlorinated nitrobenzenes on the sprouting of potato tubers. *Ann. Appl. Biol.*, 34.

- Brown, W. Reavill, M.J. (1954) Effect of tetrachloronitrobenzene on the sprouting and cropping of potato tubers. *Ann. Appl. Biol.*, 41 435-447.
- Burton, W.G. Horne, T. and Powell, D.B. (1959) The effect of gamma-irradiation upon the sugar content of potatoes. *Eur. Pot. J.*, 2, 105.
- Callaghan, J.J. and Grun, P. (1961) Incorporation of ^{14}C -labelled maleic hydrazide into the root tip cells of *Allium Cernuum*, *Vicia Faba*, and *Tradescantia Paludosa*. *J. Biophys, Biochem. Cytol.*, 10 567-575.
- Chan, M.T. and Karanov, E.N. (1983) Comparative tests of some growth regulators in view of prolonging the storage of potato tubers intended for seed stock. *Fiziol. Rast. (Sofia)*, 9⁽³⁾, 72-79.
- Chung, H.D. (1983) Effect of preharvest foliar applied MH on sprout inhibition and storage quality of potato, *Solanum tuberosum* C.V. Dejima tubers. *Han'guk Wonye Hakhoechi* 24⁽³⁾, 207-213.
- Cibes, H. Samuels, J. and Rodriguez, J.P. (1955) Influence of pre-harvest sprays with Maleic Hydrazide on the sprouting of potatoes. *J. Agric. Univ. Puerto Rico*, 39, 92.
- Clor, M.A. Crafts, A.S. and Yamaguchi, S. (1962) Effects of high humidity and translocation of foliar applied labelled compounds in plants. *Plant Physiol.* 37, 609-617.
- Corsini, D. Stallknecht, G. and Sparks, W. (1979) Changes in chlorpropham residues in stored potatoes. *Am. Pot. J.*, 56⁽¹⁾, 43-50.
- Cottle, W. and Kolattukudy, P.E. (1982) Biosynthesis, deposition and partial characterisation of potato suberin phenolics. *Plant Physiol.*, 69, 393-399.

- Coupland, D. and Peel, A.J. (1971) Uptake and incorporation of ^{14}C labelled MH into the roots of *Salix Viminalis* (willow). *Physiol. Plant.*, 25, 141-144.
- Coxon, D.T. and Filmer, A.A.E. (1985) The fate and distribution of chlorpropham when applied to stored potatoes as a sprout suppressant. *Pestic. Sci.*, 16, 355-363.
- Cradwick, P.D. (1975) Is maleic hydrazide a pyrimidine or purine analog? *Nature*, 258, 774.
- Crafts, A.S. Currier, H.B. and Drever, H.R. (1958) Some studies on the herbicidal properties of maleic hydrazide. *Hilgardia*, 27, 723-57.
- Crafts, C.C. and Andia, W.V. (1962) Phenolic substances associated with wound barrier formation in vegetables. *Bot. Gaz.*, 123, 211-219.
- Cunningham, H.S. (1953) A histological study of the influence of sprout inhibitors on *Fusarium* infection of potato tubers. *Phytopathology*, 43, 95-98.
- Dalziel, J. (1978) Potato sprout suppressants with particular reference to tecnazene. Ph.D. Thesis, University of Glasgow.
- Dalziel, J. and Duncan, H.J. (1974) Studies on potato sprout suppressants 1. Residual levels of tecnanzene in laboratory treated and in commercial samples of potatoes. *Pot. Res.*, 17, 215-223.
- Davis, J.R. and Groskopp, M.D. (1981) Yield and quality of Russet Burbank potato as influenced by interactions of *Rhizoctonia*, Maleic Hydrazide, and PCNB. *Am. Pot. J.*, 58, 227-237.
- Denison, E.L. (1950) Maleic Hydrazide on potatoes. Research Report 7th Ann. North Central Weed Control Conf. p 185.

Dettweiler, C. (1952) Relation between sprouting and respiration of potato tubers (I) Inhibition of sprouting by enzyme inhibitors. *Planta*, 41, 214-39.

Duncan, H.J. (1965) Studies on nucleotides and related compounds in plants. Ph.D. Thesis, University of Glasgow.

Duncan, H.J. Beglan, M. and Boyd, I.M.G. (1986) Optimising the use of sprout suppressant chemicals. *Asp. App. Biol.*, 13, 473-479.

Fox, R.T.V. Manners, J.G. and Myers, A. (1971) Ultrastructure of entry and spread of *Erwinia Cavatovera* var. *atroseptica* into potato tubers. *Pot. Res.*, 14, 61-73.

Franklin, E.W. and Thompson, N.R. (1953) Effects of MH on stored potatoes. *Am. Pot. J.*, 30, 289-95.

Franklin, E.W. and Loughed, E.C. (1964) The rate and amount of absorption of Maleic Hydrazide by potato tubers. *Am. Pot. J.*, 41, 191-195.

Frear, D.S. and Swanson, H.R. (1970) Biosynthesis of S-(4-Ethylamino-6-isopropylamino-2-S-triazino) glutathione. Partial purification and properties of a Glutathione-S-Transferase. *Phytochem.*, 9, 2123-2132.

Frear, D.S. and Swanson, H.R. (1978) Behaviour and fate of (^{14}C) MH in tobacco plants. *J. Agric. Food Chem.*, 26, 660.

Germane, S. and Kimenis, A. (1973) Initial toxicological appraisal of Maleic Acid Hydrazide in the form of its triethanolamine salt. *Gidrazid Maleinovo Kislotoy Regul. Rosta Rast.* (ed. Rakitin, Yu.V.) Nauka, Moscow, USSR, 335-40.

- Greulach, V.A. and Haesloop, J.G. (1954) Effects of MH on internode elongation, cell enlargement and stem anatomy. *Am. J. Bot.*, 41, 44-50.
- Haeberer, A.F. Nichols, B.C. and Chartyk, O.T. (1978) Effects of application rates on maleic hydrazide residues in Burley tobacco. *J. Agric. Food Chem.*, 26⁽²⁾ 385-388.
- Haeberer, A.F. Schlotzhauer, W.S. and Chartyk, O.T. (1974) A rapid quantitative method for MH. *J. Agric. Food Chem.*, 22, 328-330.
- Hansen, F. (1960) MH as a sprout inhibiting agent for potatoes. *Tidsskr. Planteavl* 64, 417-448.
- Harris, W.D. and Schoene, D.L. (1951) United States Rubber Co. C.A. 46:P6161g.
- Hegazy, A.T. Radwan, A. El-Fouley, M.M. and Garas, N.I. (1978) Effect of 2,4-D, maleic hydrazide (MH) and methyl ester of naphthalene acetic acid (MENA) on potato yield and tuber sprouting under Egyptian conditions. *Agrochimica*, 22⁽²⁾, 88-97.
- Higginbotham, P.G. (1985) Microtab, an all-purpose statistical package. Edward Arnold, pp58-62.
- Highlands, M.E. Licciardello, J.J. and Cunningham, C.E. (1952) Reducing sugar content of Maine grown potatoes treated with Maleic Hydrazide. *Am. Pot. J.*, 29 225-227.
- Hughes, C. and Spragg, S.P. (1958) The inhibition of mitosis by the reaction of Maleic Hydrazide with sulphhydryl groups. *Biochem. J.*, 70, 205.
- Hunt, T.W. Wojeck, G.W. and Sheets, T.J. (1985) Applicator exposure to maleic hydrazide (MH) in flue-cured tobacco. *Bull. Environ. Contam. Toxicol.*, 34, 403-406.
- Jarvis, M.C. and Duncan, H.J. (1979) Water loss from potato tuber discs: a method for assessing wound healing. *Pot. Res.*, 22, 69-73.

- Isenberg, F.M.R. (1954) Effects of foliar sprays of maleic hydrazide on the storage and respiration of certain vegetable crops. Penn. State Univ. Bull., 584, 12.
- Josephson, E.S. and Peterson, M.S. (1983) Sprout inhibition in tubers and bulbs : in Preservation of food by ionizing radiation. CRC press inc., B Raton, Florida, 159-213.
- Kolattukudy, P.E. and Dean, B.B. (1974) Structure, gas chromatographic and function of suberin synthesized by potato tuber slices. Pl. Physiol., 54, 116.121.
- Kashafutdinov, G.A. and Tsarev, S.G. (1973) Effect of silage obtained from beet tops and treated with the maleic acid hydrazide in the form of its sodium salt on animal organisms. Gidrazid Maleinovo Kisloty Regul. Rosta Rast. ed Rakitin Yu.V., Nauka, Moscow, USSR, 340-343.
- Kennedy, E.J. and Smith, O. (1951) Response of potatoes to foliar applications of maleic hydrazide. Am. Pot. J., 28, 701-712.
- Kennedy, E.J. and Smith, O. (1953) Response of seven varieties of potatoes to foliar applications of MH. Proc. Am. Soc. Hort. Sci., 61, 395-403.
- Kuc, J. (1972) Metabolites accumulating in potato tubers following infection and stress. Phytoalexins Rev. of Phytopath., 10, 207-232.
- Lamareaux, G.L. Shimabukuro, R.H., Swanson, H.R. and Frear, D.S. (1970) Metabolism of 2 Chloro-4-ethylamino-6-isopropylamino-S-triazine (atrazine) in excised sorghum leaf sections. J. Agric. Food Chem., 18, 81-86.
- Lamareaux, G.L. Stafford, L.E. and Shimabukuro, R.H. (1972) Conjugation of 2-chloro-4,6-Bis(alkylamino)-S-traizines in higher plants. J. Agric. Food Chem., 20, 1004-1010.

Lamareaux, G.L. Stafford, L.E. and Tanaka, F.S. (1971)
Metabolism of 2-chloro-N-isopropylacetanilide (Propachlor) in the
leaves of corn sorghum sugarcane and barley. J. Agr. Food Chem., 19,
346-350.

Lane, J.R. (1963) Collaborative study of maleic hydrazide residue
analysis. J. Ass. Off. Agric. Chem., 46, 261-268.

Lane, J.R. Gullstrom, D.K. and Newell, J.E. (1958) Extension of
the residue methods for 1,2-Dihydro-3,6-pyridazinedione (MH) and N-1-
Naphthylphthalamic acid (alanap). J. Agric. Food Chem., 6, 671-678.

L'Annunziata, M.F. and Legg J.O. (1984) Animals, plants, food and
the environment : in Isotopes and radiation in agricultural sciences,
Vol. 2. Academic Press.

Leonard, M.J. McGee, E. Jarvis, M.C. and Duncan, H.J. (1986)
The effect of sprout suppressant chemicals on wound healing. Asp.
App. Biol., 13, 481-490.

Leopold, A.C. and Klein, W.H. (1952) MH as an anti-auxin in
plants. Physiol. Plant, 5, 91.

Lipton, W.J. (1967) Some effects of low oxygen atmospheres in
potato tubers. Am. Pot. J., 44, 292-299.

Lutman, P.J.W. (1986) The biology and control of groundkeeper
potatoes : a review. Asp. App. Biol., 13, 177-185.

Marshall, E.R. and Smith, O. (1951) Maleic hydrazide as a sprout
inhibitor for potatoes. Bot. Gaz., 112, 329-330.

Marth, P.C. and Schultz, E.S. (1950) Sprout inhibitor for potato
tubers. Am. Pot. J., 27, 23-32.

Matlob, A.N. (1979) Effect of preharvest foliar spray of MH on the sprouting of potatoes during storage. *Mesopotamia J. Agric.*, 14⁽¹⁾, 153-162.

Meigh, D.F. Filmer, A.A.E. and Self, R. (1973) Production of growth suppressing volatile substances by stored potato tubers. II, Growth inhibitory volatile aromatic compounds produced by *Solanum tuberosum* tubers. *Phytochem.*, 12, 987-993.

McGee, E. (1984) A study of wound healing and associated problems in potatoes. Ph.D. Thesis, University of Glasgow.

McGee, E. Jarvis, M.C. and Duncan, H.J. (1985a) Wound healing in potato tuber. I, Effects of maturity at harvest. *Pot. Res.* 28, 91-99.

McGee, E. Jarvis, M.C. and Duncan, H.J. (1985b) Wound healing in potato tuber tissue. II, varietal and anatomical variation. *Pot. Res.*, 28, 101-108.

Miller, D.M. (1955) The polarography of MH. *Can. J. Chem.*, 33⁽²⁾, 1806-1813.

Miller, D.M. and White, R.W. (1956) The structure of MH as inferred from the ultraviolet spectra of its methyl derivatives. *Can. J. Chem.*, 34⁽²⁾, 1510-1512.

Moll, A. (1973) Effect of maleic hydrazide (MH) on the hexose content of potato tubers. *Nahrung*, 17, 1-10.

Moorby, J. (1977) Ion transport and crop growth. *Physiol. Aspects Crop. Nutr. Resist.* pp35-73.

Morgan, E.J. and Friedmann, E. (1938) Interaction of Maleic Acid with thiol groups. *Biochem. J.*, 32, 733-742.

- Muir, J.W. Boyd, I.M.G. and Duncan, H.J. (1987) A comparison of the effect of irradiation and chemical sprout suppressant treatments on sugar levels in tubers. EAPR, Abstracts of conference papers and posters, 164-165.
- Mukhorina, K.V. (1973) Action of maleic hydrazide on animal organisms. *Gidrazid Maleinovoï : Kisloty Regul. Rosta Rast.* ed Rakitin, Yu.V., Nauka, Moscow, USSR, 318-332.
- Newsome, W.H. (1974) A method for determining Ethylenebis (dithiocarbamate) residues on food crops as Bis (trifluoroacetamido) ethane. *J. Agric. Food Chem.*, 22, 886-889.
- Newsome, W.H. (1980a) A method for the determination of maleic hydrazide and its B-D-glucoside in foods by High Pressure Anion exchange liquid Chromatography. *J. Agric. Food Chem.*, 28, 270-272.
- Newsome, W.H. (1980b) Residues of maleic hydrazide in field treated potatoes. *J. Agric. Food Chem.*, 28, 1312-1313.
- Nielson, N.K. (1973) A quick microtechnique for inspection of potato periderm or wound periderm formation. *Pot. Res.*, 16, 180-182.
- Nooden, L.D. (1969) The mode of action of Maleic Hydrazide : Inhibition of growth. *Physiol. Plant.*, 22, 260-270.
- Nooden, L.D. (1970) Metabolism and binding of ^{14}C -MH. *Plant Physiol.*, 45, 46.
- Nooden, L.D. (1972) Inhibition of nucleic acid synthesis by maleic hydrazide. *Plant Cell Physiol.*, 13, 609.
- O'Keefe, R.B. (1974) Effect of maleic hydrazide sprout inhibitor on potato quality. *Am. Pot. J.*, 51, 311.

- Orfanos, A.P. Naylor, E.W. and Guthrie, R. (1980)
Ultramicromethod for estimation of total glutathione in dried blood spots on filter paper. *Anal. Biochem.*, 104, 70-74.
- Paterson, M.I. and Gray, E.G. (1972) The formation of wound periderm and the susceptibility of potato tubers to gangrene (caused by *Phoma exigua*) in relation to rate of fertiliser application and time of planting. *Pot. Res.*, 15, 1-11.
- Paterson, D.R. Wittwer, S.H. Weller, L.E. and Sell, H.M. (1951)
The effect of preharvest foliar spray of MH on sprout inhibition and storage quality of potatoes. *Plant Physiol.*, 27, 135-142.
- Payne, M.G. and Fults, J.L. (1955) The effect of Maleic Hydrazide and 2,4-D on reducing sugars and sucrose of red McLure potatoes. *Am. Pot. J.*, 32, 144-149.
- Peddie, A.S. Bartlett, D.H. and Taverner, P.B. (1986) Preharvest application of potassium maleic hydrazide to potatoes to suppress volunteers in succeeding crops. *Asp. App. Biol.*, 13, 219-225.
- Peterson, E.L. and Naylor, A.W. (1953) Metabolic changes in tobacco stem tips accompanying maleic hydrazide treatment and the appearance of frenching symptoms. *Physiol. Plant.*, 6, 816.
- Poapst, P.A. and Genier, C. (1970) A storage disorder in kennebec potatoes caused by high concentrations of MH. *Can. J. Plant Sci.*, 50⁽⁵⁾, 591-593.
- Ponnampalam, R. Mondy, N.I. and Babish, J.G. (1983) A review of environmental and health risks of maleic hydrazide. *Regul. Toxic. and Pharm.*, 3, 38-47.
- Priestley, J.H. and Woffenden, L.M. (1923) The healing of wounds in potato tubers and their propagation by cut sets. *Ann. App. Biol.*, 10, 96-115.

Pudova, R.A. Peredirei, E.N. and Garlov, V.T. (1985) Content of phenolic compounds in potatoes treated with maleic hydrazide and 2,4-D. Vestn. S-kh. Nauki Kaz, 1, 47-50.

Rakitin, Yu.V. Povulotskaya, K.L. and Khovanskaya, I.V. (1973) Distribution and translocation of maleic acid hydrazide in plants. Gidrazid Maleinovo Kisloty Regul. Rosta Rast. ed Rakitin Yu.V., Nauka, Moscow, USSR, 281-290.

Rao, S.N. and Wittwer, S.H. (1955) Further investigations on the use of maleic hydrazide as a sprout inhibitor for potatoes. Am. Pot. J. , 32, 51-59.

Reeve, R.M. Forrester, L.J. and Hendel, C.E. (1963a) Histological analysis of wound healing in potatoes treated to inhibit sprouting. I. CIPC (isopropyl-N-3-chlorophenyl carbamate) treatments. J. of Food Sci., 28, 649-654.

Reeve, R.M. Forrester, L.J. and Hendel, C.E. (1963b) Histological analysis of wound healing in potatoes treated to inhibit sprouting, II. Puncture wounds and Fusarium inoculates. J. of Food Sci., 28, 655-662.

Rhodes, A. Sexton, W.A., Spencer, L.G. and Templeman, W.G. (1950) Isopropylphenylcarbamate to reduce sprouting of potato tubers during storage. Research, 3, 189-90.

Ritchie, W. Boyd , I.M.G. and Duncan, H.J. (1983) A method for the determination of chlorpropham residues in crisps and crisp frying oil. Pot. Res., 26, 73-77.

Salunke, D.K. Wittwer, S.H. Wheeler, E.J. and Dexter, S.T. (1953) Effect of preharvest folian spray of maleic hydrazide on the specific gravity of potatoes and the quality of potato chips. Food Res., 18, 191-196.

Sawyer, R.L. and Dallyn, S.L. (1957) Vaporized chemical inhibitors and irradiation, two methods of sprout control for tubers and bulb crops. *Farm Res.*, 23⁽⁶⁾, 6.

Sawyer, R.L. and Dallyn, S.L. (1958) Timing of MH sprays to stage of plant development. *Am. Pot. J.*, 35, 620-625.

Schoene, D.L. and Hoffman, O.L. (1949) MH, a unique growth regulant. *Science*, 109, 588.

Shapalov, M. and Edson, H.A. (1919) Wound cork formation in the potato in relation to seed - piece decay. *Phytopath.*, 9, 483-496.

Simonds, A.O. Johnson, G. and Schaal, L.A. (1953) Comparative effects of catechol, some related compounds and other chemicals on suberisation of cut potato tubers. *Bot. Gaz.*, 115, 190-195.

Smith, A.E. (1955) Absorption studies of maleic hydrazide by plants. Bethany Info. Sheet No. 88 US Rubber Co., Naugatuck Chem. Div., Naugatuck, Conn.

Smith, A.E. Zuckel, J.W. Stone, G.M. and Riddell, J.A. (1959) Factors affecting the performance of MH. *J. Agric. Food Chem.*, 7, 341-344.

Sparrow, A.H. and Christionsen, E. (1954) Improved storage quality of potato tubers after exposure to Co⁶⁰ gammas. *Nucleonics*, 12⁽⁸⁾, 16.

Struckmeyer, B.E. Weiss, G.G. and Schoenemann, J.A. (1981) Effect of two forms of maleic hydrazide on the cell structure at the mid section, stem and bud ends of the cortical and perimedullary regions of Russet Burbank tubers. *Am. Pot. J.*, 58, 611-618.

- Thomas, P. (1982) Wound induced suberisation and periderm development in potato tubers as affected by temperature and gamma irradiation. *Pot. Res.*, 25, 155-164.
- Thomas, P.E. and Smith, D.R. (1983) Relationship between cultural practices and the occurrence of volunteer potatoes in the Columbian basin. *Am. Pot. J.*, 60, 289-294.
- Timm, H. Bishop, J.C. and Hoyle, B.J. (1959) Investigations with MH on potatoes and effects of time and application and concentration on potato performance. *Am. Pot. J.*, 36, 115-123.
- Towers, G.H.N. Hutchinson, A. and Andreae, W.A. (1958) Formation of a Glycoside of MH in plants. *Nature (London)*, 181, 1535.
- Victor, D.M. Hall, R.E. Shamis, J.D. and Whitlock, S.A. (1984) Methods for the determination of MH, ethoxyguin, and thiabendazole in waste waters. *J. of Chrom.*, 283, 383-389.
- Weast, R.C. (1979) ed : Handbook of Chemistry and Physics. CRC Press, 60th edition.
- Weiss, G.G. Schoenemann, J.A. and Grosskop, M.D. (1980). Influence of time of application of maleic hydrazide on the yield and quality of Russet Burbank potatoes. *Am. Pot. J.*, 57, 197-204.
- Wigginton, M.J. (1974) The effects of temperature, oxygen tension and relative humidity on the wound healing process in the potato tuber. *Pot. Res.*, 17, 200-214.
- Wittwer, S.H. and Paterson, D.R. (1951) Inhibition of sprouting and reduction of storage losses of onions, potatoes, sugar beets, and vegetable root crops by spraying plants in the field with maleic hydrazide. *Mich. Agr. Exp. Quart. Bull.*, 34, 3-8.

Wood, P.R. (1953) Determination of maleic hydrazide residues in plant and animal tissue. Anal. Chem., 25, 1879-1883

Young, N.A. (1981) Chapter IV The potato processing industry of the United Kingdom : in Potato products : Production and markets in the European Communities No. 75, EEC, 136-160.

Zaehring, M.V. Cunningham, H.H. and Sparks, W.C. (1966) Effect of storage temperature and sprout inhibitors on mealiness, sloughing, and specific gravity of Russet Burbank potatoes. Am. Pot. J., 43, 305-314.

Zukel, J.W. (1950) Use of MH as a plant growth inhibitor. Agric. Chem., 5, 35-36.

