A STUDY ON RESIDUES OF MALEIC HYDRAZIDE IN POTATOES AND POTATO PRODUCTS

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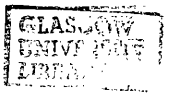

This thesis is dedicated to

my

MOTHER *and* **FATHER**

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CHAPTER 3 : **EFFECT OF PROCESSING ON MALEIC HYDRAZIDE (MH) RESIDUES IN POTATOES**

A list of abbreviations used in this thesis

a.i.	active ingredient
aq.	aqueous
AR	analytical reagent
^{0}C	degrees Celsius
cm	centimetre
conc.	concentration
cv.	cultivar
dm	decimetre
DNA	deoxyribonucleic acid
g	gram
h	hour
ha	hectare
HPLC	high performance liquid chromatograph (y) (ic)
i.d.	internal diameter
i.e.	that is
Kg	kilogram
l	litre
lb.	pound
LD50	lethal dose required to kill 50% of population
Ltd.	Limited
m	meter
M	Molarity
mg	milligram
min	minutes
ml	millilitres
N	Normality
ng	nano grams

nm	nanometers
pp	pages
p	page
ppm	parts per million
R.B.	Round bottom
RNA	Ribonucleic acid
SAX	Strong anion exchange
SCX	Strong cation exchange
UK	United Kingdom
UV	ultra violet
v/v	volume by volume
w/w	weight by weight
%	percentage
<	less than
>	more than
µg	micro grams
µl	micro litres
*	multiplication sign

Summary

The objective of this thesis was to study the fate of pesticides in food, both raw and processed, resulting from the deliberate application of pesticides to agricultural crops. The example chosen for this study was maleic hydrazide (MH) which is regularly used in the USA as a growth regulant and sprout suppressant and only recently introduced into the UK on a staple crop- potatoes as a sprout suppressant.

In Chapter 1 the importance of analysing pesticide residues in agricultural produce and some background information on the potato crop in the UK is discussed. Sprout suppressant methods used on potatoes are emphasised and the chemistry, properties and uses of the chemical MH are presented.

Chapter 2, describes existing analytical methods for the analysis of MH residues in various commodities with the aim of finding a suitable method to analyse free MH residues in potatoes. An HPLC method was developed and the validity of the method was determined. A recovery of 88.69% with a standard deviation of 4.19% was obtained using this method. This method was used to determine the MH residues in MH treated potatoes. The amount of MH in the total tuber, the amounts of MH present in various sized tubers and the distribution of MH residues within a tuber were determined. It was found that between the tubers the MH contents varied and within the tuber MH residues were evenly distributed.

In Chapter 3, the effect of processing such as frying and boiling on MH residues in potatoes was studied. To study the effect of frying on MH, before analysing MH treated potatoes, a series of model experiments with MH treated filter papers were carried out. From these experiments it was found that frying has no effect on MH. Also it was observed that during frying no MH went either into the oil fraction of the fried material or into the fryer oil. However in the presence of potato material there was some effect on MH during frying. Using this filter paper model a suitable method for the analysis of MH residues in fried potatoes was developed. Following this method 65-80% of the amount of MH which was present in the raw potato was recovered from the fried potato material.

In Chapter 4, the possible presence of bound MH residues in potatoes was studied An acid hydrolysis method and an enzyme hydrolysis method were used to release MH from any bound forms of MH residues present in potatoes. By acid hydrolysis an increase in the MH content in potatoes was observed while enzyme hydrolysis did not result in any increase in the MH content. The enzyme used was beta-glucosidase and since there was no increase in MH content observed by the reaction with this enzyme it was suggested that no MH was present in potatoes as the beta-glucoside. The activity of the enzyme in the presence of potato extract was verified.

The amount of MH released by acid hydrolysis was more in old potatoes than in new potatoes. This suggested that during storage more MH may become bound, which can be released with an acid treatment.

CHAPTER 1

INTRODUCTION

1.1. Pesticide use and the importance of residue analysis

Pests destroy up to one third of the world food crops during their growth, harvesting and storage. In developing countries like Sri Lanka crop losses are even higher. Therefore large scale use of pesticides is already one of the main factors in eliminating such losses. Pesticides are important not only in agriculture, but also in public health programmes such as control of Malaria and Filariasis. Because of this important role played by pesticides in agriculture and public health their usage has increased gradually. For instance Table 1 and Table 2 show some figures on imports of pesticides to Sri Lanka a few years ago. Although the information is dated it does show that the quantities of pesticides used annually are increasing significantly. More recent information supports this trend.

Table 1: Imports of technical grade material* to Sri Lanka.

	Quantity in tonnes or Kilolitres		Value in $ US' 000	
	1982	1984	1982	1984
Fungicides	4	10	35	60
Herbicides	119	160	392	428
Insecticides	76	122	459	752
Rodenticides	0	0	0	0
Others	1	4	8	8
Total	200	296	894	1248

* Active ingredient only, Preparation of formulations carried out industrially in Sri Lanka.

Table 2: Imports of formulated products to Sri Lanka.

	Quantity in tonnes or Kilolitres		Value in $ US' 000	
	1982	1984	1982	1984
Fungicides	449	508	600	872
Herbicides	853	1456	2512	2742
Insecticides	381	422	2038	1780
Rodenticides	1	1	4	1
Others	14	22	32	50
Total	1698	2409	5186	5445

Source: Agriculture Economics Division, Dept. of Agriculture, Sri Lanka.

As shown in the Tables 1 &2, from 1982 to 1984 there is an increase in the amounts of technical grade and formulated products of pesticides imported to Sri Lanka, which indicates an increase in usage. With the increase in use of pesticides there is a great concern about the residues of these chemicals in agricultural products as well as in the environment.

The term residues of pesticides can be defined as "any substance in food, agricultural commodities, animal feed or in the environment resulting from the use of pesticides. It includes any derivative of pesticide such as conversion products, metabolites or reaction products"(Anon 1989). As they are perceived as being toxic by the general public there is a growing interest in the detection and determination of these pesticide residues in agricultural produce intended for human consumption to minimise the possible health hazards to man. This great concern has been reflected in the joint publication by the Food and Agricultural Organisation (FAO) with World

Health Organisation (WHO) of the Maximum Residue Limits (MRL) for pesticides in a variety of agricultural produce.

Relevant definitions are given below.

Maximum residue Limit (MRL):

'MRL is defined as maximum concentration of a pesticide residue resulting from the use of a pesticide according to Good Agricultural Practice (GAP), that is recognised by the Codex Alimentarius Commission to be legally permitted or acceptable in or on a food, agricultural commodity or animal feed. The MRL is expressed in milligram(mg) of the residue for kilogram(kg) of commodity.'(Anon 1989)

Good Agricultural Practice (GAP):

'Good Agricultural Practice in the use of pesticides is the officially recommended or authorised use of such substances, under practical conditions, at any stage of production, storage, transport, distribution, or processing of food, agricultural commodities, or animal feed, bearing in mind the variations in requirements within and between regions. This takes into account the minimum quantities necessary to achieve adequate control, applied in such a manner that the amount of residues is the smallest practicable and which is toxicologically acceptable.'(Anon 1989).

Accurate measurement of the pesticide residue concentration in food and the environment is important to evaluate the possible toxicologic risk. Lack of knowledge on the residues resulting from the use of pesticides may lead to either an under estimation or a superficial over estimation of the real damage. Some of the food

importing countries now apply legally enforceable MRL's. Therefore to comply with these levels when importing the agricultural produce it is essential to monitor residue levels in these commodities frequently.

On the other hand pesticide residue analysis is important in pesticide legislation as well. To register a new pesticide with the authorities it is necessary to supply data on degradation studies on the residues and metabolites of the chemical in the agricultural produce, as well as the impact on environment, apart from the other requirements for registration.

In light of all these facts, it can be seen that it is very important to study the fate of these pesticides in agricultural produce. Therefore the aim of this thesis was to study the fate of pesticides in food both raw and processed resulting from the application of pesticides to agricultural crops. The example of the chemical chosen to investigate its behaviour in agricultural produce, which is described in this thesis is **maleic hydrazide** (MH).

MH is a growth regulant and it is used in many countries although not yet in Sri Lanka, but especially in USA and Australia, on potatoes to inhibit sprouting during storage. This chemical has been used in the UK for many years on onions as a sprout suppressant chemical. But, only very recently it has been introduced as a sprout suppressant for use on potatoes in the UK. As potato is a staple crop in the UK the introduction of this chemical has led to several questions regarding its residues in both fresh and processed potatoes, concerning possible health effects. Limited information is available about MH residues and metabolism of MH in potatoes. Therefore, this study was initiated to investigate the residues of MH in both raw and processed

potatoes, to develop a suitable method to analyse MH residues especially in processed products high in oil and also to search for bound residues.

Before discussing experimental work, some background information on the potato crop, particularly the control of sprouting of potatoes during storage and some background information about the chemical MH will be discussed in this chapter.

1.2. **Potato crop:**

Potato is a main crop grown in the UK as it is the staple food stuff in Europe. Each year approximately 6-7 million tonnes of potatoes are harvested in the UK. Following table shows some potato statistics for the UK.

Statistics on potato cultivation in the UK:

Year	Area '000 ha	Production '000 tonnes
1990	153	6198
1991	154	6032
1992	154	7481
1993	152	6808
1994	152	6263

(Anon 1995)

Over 24% of potatoes grown in the UK are processed in some way and over 10% goes to the crisping industry alone (Anon 1994a). As potato is the staple food in this country there is a great demand for raw and processed potatoes throughout the year. Due to the limitations of climate a continuous growing season is not possible.

Therefore it is necessary to store harvested potatoes for prolonged periods until they are required.

After lifting, potato tubers normally go through a period of dormancy of between 1-15 weeks during which time sprouting will not take place. After the dormancy period sprouting will start depending on the temperature under which the tubers are stored. At very low temperatures such as 4 ^{0}C sprouting will not take place. But the potato tubers will be of a poor processing quality due to the build up of sugars. The recommended storage temperature for ware crop is 7 ^{0}C and crops for processing is 7-10 ^{0}C (Anon 1994b). But at this temperature sprouting will take place unless it is controlled using sprout suppression methods.

It has been estimated that in the absence of sprout suppression, sprouting losses in England would average over 1% of the weight of potatoes stored (Burton 1989).
In Sri Lanka the situation is different. There the staple crop is rice and potato is mainly a commercial vegetable crop grown especially in highland areas of the country. The extent of potato cultivation and the production of potatoes is very low compared to the UK. Following table shows some potato statistics for Sri Lanka.

Statistics on potato cultivation in Sri Lanka.

Year	Area '000 ha	production '000 tonnes
1990	11.15	86.30
1991	11.46	91.09
1992	9.66	76.97
1993	9.98	77.16
1994	7.49	68.40

Source: Department of Agriculture, Sri Lanka.

As there is only a limited production of potatoes due to many limiting factors in potato production in Sri Lanka, no potatoes are processed as an industry in Sri Lanka. They are consumed only as a fresh vegetable and used in a curry. Only a very small percentage goes for the processing in the form of chips (Sathiamoorthy *et al.* 1985). Therefore potatoes meant for consumption are not stored for a long period of time. Only the storage of seed potatoes is practised in Sri Lanka. Therefore so far there is no necessity to use any post harvest chemicals on potatoes which are meant for consumption.

But in future if the production of potatoes in Sri Lanka is increased by overcoming the limiting factors such as unavailability of good seeds, diseases, poor storage conditions etc. and also by increasing the area under cultivation the necessity for storing potatoes for prolonged periods of time to use in consumption and processing purposes may arise. Then the use of sprout suppressant chemicals such as MH would be required to maintain the quality of potatoes during storage.

1.3. **Sprout suppression of potatoes during storage**:

There are several methods for controlling sprouting of potatoes during storage such as controlling the storage temperature, control by diffused light, use of irradiation and use of chemical sprout suppressants.

Inhibition of sprouting by controlling storage temperature:

Potatoes do not sprout immediately after harvest. They undergo a period of dormancy during which time sprouting will not take place. At very low temperature (below 5 ^{0}C) the break of dormancy and subsequent sprouting will be very low.

Therefore it is possible to control sprouting by storing potatoes at a lower temperature. But at lower temperatures the sugar content of stored potatoes will increase resulting in a poor processing quality which is a major draw back to the use of this method. And also it is an expensive method of storage as refrigeration of large stores will normally be necessary.

Inhibition of sprouting using diffused light:

This is based on using natural indirect light instead of low temperature to control excessive sprout growth and storage losses. Diffuse light storage (DLS) method is a useful method for inhibiting the sprouting during storage of seed potatoes since it is a reversible process. This DLS method is used in Sri Lanka as well for storage of seed potatoes. This method has been introduced to Sri Lanka in early 1980 (Anon 1984). One of the disadvantage of this method for use in storage of potatoes meant for consumption is that tuber greening takes place due to the build up of solanine and chlorophyll. This makes the potatoes unacceptable for eating (Duncan *et al.* 1992).

Irradiation:

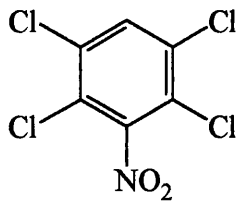
There is an interest in the commercial use of gamma irradiation as a means of sprout suppression. By irradiating the tubers with doses in the range 30-150 Gy sprouting can be delayed or prevented. The usual dose rate is 100 Gy.(Duncan *et al.* 1992). But, at the same time it has been found that gamma irradiation causes a marked and rapid though temporary increase in the content of reducing sugars which is dose dependant. This can be a cause of concern to processors. There are no

nutritional or public health hazards in potatoes due to irradiation. But, there are many practical and commercial difficulties in using this method (Burton 1989). This process is not suitable for farm use as it needs many facilities. A great tonnage of potatoes stored has to be transported to the reactor for irradiation treatment and then back to the store. Irradiation could also inhibit the wound healing process which causes evaporative loss and risk of rotting of potatoes. Because of these difficulties irradiation has not become an established method for sprout suppression.

Chemical control:

Main sprout suppressant chemicals used on potatoes in the UK are tecnazene, chlorpropham and propham. The chemical MH has been introduced very recently.

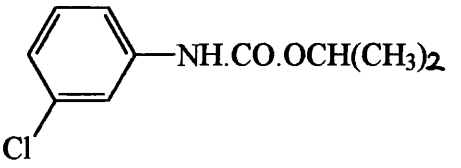
Tecnazene:



1,2,4,5 - tetrachloro-3-nitrobenzene (TCNB) is a colourless crystalline compound which is volatile at room temperature. This controls sprouting of potatoes during storage by prolonging dormancy. TCNB will not control sprouting if dormancy has already broken or about to break. This chemical also has fungicidal properties and gives control of dry rot and partial control of skin spot, silver scurf and gangrene. TCNB is applied to potatoes at the time of store loading. Application is done as a dust, granular or spray formulation. As the chemical does not inhibit wound healing it can be applied to potatoes, stored soon after harvesting.

At present there is only a minor use of this chemical due to some suspicions regarding the persistence of this chemical and its metabolites in the environment. But recently this chemical has been cleared by Pesticide Safety Division in the UK on its safety point of view and approved for use for another three years. They have laid down a MRL of 10 ppm and have raised the Acceptable Daily Intake (ADI) value to 0.05 mg/Kg body weight per day on receipt of more up to date data..

Chlorpropham:

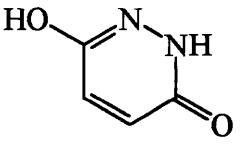


isopropyl-N-(3-chlorophenyl) carbamate (CIPC) is a crystalline compound having appreciable volatility at room temperature. This controls sprouting of potatoes during storage by stopping the cell division. It also delays or prevents the wound healing of potatoes. Therefore the chemical should be applied to potatoes after the wound healing has been completed. CIPC is usually applied as a fog. At present this chemical is the most commonly used potato sprout suppressant in the UK. and world wide.

Propham:

isopropyl-N-phenyl carbamate (IPC), has similar properties to CIPC but it is less commonly used. It is only available as a formulation with CIPC.

Maleic hydrazide:



6-hydroxy-3-(2H)-pyridazinone(MH) is the recently introduced chemical into the UK for controlling sprouting of potatoes during storage. This chemical is different from other sprout suppressant chemicals. It has to be applied during the growing season to the foliage where it is translocated down to the tubers to inhibit sprouting during storage. This chemical is available in a water soluble powder formulation and applied to the crop as a foliar spray 3-5 weeks before harvesting.

Apart from the sprout inhibition, another benefit offered by this chemical is, it acts as a growth regulator to control the regrowth of volunteers(ground keepers). Ground keepers are the tubers remaining in the field after harvesting the potato crop. They can come out as a weed during the next crop which is obviously not potatoes. Normally the potato crop is not grown continuously due to the development of potato eel worm in the soil. Therefore potato is grown only once in three years in the same field as a minimum. In the meantime grass or a cereal crop will be grown. Therefore it is important to control the volunteer potatoes growing from ground keepers during succeeding crops. It has been found that MH has the ability to control growth of ground keepers (Peddie *et al.* 1986).

Beside these chemicals there are several other secondary plant products which have sprout inhibiting properties (Hartsman and Van ES 1986).

Examples:

DMN (Dimethylnaphthalene), TMN (Trimethylnaphthalene) and DPA (Diphenylamine).- These are volatile components from the potato tuber itself.

Pulegon and Carvone - These are volatile components from other plants.

Use of these compounds are still at the research level.

Another storage method used specially in storage of fruits is the use of controlled environment. In this case the CO_2 level in the store will be increased so that it will lower the respiratory activity of the stored material. When the respiratory activity is lowered, it will control the fast development of the stored product. Therefore it is possible to apply this method to control sprouting of potatoes as well. But there are many disadvantages to using this method. This is a quite expensive method to use in large stores. And also when the oxygen level in the store is too low and carbon dioxide level is too high anaerobic respiration will take place and this will accelerate deterioration, internal rots and also resulting in off flavour or taint in the product. But this is a suitable method to use in storage of fruits such as apples which give a higher return.

1.4. **Introduction of MH to the UK**:

Since CIPC was the only sprout suppressant chemical universally approved for processing potatoes there was a necessity for alternative chemicals. MH is a very popular chemical in USA and Australia as a potato sprout inhibitor. This is an accepted sprout suppressant in the UK for many years on onions which is another edible crop. Therefore because of its sprout suppressing activity in root crops during storage and its capability of controlling ground keepers and also its popularity

elsewhere, it was introduced into the UK for use as a potato sprout suppressant on a restricted basis.

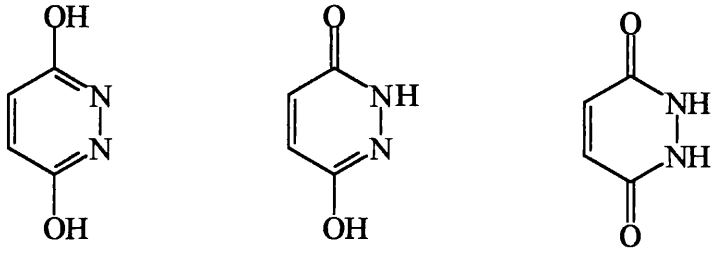
1.4.1. Chemistry of Maleic hydrazide:

Maleic Hydrazide(MH) is the common name for 6-Hydroxy-3-(2H)-pyridazinone.

Synonyms: 1,2-Dihydro-3,6-pyridazinedione (Howard 1991)

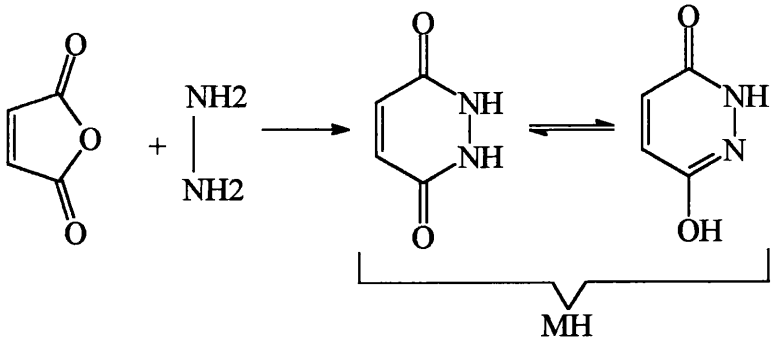
Molecular formula: $C_4H_4N_2O_2$

There are three possible tautomeric forms for Maleic Hydrazide (I-III) and the accepted structure is the form II.(Miller and White 1956)



I II III

MH is obtained by condensation of maleic anhydride and hydrazine (Cremlyn 1991).



It can also be prepared by reacting hydrazine sulphate with maleic anhydride in aq. NaOH, by reacting maleic anhydride with hydrazine salts of strong inorganic acids, by reacting hydrazine anhydrate with maleic anhydride in glacial acetic acid or by condensing maleic anhydride and dihydrazine sulphate in aq. medium (Ponnampalam *et.al.* 1983).

Chemical and Physical properties:

The dry technical grade (>99% pure) MH is a colourless non volatile, crystalline solid.

Molecular weight = 112.1

Melting point = 292-298 ^{0}C

Solubility at 25 ^{0}C

6g/Kg water

24g/Kg Dimethylformamide

1g/Kg Ethanol

(Worthing and Walker 1987)

MH is a very stable compound and it is not decomposed by concentrated mineral acids and alkali even during heating to 200 ^{0}C. (Rakitin *et al.* 1971). But it is decomposed by concentrated oxidising acids with formation of nitrogen (Worthing and Walker 1987).

MH exhibits acidic properties and has been shown to have a pKa value of 5.65 (Miller 1955). As it is a monobasic acid it forms water soluble alkali metal and amine salts. For this reason these salts are used instead of crystalline MH, in research and practical horticulture (Rakitin *et al.* 1971)

Structurally MH closely resembles Uracil, one of the pyrimidine bases in RNA. On the basis of its crystal structure and possible formation of base pairs with nucleic acid bases (purines and pyrimidines) Cradwick (1975) suggested that MH can be incorporated into RNA. Appleton *et.al.* (1981) proved the incorporation of MH into RNA in yeast cells.

1.4.2. Toxicology of MH:

There was a great concern over the possible toxic and carcinogenic activity of MH in animals and man, after the discovery of its potential chromosome breaking activity (Darlington and McLeish 1951) in plant cells. The work carried out by Barnes *et al.* (1957) on carcinogenic properties of MH on laboratory animals, mice and rats gave negative results. But their findings have not been confirmed. Epstein and Mantel (1968) reported the high carcinogenicity of MH for male mice. Akin (1976) found MH was not tumorigenic to mouse skin and decreased the tumour initiating activity of the carcinogenic hydrocarbon 7,12 - dimethylbenz[a]anthrazene (DMBA), by about 50%. Van der Heijden (1981) carried out carcinogenicity studies on rats and found that tumour incidence and tumour type in the treated animals did not differ from the control group. When the same batch of MH was tested for oncogenicity on mice, no oncogenic effects were observed (Cabral and Ponomarkov 1982). Both authors concluded that MH itself is not a carcinogen in mammals. It was suggested that the presence of relatively high levels of hydrazine (which is a well known carcinogen and mutagen) as an impurity was responsible for the contradictory results in studies previously done. Bakker *et al.* (1983) determined the hydrazine content in 15 commercial formulations and found that the hydrazine content ranged from 0.20 to 53

mg/kg of MH. This also implied that the contradictions found in earlier carcinogenicity studies may have been due to the high levels of hydrazine impurity. Biswas et al (1967) found hydrazine as a possible metabolite in tea plants. But it was not convincingly proved. Hunter (1973) administered MH and its diethanolamine salt to rats and examined the tumour incidence . His findings did not lend support to the view that MH or its diethanolamine salt might constitute a carcinogenic hazard to use.

Mutagenicity of MH has not been studied extensively. In a few systems it appeared to be mutagenic but not in others. Experiment carried out by Nasrat (1965) with *Drossophila* indicated MH as a mutagenic agent. Sandhu and Waters (1980) also reported MH as a mutagenic agent after reviewing the data reported in literature.

Ponnampalam *et al.* (1983) have reviewed the experimental data on toxicity of MH. A brief summary of the relevant toxicology is given below.

Acute oral LD_{50} for MH technical grade for rabbits was greater than 4g/Kg.

Acute oral LD_{50} for Diethanolamine salt of MH to rats was 2.35g/Kg.

Acute oral LD_{50} for Sodium salt of MH to rats was 6.95g/Kg.

MH diethanolamine salt does not cause primary irritation of the eyes or skin of rabbits and is nonallergenic in guinea pigs. In subchronic studies, a dairy cow consumed 8.8 lb of MH in her diet over a 3 month period with no apparent physiological or pathological effects. Two dogs fed 1g of sodium salt of MH for 1 month showed no adverse effects in the liver, spleen, kidney or bone marrow, but one animal showed a decrease in erythrocyte count.

Although, the toxicity of MH has been studied for more than three decades still an uncertainty remains about its chronic toxicity to non target organisms due to conflicting research results (Swietlinska and Zuk 1978; Ponnampalam *et al.* 1983).

Hence, further studies should be carried out on laboratory animals so that it can be extrapolated with confidence to human cases.

1.4.3. Uses and Mode of action of MH:

MH was the first synthetic growth suppresser to be described. Schoene and Hoffmann (1949) found the effect of MH on tomato plants. It has inhibited the stem elongation and destroyed the apical dominance which is an auxin mediated phenomenon. Leopold & Klein (1952) reported the anti auxin effect of MH. Brian an Hemming (1957) studied the effect of MH on growth response of plants to giberrellic acid (GA) and stated that the effects of MH and GA on plant growth and development are opposite.

Due to its potential growth inhibiting effects it has been widely used in agriculture as a weedkiller and a depressant in plant growth in various circumstances such as suppression of sprouting of vegetables and stored root crops such as beets, carrots, onions, potatoes and rutabagas (Worthing and Walker 1987), control of sucker growth on tobacco plants, retardation of flowering and prolongation of dormancy period, inducing male sterility in plants etc.(Swietlinska and Zuk 1989). It is also used to retard growth of hedges and trees. MH is an extensively used chemical throughout Europe on a variety of crops as a growth inhibitor (Anon 1986). It is readily taken up by plants and translocated through phloem or xylem to the areas of active growth. It is generally considered that effectiveness of MH lies in its ability to inhibit cell division. Nooden (1969) reported that sprout inhibition of MH treated potatoes was due to the prevention of cell division in the active tissues. Inhibition of cell division might have resulted from the functional loss of spindle (Darlington and

McLeish 1951) in the metaphase, or functional damage of nuclei (Nooden 1970). However MH does not inhibit the enlargement of differentiated cells (Nooden 1969).

1.4.4. Use of MH on potatoes:

Sprout inhibiting effects of MH on potatoes was first reported by Zukel (1950). In USA MH is the major sprout suppressant chemical used in storage of potatoes. Formulated MH for agricultural use is available under different trade names. 'Fazor' which is formulated as water soluble powder and supplied by DowElanco is the product recommended for use on potatoes in the UK (Anon 1994/95).

Active ingredient content (a.i.) is 80% w/w potassium salt of MH. Recommended rate of use is 5 Kg in 350-500 litres. water per ha. It is applied to the foliage of the potato crop when the smallest tubers required to reach marketable size are less than 25 mm long and starting to expand. i.e. normally 3-5 weeks before defoliation.

1.4.5. Some biological effects of MH on potato crop:

During the past, many studies have been carried out on effectiveness of MH in inhibiting the post harvest sprouting and the quality parameters such as yield, specific gravity, sugar content and chip colour etc. of potatoes. Anglin and Mahon (1958) reported that the tubers obtained from MH treated plants hardly sprout when stored in ordinary storage. Kennedy and Smith (1951), Rao and Wittwer (1955), Cibes *et al.* (1955), Bishop and Schweeres (1961), Kumar and Mukerjee (1989), Gul *et al.* (1990) and Yada *et al.* (1991) also reported the effectiveness of MH as an antisprouting agent for potatoes. Matlob (1979) reported that tubers from potato plants treated with

MH retained a higher quality after storage than untreated tubers. Weis *et al.* (1980) studied the effect of time of application of MH on yield and quality of the potato variety Russet Burbank and identified that, the timing of foliar application of MH is essential resulting in an improvement in quality and a favourable alteration in the shape of tubers, and also results in the most effective sprout control during storage. Chung (1983) showed that there is a decreasing pattern of total soluble sugar content and invertase activity in the tubers with storage period. Baijal (1978) reported that treatment of MH decreases the tuber yield of potatoes. Yada *et al.* (1991) observed that MH has no detrimental effect on marketable or total yield of potatoes and no effect on the sugar content of potatoes newly harvested and after 6 month storage. No consistent difference between the colour of chips made from treated and untreated potatoes was observed. Klein *et al.* (1984) found that MH significantly increased the total and non protein nitrogen of cortex tissue of the tubers. And also found a significant increase in the amounts of Ca, Mg, Mn, and Zn in sprouts by MH treatment. They suggested that the growth inhibiting effect of MH may be due to this excessive accumulation of trace elements which are toxic to plants when present in more than usual quantities. Mukerjee and Chava (1988) also studied the influence of pre harvest application of MH on sprouting and quality of potatoes during storage and found that rotting was higher in treated tubers than in control. Some reduction in weight loss and starch breakdown in tubers also was recorded. Mondy *et al.* (1978) studied the effect of MH on glycoalkaloid formation in potato tubers and found that while MH is effective in controlling the sprouting, it is also effective in reducing the glycoalkaloid content of the tubers (Glycoalkaloids are naturally occuring steroid alkaloids in potatoes which are toxic to human and they accumilate in potato tubers

due to physiological stress induced by mechanical injury, the interaction with microorganisms or by sprouting of the tubers).

1.4.6. Residues of MH:

MH which is generally translocated from the treated leaves down to the rest of the plant is relatively stable. Residues occur in potatoes as long as 8 months after treatment (Smith *et al.* 1959)

Anglin and Mahon (1958) studied the total MH residues in fruits and vegetables after pre harvest foliar spray of MH and reported that in potatoes MH was distributed uniformly within the tuber. Hoffman and Carson (1962b) also studied the total MH residues in fruits and vegetables (potato, carrot, onions and apples) after foliar application of MH and storing them for 4-5 months. They indicated that the amounts of MH residues found in the samples depended on the rates of application of MH and the residues tended to accumulate more in the regions of cell division. They found that peelings of the potato tubers had more MH residues than the remainder.

McKenzie (1989) reported that MH residues are distributed throughout the tubers and the maximum concentration would seem to be in the outer flesh including the peel compared to other portions.

Newsome (1980b) monitored the residue level of MH in potatoes after field treatment with MH and found that there was an initial decline in the residue level until 6 weeks after treatment. When the potatoes were stored after harvesting seven weeks after treatment he found that at least for 8 weeks in the store there was no significant change in the residue level of MH.

Maximum recommended Limits(MRL's) for potatoes:

Because MH is a comparatively non toxic substance very few countries have established detailed residue tolerances governing its use (Hoffmann and Parups 1964). In the USA, tolerances of MH have been set at 50 ppm in or on potatoes and 15 ppm in or on onions (Dry bulbs).

For potato chips it is recommended that the residue amount must not exceed 160 ppm by weight of the finished product.

Canada Food and Drug Act sets tolerances of 15 ppm on onions, 20 ppm on potatoes, 30 ppm on beets carrots turnips and rutabagas (Hoffmann and Parups 1964).

The MRL for MH, followed in the UK is the level recommended by CAC (Codex Alimentarious Commission) i.e. 50 ppm for fresh potatoes (revised target MRL is 30 ppm).

1.4.7. Problems associated with the use of MH on potatoes:

As mentioned in a previous section sprout suppressant chemicals normally used on potatoes in the UK are chlorpropham (CIPC), propham (IPC) and tecnazene (TCNB). They are usually applied on to the surface of the potato tubers at the time of store loading in the case of TCNB or after the wound healing in the case of CIPC/IPC. These chemicals mainly remain on the peel and very little will penetrate into the tubers (Dalziel and Duncan 1980; Ritchie *et al.*1983; Mondy *et al.* 1992). Therefore when the tubers are washed and peeled most of the chemical can be removed thereby minimising substantially the residue level in the final product. However environmental problems can still arise when dealing with the waste material. Application of MH is not the same as other sprout suppressant chemicals. It is usually

applied to the growing crop as a foliar spray, 3-5 weeks before defoliation, to control spouting during storage. When MH is applied as a foliar spray the chemical will be taken up by the leaves and translocated through the plant into the tubers. As a result residues are distributed throughout the tubers and by washing and peeling its concentration in the tubers cannot be reduced. Hence at the time of consumption substantial residues of MH will be present in potatoes. Therefore it is important to study the fate of this chemical in potato tubers with regard to public health.

1.5. Objectives of the thesis:

Therefore as summary the objectives of this thesis are

1) To find out a suitable method to determine quantitatively the amount of free MH present in both potato tubers and in processed products resulting from the pre-harvest foliar treatment of potato crop with MH.
2) To identify the distribution of MH residues within the potato tuber.
3) To find out the effect of processing on MH content in tubers.
4) To search for the possible presence of bound forms of MH in potatoes.

CHAPTER 2

RESIDUE ANALYSIS OF MALEIC HYDRAZIDE (MH) IN POTATOES

2.1. **Introduction**

As described in the previous chapter, unlike other sprout suppressant chemicals, MH is applied to the potato crop as a pre harvest foliar spray. Then it is taken up by the leaves and translocated through the plant into the tubers. When this chemical enters into the potato tuber it can remain either as the parent compound (free MH) or as a metabolite or in a bound form. In this chapter main interest was on the determination of free MH residues present in the tubers resulting from the pre harvest foliar treatment of potato crop with MH. Therefore to determine the amount of free MH residues in potato tubers a sensitive reliable and concise method was required. Various methods were available in the literature for the determination of MH residues in various commodities and hence, it was decided to develop a suitable method from the available methods.

2.1.1. Existing methods for analysis of MH residues

During the past several years various procedures have been reported for the determination of 1,2-dihydro-3,6-pyridazinedione (Maleic hydrazide [MH]) residues in various commodities. The initial method described by Wood (1953) for the determination of Maleic hydrazide (MH) in plant and animal tissues involved alkaline digestion of the sample, rapid steam distillation of the sample with zinc as a reductant and the determination of the hydrazine in the distillate by means of the yellow colour formed with p- dimethylaminobenzaldehyde. Residue determination of below 1 ppm was possible using this method. Lane *et al.*(1958) tried out this method with tobacco and observed the development of a strong red interference colouration and the amount of p-dimethylaminobenzalazine having a yellow colour produced by reacting with MH was also less. They assumed that high nitrate

content of tobacco was causing oxidation of the hydrazine liberated from MH before it could distil over and therefore they made a modification in the method by addition of a small amount of ferrous chloride to the caustic digested sample prior to distillation. This gave essentially a quantitative recovery of MH. Other reducing agents such as stannous chloride, sodium sulphite, sodium thiosulphate, hydroquinone etc. did not aid the recovery. Anglin and Mahon (1958) and Hoffman (1961) also made modification of the distillation-spectrophotometric procedure to obtain a method with increased sensitivity and free from interferences from other compounds. The method modified by Hoffman (1961) was used successfully to analyse MH residues in fruits and vegetables (Hoffman *et al.*1962a) and tobacco and soil (Hoffman *et al.*1962b). Hoffman *et al.*(1965) made another modification in the spectrophotometric method using a charcoal treatment step to increase its specificity. Ihnat *et al.*(1973) improved the recovery of this method using two distillation steps rather than one. But after a collaborative study they found that it was not a successful method (Ihnat *et al.*1975).

After a collaborative study Lane (1963) recommended that the colorimetric detection is the only chemical test for determining MH residues. Later Haeberer *et al.*(1974) also reported that when the colorimetric method was applied to tobacco the colour detection was interfered with other leaf constituents such as pyrole, resorcinol and tryptophan. And also he claimed that any hydrazine remaining in the MH preparation itself, would be analysed and not be distinguishable from the hydrazine produced by the reduction of MH. He developed a rapid quantitative gas chromatographic (GC) method to analyse residues of MH in tobacco and tobacco products, which involved formation of a bis(trimethylsilyl) derivative. This method was not successful in the analysis of MH in crude Cigarette Smoke Condensate (CSC) due to the interferences from other constituents in CSC which were not present in tobacco extract. He improved this method by including a cleaning up step using alumina in a

microcolumn (Haeberer and Chortyk 1974). Liu and Hoffmann (1973) and Haeberer and chortyk (1979) also developed more specific GC methods where derivatisation was involved. King (1983) developed a method to determine MH residues in potato tubers, using GC with electron capture detection, by making a Diels-Alder adduct of MH with cyclopentadiene. Renaud *et al.*(1992) extracted free and bound MH in cured tobacco using hydrochloric acid, by which MH glycoside is simultaneously hydrolysed. Free MH was methylated and detected by capillary GC with an NP detector.

Harrison and Nelson (1990) analysed MH residues in potatoes by competitive inhibition Enzyme-Linked Immunosorbant assay (ELISA) based on two monoclonal antibodies specific for MH. The limit of detection of the system was approximately 1 ppm.

A high performance liquid chromatographic (HPLC) method was developed by Newsome (1980a) to determine MH and its beta-D-glucoside in foods. It did not involve any derivatisation and hence, the sample preparation was quicker than the previous methods. Victor *et al.*(1984) developed a HPLC method for analysis of MH in waste water by direct aqueous injection after filtering through 0.45 μm filter and by electrochemical detection. Vadukul (1991) reported a liquid chromatographic method to determine methanol extractable MH residues in onion and potatoes using solid phase extraction and anion exchange high performance liquid chromatography with UV absorption at 313 nm. Cessana (1991) also developed a more or less similar HPLC method with UV detection at 303 nm for the determination of MH residues in cured garlic bulbs.Yang (1992) developed a method to determine both free and bound MH residues in tobacco using HPLC. He had tried out different methods of sample preparation prior to the detection of MH by HPLC.

2.1.2. Selecting a method for residue analysis of MH in potatoes

As described in section 2.1.1. there were several methods developed by various authors to determine the MH residues in different commodities especially in tobacco and tobacco products. To analyse MH residues in potatoes it was decided to select a simple, quick, reliable and sensitive analytical method from the reported methods, which can be used to quantify the free MH residues present in potatoes. The colorimetric method initially developed by Wood (1953) and later modified by others was the most popular method of analysing MH residues. But using this method it is not possible to quantify the amount of free MH present in the sample as it gives the total amount of MH present. This includes both free and bound forms of MH. And also the colour development is susceptible to interferences by other constituents in the reaction mixture.

Although gas chromatographic (GC) methods developed by Haeberer *et al.*(1974); Haeberer and chortyk (1979); King (1983); and Renaud *et al.*(1992) can be used to quantify individual forms of MH present, it requires derivatisation before injecting into the GC as MH is not a volatile compound. Hence these procedures consume lots of time and are open to inaccuracies. Other available methods for analysis of MH residues were based on high performance liquid chromatography (HPLC). Three HPLC methods were found in the literature to analyse for MH residues in plant materials (Newsome (1980a), Cessana (1991), Vadukul (1991)).

Advantages offered by use of HPLC are that derivatisation was not necessary and the analysis could be carried out under normal laboratory conditions at room temperature. Compared to the other methods these HPLC procedures seemed to be simple and quick and have been used to quantify the free MH present in the samples. Newsome's (1980a) method involved an extensive preparation of a cation exchange resin for the cleaning up of extracts before injecting into the HPLC. But in the methods developed by Cessana (1991) and Vadukul (1991) cleaning up of the extracts was carried out using solid phase

extraction cartridges which is an easy and less time consuming approach. Cessana (1991) had developed his method to determine the MH residues in cloves of garlic bulbs and he used C_{18}-Sep Pack cartridges for the cleaning up where as Vadukul (1991) reported the method for residue determination of MH in onions and potatoes for which he used ion exchange solid phase extraction cartridges for the cleaning up of extracts. As it was felt that the method reported by Vadukul (1991) was easier than other methods and also since an HPLC instrument was readily available in the laboratory it was decided to concentrate on his method for the analysis of MH residues in treated potatoes in order to study the fate of MH within the potato tuber.

In the procedure developed by Vadukul (1991), samples have been extracted with methanol, cleaned up (the extracts) by passing through strong cation exchange (SCX) cartridges and the MH content determined by anion exchange high performance liquid chromatography with UV detection at 313 nm. Using this method the amount of methanol extractable free MH present in the sample can be quantified. To identify other forms of MH (bound or conjugated) present in the samples, other methods have to be followed to release MH before injecting in-to the HPLC.

As the selected method involves HPLC it was thought that reviewing some knowledge about this technique and its use in pesticide analysis would be useful if not essential to the project.

2.1.3. High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography is found to be the most powerful of all the chromatographic techniques. This has arisen from the classical liquid chromatography. Liquid chromatography (LC) refers to any chromatographic procedure in which the moving phase is a liquid in contrast to the moving gas phase in gas chromatography. Compared to GC, HPLC can be applied to almost any substance. For GC analysis molecules must be volatile and

also it is no use for heat-labile compounds. It has been estimated that only 20% of known organic compounds can be satisfactorily separated by GC without prior chemical modification of the sample (Snyder and Kirkland 1979). In practice HPLC and GC methods are complimentary. For a lot of separations either of them can be applied. But it is rare for both to be equally suitable.

Basic parts of HPLC:

Basic parts of HPLC consist of solvent reservoir and pump, injection unit, column, detector and chart recorder or computing integrator to display the detector signal.

Solvent reservoir and pump:

The solvents (mobile phase) used in HPLC can be either water, organic solvent or buffers on their own or mixed with one another. The function of the pump is to pass mobile phase through the column at a controlled flow rate. Before solvents get near to the pump they must be completely free from dust and dissolved air. Dirt in the solvents can damage the pump. And also such materials can collect on top of the column causing irregular behaviour or blockages. It is also important to remove dissolved air because pockets of air can collect in the pump and other places, causing strange behaviour from the detector and irregular pumping action. Therefore the common practice is to filter the solvents through a membrane filter under vacuum. Then it is degassed by ultrasonicating or by bubbling Helium (He) gas through it (purging) for a few minutes to flush out dissolved air. When a UV detector is being used, the solvents must have a low UV absorbance. Since most of the commercial grade solvents have impurities that absorb at low UV wavelengths it is preferable to use purified HPLC grade solvents, if the absorbance range for detection is below 250 nm.

Injecting unit:

Injecting unit is for introducing samples on to the column. Samples can be injected either by microlitre syringe or using a sample injecting valve depending on the type of instrument.

Column:

Columns used in HPLC are usually made with stainless steel. Common dimensions are 25 cm long and 4.6 mm internal diameter. Virtually all modern HPLC columns are packed with the same form of fully porous silica particles with spherical or irregular shape with particle diameters of 10, 5 or 3 μm. Different kinds of surface chemistry give different forms of chromatography.

Detector:

Different kinds of detectors are being used in HPLC. UV absorbance detector is the most common and most satisfactory detector. Here the mobile phase from the column is passed through a small flow cell held in the radiation beam of a UV/VIS spectrophotometer. Compounds that absorb UV or visible radiation can be detected using this detector. Another detector is the fluorescence detector. Some substances when UV irradiated emit either UV or visible light. This fluorescence can be detected in this modified spectrophotometer which is called a fluorescence detector. A refractive index detector monitors the difference in refractive index between the column eluent and a reference stream of pure mobile phase. This is the only HPLC detector that can detect any solute as long as there is a difference in refractive index between the solute and the mobile phase. Photodiode array detectors and electrochemical detectors are the other possible detectors but they are not very commonly used.

Chart recorder and integrator:

Normally the HPLC detector is connected to a chart recorder via an amplifier and the output is a trace where the peak area and peak height measurements give the measure of concentration of the components in the sample.

2.1.4. Use of HPLC in pesticide residue analysis

High performance liquid chromatography has found increasing use for separation of pesticides and their conversion products compared to gas chromatographic methods (Sieber 1974). This requires less extensive clean up of the samples. HPLC methods also can be used to monitor many chemically different pesticides namely carbamates or organophosphates(OP) or organochlorines(O-Cl). This method is particularly useful for pesticides which are not directly amenable to GC determinations such as, the compounds which are not thermally stable (e.g. methyl carbamate insecticides such as carbofuran, or phenylurea herbicides such as diuron and monuron) or cannot be analysed directly (e.g. phenoxyacetic acids such as 2,4 D and MCPA) or are not sufficiently volatile (e.g. some benzimidazole fungicides such as carbendazim and benomyl) without further derivatisation (Ambrus and Thier 1986).

Therefore, as HPLC was found to be an important technique in pesticide residue analysis, it was decided to concentrate on using an HPLC method in the residue analysis of MH in potatoes. And also it was thought that the experience that would be gained by working with this technique would be helpful in undertaking future research work on residues of other pesticide chemicals.

2.2. **Experimental**

2.2.1. Application of HPLC to determine MH

Before extracting potatoes, the performance of the HPLC instrument available in the laboratory to determine MH was studied.

The Liquid chromatograph and the conditions used by Vadukul (1991) for the determination of MH were as follows:

The liquid chromatograph consisted of a Shimatzu LC 6A pump, a Waters 490 programmable ultraviolet (UV) detector operating at 313 nm and a Rheodyne 7125 sample injection valve with a 5 mm^3 sample loop.

analytical column - 25 cm * 4.6 mm i.d. packed with Nucleosil 5 SNB

Guard column - 5 cm * 4.6 mm i.d. packed in house with pellicular anion exchange material

Mobile phase - 0.1 mol dm^{-3} acetic acid adjusted to pH 4.8 with tetramethylammonium hydroxide

The chromatograms were recorded on a chart recorder and integrator.

The liquid chromatograph available in the laboratory here, consisted of a Perkin Elmer series 400 solvent delivery system, Perkin Elmer LC 90 UV spectrophotometric detector with operating range from 195 nm to 390 nm, Perkin Elmer ISS-100 injecting system and a Perkin Elmer LCI-100 laboratory computing integrator.

An analytical column and a guard column self packed with another anion exchange material, Spherisorb SAX 5 μm similar to Nucleosil 5 SNB was used for the study carried out here as that material was readily available. Other HPLC conditions used were similar to the Vadukul (1991) method as given above.

Packing of analytical column and the guard column with Spherisorb SAX 5 μm was carried out as follows.

i) Packing the analytical column and the guard column:

The analytical column was packed using a Shandon column packing instrument.

A dilute slurry of the packing material was packed into the column in upward configuration at high pressure. 25 cm * 4.6 mm i.d. column was used in this case 3.8 g Spherisorb SAX 5 μm packing material was added to 30 ml acetone while stirring. The slurry was ultrasonicated in a low power ultrasonic bath for 5 min before being placed in the solvent reservoir. After the column had been fitted to the top of the reservoir, the pump was switched on to give an instantaneous packing pressure of 6500 psi (44828 KPa), which powered the slurry up into the column at speed to give a compact, stable bed. The packed column was taken out and excess material remaining outside the column was scraped off using a razor blade.

The column was connected to the pump in the HPLC and methanol/water mixture 50:50 (v/v) (conditioning solvent) was passed through the column for 30 min without connecting the detector.

The guard column (precolumn) also was packed as follows using the same column material (Spherisorb SAX 5 μm). The outlet column end fitting of the precolumn body was hand tightened and the inlet end fitting removed. The funnel was screwed onto the precolumn body. About 20 mg packing material was added and the precolumn was tapped vertically on a hard surface at a rate of 1-3 taps per second for 10 min. The procedure was repeated until the column was full (about 60 mg material was needed). The funnel was removed and the excess packing material was scraped off from the top of the column using a razor blade. The inlet end fitting was screwed onto the column and both end fittings were tightened.

The packed guard column was connected to the pump without the analytical column and also without connecting to the detector. Methanol/water 50:50 (v/v) mixture was passed through the column for 15 min. The guard column

was disconnected, the inlet end fitting removed and inspected for a void. Since no void had developed, the inlet end fitting was screwed on again to the column body and connected to the HPLC pump. The other end was connected to the analytical column and in turn to the detector.

ii) Preparation of maleic hydrazide (MH) standard solution:

500 ppm stock solution of MH was prepared as follows, using maleic hydrazide (98.0% purity) obtained from Chem. Services Inc., West Chester, PA. 0.05 g maleic hydrazide was dissolved in boiling pure water (about 80 ml) in a 100 ml volumetric flask. After cooling down, the volume was made up to 100 ml with pure water at room temperature.

iii). Preparation of mobile phase:

0.1 mol dm^{-3} acetic acid solution was prepared by diluting 6.0 g of glacial acetic acid (pronalys'AR, May & Baker Ltd. mol.wt. 60.05) to 1 litre with pure water. pH was adjusted to 4.8 by adding a 10% aq. solution of tetramethylammonium hydroxide (Lancaster).

Before using in HPLC the solution was filtered through a milipore HVLP 0.45 μm membrane filter and degassed for 10 min in an ultrasonic bath. (In later HPLC analyses the mobile phase was degassed by purging with Helium gas.)

iv) Detection of Maleic hydrazide by HPLC:

1 ml of the above stock solution was diluted to 50 ml with pure water in a volumetric flask to give a standard solution containing 10 μg/ml. 10 μl of this solution was injected on to the HPLC under the following conditions.

Analytical column - 25 cm * 4.6 mm i.d. packed with Spherisorb SAX 5 μm
Guard column - 5 cm * 4.6 mm i.d. packed with Spherisorb SAX 5μm
Detector - UV detector operating at 313 nm

Mobile phase - 0.1 mol dm^{-3} acetic acid adjusted to pH 4.8 with tetramethylammonium hydroxide, flow rate 1.0 ml/min

Under these conditions the retention time (R.T.) of MH was about 5 min.

v) Determination of the reproducibility of the integrating system:

Two standard solutions (50 ppm and 10 ppm) of MH were prepared by diluting 5 ml and 1 ml of the 500 ppm stock solution to 50 ml with water separately.

To check the reproducibility of the peak area and peak height at 50 ppm level, fifteen, 10 µl injections of 50 ppm standard was made to the HPLC. Peak area and peak height values were obtained from the integrating system and percent variabilities were calculated.

Same procedure was followed with 10 ppm standard solution.

At 50 ppm level:

% variability of Peak area = 0.224

% variability of peak height = 0.304

At 10 ppm level:

% variability of peak area = 3.227

% variability of peak height = 0.728

At higher concentrations peak area measurement and peak height measurement did not show much variability. But at lower concentrations peak height measurement was more consistent than the peak area measurement. Therefore it was preferred to use peak heights rather than peak area values.

vi) Linear response of the detector:

Series of diluted standard solutions of MH was prepared using the 500 ppm stock solution.

0.1, 0.5, 1.0, 2.5, 5.0 and 10.0 mls of stock solution were transferred to six 50 ml volumetric flasks and the volumes made up to 50 ml with pure water to give concentrations of 1 ppm, 5 ppm, 10 ppm, 25 ppm, 50 ppm and 100 ppm respectively.

From each standard solution two 10 µl injections were carried out on the HPLC under the conditions given in sub section (iv) of Section 2.2.1., and the peak area and peak height values were obtained from the integrating system.

Response curve is given in Fig 2.1.

Each point in the calibration curve represents the average value for two replicate injections.

The straight line calibration curve demonstrated that the detection system is linear from 1-100 µg/ml with UV detection.

Calibration graph obtained by plotting peak area vs concentration of MH (µg/ml) corresponds to the equation

$Y = 95.1X + 235$

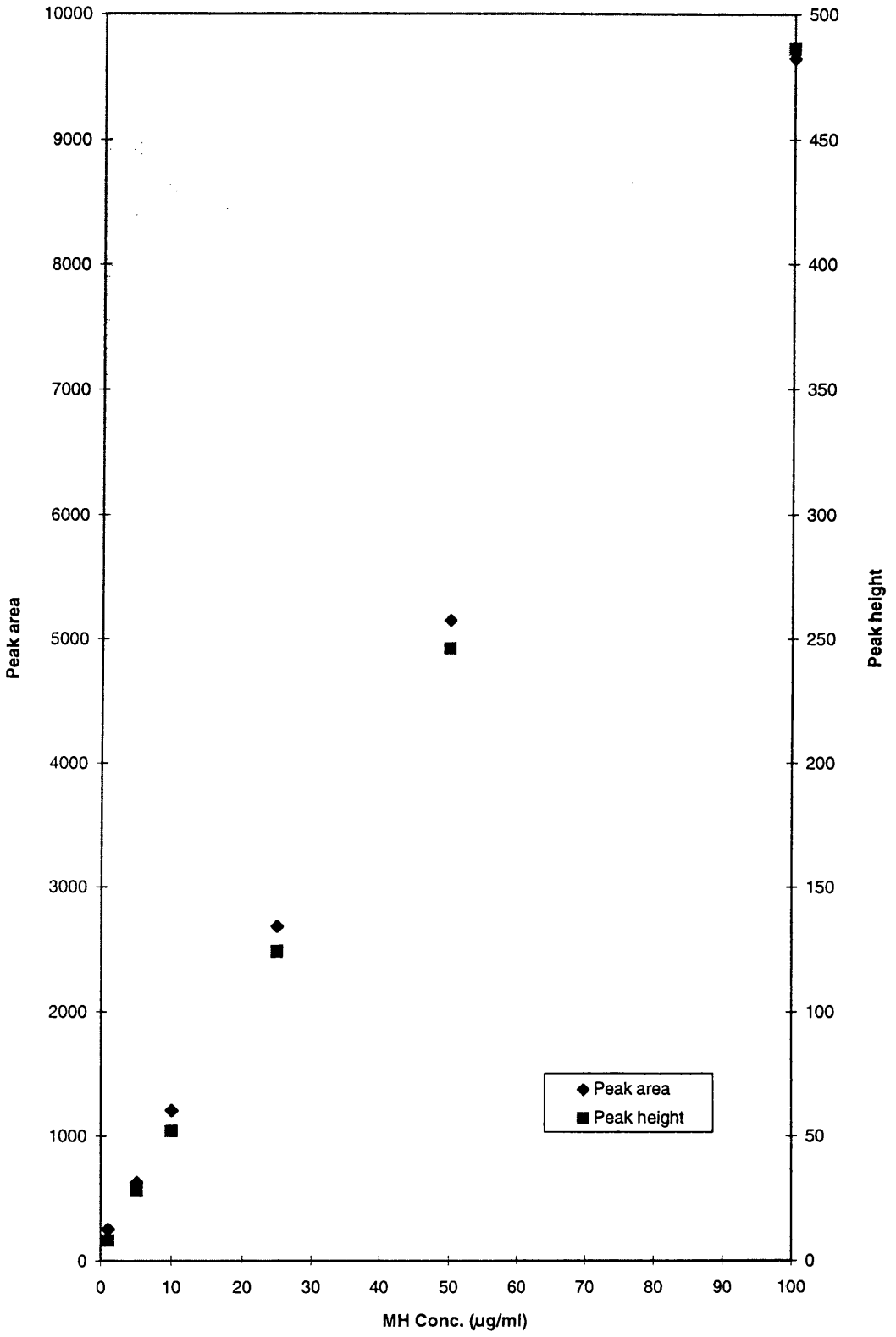
The correlation coefficient of the linear regression=0.999

Calibration graph obtained by plotting peak height vs concentration of MH (ug/ml) correspond to the equation

$Y = 4.82X + 3.79$

The correlation coefficient of the linear regression=1.000

Fig. 2.1 Linear response of the HPLC-UV Detector



(Refer to Section 2.2.1. sub section (vi) for details)

2.2.2. Use of HPLC method to determine free MH residues in potatoes

For the determination of free MH residues in potatoes, the HPLC method described by Vadukul (1991) was used. According to the method the sample was extracted with methanol and cleaned up by passing the extract through a Bond Elut SCX cartridge and eluting with water. Detection of MH was by anion exchange high performance liquid chromatography with UV detection at 313 nm.

Before analysing treated potatoes the method was tried out with a sample of untreated potato to obtain a chromatogram for a MH free potato extract and for a potato extract spiked with MH. The reason for doing so was to find out whether there could be any interfering peaks due to the other constituents in the potato extract at the retention time of MH. The method described by Vadukul (1991) was followed with few modifications. The sample weight used was 20 g, instead of 10 g because the latter sample size was too small to be homogenized in the blender available in the laboratory. The volume of methanol used was also changed accordingly.

2.2.2.1 Extraction and analysing a untreated potato using HPLC

2.2.2.1.1 Material and method

Reagents:

Methanol (HPLC grade, Rathburn chemicals Ltd.)

Glacial acetic acid (Pronalys'AR, May & Baker Ltd.)

Tetramethylammonium hydroxide (Lancaster)

Bond Elut SCX (Varian)

Procedure:

Extraction:

An untreated potato sample (20 g) was blended in a Waring blender with 80 ml methanol for 1 min. Another 20 ml of methanol was added to rinse the sides of the blending cup and then blended for a further 1 min. The homogenized mixture was filtered through Whatman No.1 filter paper on a 10 cm Buchner funnel using a vacuum. The blending cup was rinsed with 20 ml of methanol and transferred into the filter funnel in order to wash the residue. The residue was further rinsed with 10 ml of methanol and the combined filtrate was transferred into a 250 ml R.B. flask. The solution was concentrated to 4-6 ml on the rotary evaporator at 40 ^{0}C and then transferred quantitatively into a 10 ml volumetric flask. The total volume was adjusted to 10 ml with water.

Clean-up:

Two, 2 ml aliquots of the extract were passed through two Bond Elut SCX cartridges separately and each was eluted with 2 ml water. Eluates were collected in 5 ml volumetric flasks. Volume of one eluate was made up to 5 ml with water.(blank sample) and the other one was made up to the volume after spiking with 50 µl of 500 ppm MH stock solution (spiked sample).

Detection:

10 µl aliquots from both solutions (blank sample and spiked sample) were injected on to the HPLC system separately, under the following conditions.

Column - 25 cm * 4.6 mm i.d. packed with Spherisorb SAX 5 µm

Mobile phase - 0.1 mol dm^{-3} acetic acid adjusted to pH 4.8 with tetramethylammonium hydroxide. Flow rate 1 ml/min.

Detector - UV spectrometric detector operating at 313 nm.

2.2.2.1.2 Results:

The chromatograms obtained are given in Fig. 2.2.

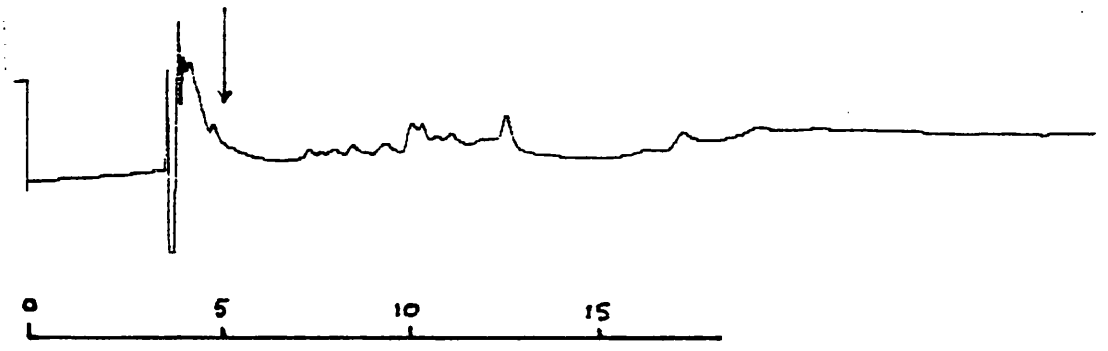
2.2.2.2. Finding out the efficiency of SCX cartridges

Two, 2 ml aliquots of the uncleaned potato extract from the above experiment (Section 2.2.2.1) was taken and one aliquot was spiked with 50 µl of 500 ppm MH stock solution before passing through the Bond Elut SCX cartridge and the other aliquot was spiked with a similar amount of MH after passing through another SCX cartridge. Volumes of both cleaned extracts were made up to 5 ml with water and injected into the HPLC separately under the same conditions as in Section 2.2.2.1.

Chromatograms obtained are given in Fig. 2.3.

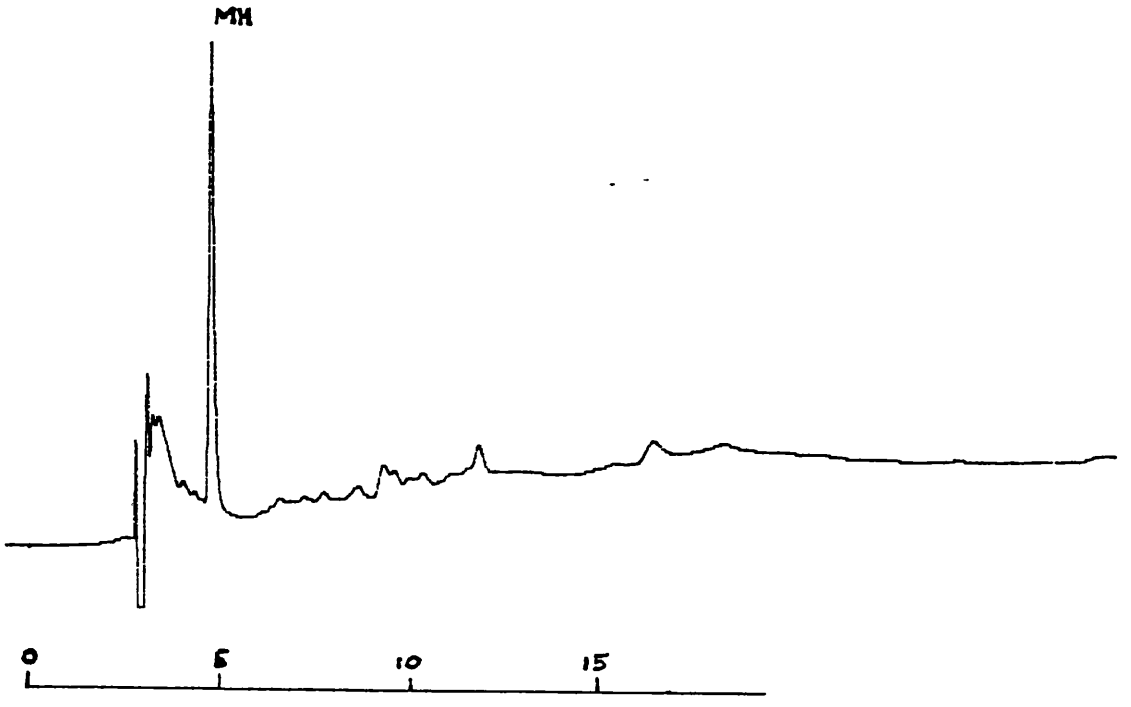
Fig. 2.2. Comparison of HPLC chromatograms of potato extracts unspiked and spiked with MH (HPLC conditions are given under Section 2.2.2.1.)

a) Unspiked sample (blank sample)



minutes

b) Spiked sample (5 ug MH/ml)

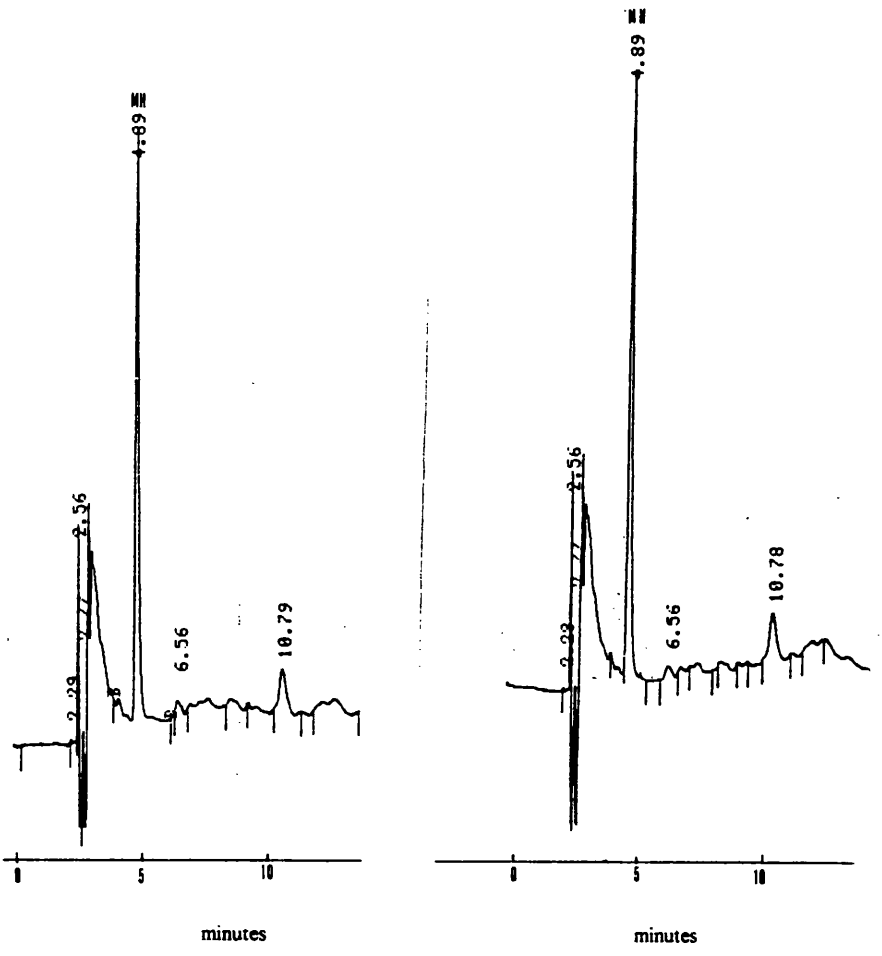


minutes

Fig. 2.3. HPLC chromatograms of cleaned up potato extracts spiked with MH before and after clean up using SCX cartridges

a) spiked with 25 µg MH before clean up

b) spiked with 25 µg MH after clean up



HPLC conditions:

column : 25 cm * 4.6 mm i. d. packed with Spherisorb SAX 5 µm

mobile phase: 0.1 mol dm-3 acetic acid adjusted to pH 4.8 with tetramethylammonium hydroxide Flow rate 1 ml/ min

detector : UV spectrophotometric detector operating at 313 nm

2.2.2.3. Determination of the limit of detection (LOD)

Several 10 µl aliquots of the cleaned up potato extract spiked with different amounts of MH were injected into the HPLC to find out the concentration at which a peak for MH can be recognised with signal/noise ratio 3:1. This level is defined as the limit of detection (Dyson 1990) and it was found to be 2.5 ng.

2.2.3. Validation of the HPLC method

The efficiency of the method described by Vadukul (1991) for the determination of MH in potatoes, was assessed by determining the amount of MH recovered after the addition of known amounts of the chemical to potato samples which were known to be free from MH. For the recovery study, 20 g samples of untreated potatoes were spiked with 3 levels of MH as follows.

i) 10 µg MH/g potato (10 ppm)
ii) 25 µg MH/g potato (25 ppm)
iii) 50 µg MH/g potato (50 ppm)

Five replicate samples were spiked at each level. Spiking the samples with MH and the determination of the recovery of MH was carried out as follows.

Procedure:

Spiking at 10 ppm level:

20 g of a MH free potato sample was blended with 80 ml methanol for 1 min in the Waring blender and 0.4 ml of 500 ppm MH stock solution (= 200 µg MH) was added to this homogenized mixture. Another 20 ml methanol was added for rinsing the sides of the blending cup and the contents blended for a further 1 min. The extract was filtered through Whatman No.1 filter paper under suction. Blending cup was rinsed with another 20 ml methanol and transferred to the filter funnel in order to wash the residue. Residue was washed with another 10 ml methanol. Combined filtrate was concentrated to 4-6 ml by evaporating at 40 ^{0}C

in the rotary evaporator. Volume of the concentrated extract was made to 10 ml with water, cleaned up by solid phase extraction using Bond Elut SCX cartridges and the MH content determined using HPLC under the conditions described in the Section 2.2.2.1.

This operation was carried out with five replicate samples.

Same procedure was followed for spiking the samples at 25 ppm level and 50 ppm level by adding 1 ml and 2 ml of 500 ppm stock solution of MH respectively and analysing and testing the recovery of MH.

Results are tabulated in Table 2.1.

2.2.4. Analysis of MH residues in treated potatoes:

A batch of Fazor treated potatoes were obtained from KP AGRICULTURE, Niven House, Riseholme, Lincoln. This potato crop had been treated with Fazor (a.i. potassium salt of MH 80%w/w) at the rate of 5 Kg/ha. Harvested tubers were stored for a period of approximately 7 months before being submitted for analysis. At the time of receiving, the tubers had sprouts of about 1 mm length. All the tubers were stored at 9-10 ^{0}C until the analysis was carried out. The analyses were undertaken to find out the MH content in a total tuber, MH content in different sized tubers and distribution of MH within a tuber.

i) Determination of MH residues in total tuber

Materials and Method:

Reagents:

Methanol (HPLC grade, Rathburn chemicals Ltd.)

Glacial acetic acid (Pronalys' AR, May and Baker Ltd.)

Tetramethylammonium hydroxide (Lancaster)

Bond Elut SCX (Varian)

Procedure:

Sample preparation:

A potato tuber (169.84 g) was taken from the sample lot, washed with tap water to remove adhering soil and dabbed lightly with a tissue paper to dry. Whole tuber was cut into small pieces suitable for maceration and mixed thoroughly. 20 g, representative samples were taken for analysis.

Extraction, clean up and detection of MH:

2 * 20 g samples were extracted with methanol in the same way as described in Section 2.2.2.1. and the extracts cleaned up by passing 2 ml aliquots through Bond Elut SCX cartridges and eluting with 2 ml water. Eluates were collected in 5 ml volumetric flasks and the volumes made to 5 ml with water. 10 ul aliquots of cleaned up extracts were injected into the HPLC under similar conditions as given in Section 2.2.2.1. and the peak heights were compared with that of the MH standard, injected into the HPLC under the same conditions.

Results are presented in Table 2.2.

ii) Determination of MH residue distribution within a potato tuber

To find out the MH residue distribution within the tuber, various parts of the tuber such as skin (peel), outer flesh and inner core were taken separately and analysed.

Sample preparation:

Preparation of peel sample:

Two, MH treated potato tubers were taken (total weight 416.17 g), washed to remove adhering soil and sliced perpendicular to the rose and heel axis to give slices of about 1 cm thickness. A 2 mm thick strip was peeled off (skin) from the slices. Peel material was cut into small pieces mixed well and 2 * 20 g samples were taken for analysis.

Preparation of outer flesh samples:

10 mm thick strip (outer flesh) was cut off from each of the above peeled slices, the strips were cut into small pieces, mixed well and 2 * 20 g samples were taken for analysis.

Preparation of inner core samples:

Remaining central core of the above slices were also cut into small pieces, mixed well and 2 * 20 g samples were taken for analysis.

Analysis of the samples:

Extraction, clean up and detection of MH in the above samples were carried out in the same manner as described for the analysis of total tuber.

Results are presented in Table 2.2.

iii) Distribution of MH residues between potato tubers

20 g representative samples from MH treated potato tubers of different sizes were analysed using the HPLC method.

The amounts of MH residues detected in each tuber are presented in Table 2.3.

iv) Distribution of MH residues between two halves of a potato tuber

A MH treated potato tuber was halved by cutting along the rose and heel axis and two representative samples (each of 20 g) were taken from the two halves. MH contents in the two samples were determined following the HPLC method.

Results are presented in Table 2.4.

2.2.5. Investigating the stability of MH in the potato extract

For the analysis of MH residues in potatoes the samples were extracted with methanol. After evaporation and clean up steps the final extracts were made up in water. On many instances it was not feasible to subject all of these extracts to HPLC analysis on the same day as they were prepared. Therefore sometimes these extracts had to be stored under refrigeration for a few days before injecting into the HPLC. Cessana (1991) reported that even though aqueous solutions of MH standards are stable, MH residues in the cleaned up aqueous extracts of garlic tissues decreased with time. Therefore it was decided to find out the stability of MH in the cleaned up aqueous extracts of potatoes as well. Three replicate samples of cleaned up aqueous extract of a MH treated potato were taken and the MH contents were determined. Then they were kept in the refrigerator for 3 weeks and analysed again for the MH content.

The results obtained are given in Table 2.5.

2.2.6. Effect of evaporating temperature on MH residues in potato extract

In the analysis of MH in potatoes, the samples were extracted with methanol and the methanol extract was concentrated by evaporating at 40 ^{0}C under reduced pressure in the rotary evaporator. Because of the high boiling point of methanol it took a long time (>30 min.) to concentrate the extract to a small volume. Therefore it was decided to increase the bath temperature used for

evaporation to reduce the time of evaporation. Before using a higher temperature, the effect of the evaporating temperature on MH was determined.

First it was tried out by evaporating three methanol solutions of equal volume containing equal amounts of MH (500 μg), at three different temperatures (40, 50 and 60 ^{0}C) under reduced pressure to a volume of about 2-3 ml. The time taken for the evaporation was noted down. Volumes of the three solutions were made to 5 ml with water and 10 μl aliquots were injected to the HPLC to determine the amount of MH recovered. From the three samples evaporated at different temperatures, almost the same recovery of MH was obtained and no loss of MH due to the increase of temperature was observed. The time required to evaporate the solution at 60 ^{0}C was about half the time required at 40 ^{0}C. There was not much difference between the times required for evaporations at 40 ^{0}C and 50 ^{0}C.

Then this operation was repeated with methanol extracts of potatoes as it was felt that there could be an effect of the higher temperature on the recovery of MH in the presence of potato extract.

Two potato samples (20 g) free from MH were extracted with methanol using the same extraction procedure as for extracting potatoes for MH analysis. The extracts were spiked with known amount of MH (125 μg) and evaporated at 40 ^{0}C and 60 ^{0}C separately under reduced pressure on the rotary evaporator until the volume was reduced to about 2-3 ml. Volume of each extract was made to 5 ml with water and cleaned up by passing 2 ml aliquot through a Bond Elut SCX cartridge and eluting with 2 ml water. Eluates were made to 5 ml with water and 10 μl aliquots were injected into the HPLC to determine the MH content.

Here also it was found that there was no loss of MH, due to the evaporation of the potato extract at 60 ^{0}C. But practically it was difficult to use a higher temperature, because the extract always tended to bump up and froth when evaporating at 60 ^{0}C. Therefore continuous watching was required throughout the evaporating step. Although there was a possibility to control the bumping by

adding an antifoaming agent to the extract when evaporating, it was decided to evaporate the extracts at 40 ^{0}C as given in the method. Even though it was time consuming, it was practically much easier to carry out.

2.2.7. Use of Internal Standard (I.S.) in HPLC for the determination of MH in potatoes

During the HPLC analysis of MH, sometimes the peak area and peak height values for the same sample changed during the day. Therefore before and after injecting each sample into the HPLC, the standard MH also had to be injected to maintain the accuracy. Hence, it was decided to try out a suitable internal standard to use with MH. In the internal standard technique a known compound at a fixed concentration is added to the unknown sample to give a separate peak in the chromatogram. The known compound is used as a internal marker. This will compensate for the effects of minor variations in separation parameters on peak size including fluctuations in the sample size.

Selecting an internal standard:

For a compound to behave as an internal standard it should have following properties (Snyder and Kirkland 1979).

1. It must have a completely resolved peak - no interference.
2. It must elute close to the compound of interest.
3. It must behave equivalently to compound of interest for analysis involving pre treatments, derivative formation etc.
4. It must be added at a concentration that will produce a peak area or peak height ratio of about unity with compound of interest.
5. It must not be present in original sample.
6. It must be stable and unreactive with sample components, column packing or mobile phase.
7. It is desirable for it to be commercially available in high purity.

The compound which was selected to be tried out was another herbicide called Asulam.

Molecular formula of asulam: $C_8H_{10}N_2O_4S$

Some properties of Asulam and MH:

	Asulam	MH
Mol. Wt.	230.2	112.1
pKa value	4.82	5.65
Solubility in water	4 g/l	6 g/l

Worthing and Walker (1987).

Preparation of an asulam standard solution and investigating the performance of asulam in HPLC:

10 mg of asulam (98.0% purity) was dissolved in 10 ml hot water to make a solution containing 1000 µg/ml (=1000 ppm). Using this solution a UV spectrum was run to find out the wavelength at which it gives maximum absorbance.

At 303 nm it gave the maximum absorbance of 2.465.

Then a standard solution of asulam containing 10 µg/ml was prepared by diluting a 50 µl aliquot from the 1000 ppm standard solution to 5 ml with water. A 10 µl aliquot of this solution was injected into the HPLC under the same conditions as used for MH which is described in Section 2.2.2.1. but with UV detection at 303 nm. Retention time (R.T.) of the asulam was compared with the retention time of MH injected under the same conditions.

R.T. of asulam was 9.3 min. while the R.T. of MH was 4.5 min under those conditions. When a mixture of these two compounds (10 μg/ml each) was injected into the HPLC under the above conditions it gave two distinct peaks. 5 * 10 μl aliquots of this mixture were injected into the HPLC and the peak area ratios and peak height ratios of the two compounds were calculated using the chromatogram obtained.

Peak area ratios obtained for five replicate injections were almost the same. It was the same with the peak height ratios. Therefore it was decided to use asulam as an internal standard.

Then a potato extract free from MH was spiked with the mixture of asulam and MH, and injected into the HPLC under the above HPLC conditions. Potato extract itself gave some peaks very close to the retention time of asulam. Therefore identifying the peak for asulam was very difficult. Hence, the use of asulam as an internal standard in the analysis of potato samples was discontinued. Although further studies could have been carried out by changing the wavelength of UV detection in HPLC and also by increasing the amount of asulam added to the potato extract, it was not persevered with due to the time limitation.

2.3. **Results**:

Table 2.1. Recovery of MH from potatoes

Fortification level (mg kg^{-1})	Recovery % (± SD)			
	n	Range	Mean	Overall recovery
10	5	81.48-87.41	85.19	
25	5	91.18-95.06	93.34	88.69(± 4.19)
50	5	81.32-94.68	87.54	

n = no. of replicates

Table 2.2. MH content in Fazor treated potatoes

Sample	MH content (µg/g) (Mean)
Total tuber	11.51 (10.92, 12.09)
Peel	9.32 (8.47, 10.16)
Outer flesh	10.58 (10.36, 10.79)
Inner core	9.35 (10.21, 8.48)

values given in parentheses are the results of replicates.

Table 2.3. MH contents in individual tubers.

size of the tuber (diameter)	MH content (μg/g)
4.5 cm	8.90
4.8 cm	8.63
5.5 cm	2.11
5.5 cm	10.26

Table 2.4. Distribution of MH between two halves of a potato tuber

One half	Other half
8.53	8.52

Table 2.5. stability of potato extract during storage

MH content (μg/g) before storage	MH content (μg/g) after storage
4.95	4.74
5.07	4.85
5.43	5.23

2.4. **Discussion**

The experimental results show that MH is present in potato tubers as a result of foliar application of MH to the potato crop. The MH concentrations in various parts (peel, outer flesh and inner core) of the tuber have very similar

values (Table 2.2). Therefore it can be suggested that MH is evenly distributed throughout the tuber. Different tubers in the same lot have different concentrations of MH (Table 2.3). Therefore among the tubers the distribution of MH is not uniform and also does not appear to be any relationship between the tuber size and the MH content judged by the limited number of samples analysed here.

The experimental results of the investigation of the stability of MH in aqueous potato extracts (Table 2.5.) show that there is no significant difference between the values for the MH content in the extracts before and after storing Hence, storing in this manner is quite acceptable.

2.5. **Conclusions**:

Work in this chapter involved finding out a suitable method for the analysis of free MH residues in potatoes. An HPLC method was selected and using this method, with few modifications the amount of free MH in potato tubers obtained from a MH treated potato crop was studied. According to the results it was found that free MH is evenly distributed throughout the tubers and between the tubers the MH content varied. Two halves of the same potato tuber had equal concentrations of MH.

In this chapter the determination of free MH residues was carried out on raw potatoes. Usually potatoes are not consumed raw. They will be subjected to some kind of processing such as frying or boiling before consumption. Therefore in the next chapter the effect of processing on MH will be discussed and investigated.

CHAPTER 3

EFFECT OF PROCESSING ON MALEIC HYDRAZIDE (MH) RESIDUES IN POTATOES

Usually potatoes are processed in different ways before consumption. Two major ways of processing are boiling and frying to make chips and crisps. No literature is available on the fate of MH on processing, apart from the work of Anglin and Mahon (1958) and McKenzie (1989). Based on some unpublished data (R.I.V. 1955) Van der Heijden *et al.*(1981) has reported that MH residues resulting from the application of MH to potato crop are relatively high, the chemical is very stable and does not decompose during household cooking and frying. Thus, the aim of this study was to find out in more detail whether processing such as frying and boiling has any effect on MH content in potato tubers.

The only published method available for the determination of MH residues in oil and products containing oil or any other processed potato products was the improved spectrophotometric method (Anon 1990) initially developed by Wood (1953). Using this method it is difficult to quantify free MH present in the sample and it provides the value for total amount of MH present including bound residues as well. The detection technique used in the work done for this thesis for the determination of free, unbound MH residues in potatoes is HPLC. Therefore in the analysis of fried potato products it is essential to extract MH from the oily material into a suitable solvent which can be injected into the HPLC. As there was no published method available to extract oil from the crisps and to extract MH from the oil the extraction method used by Ritchie *et al* (1983) in the determination chlorpropham residues in potato crisps was followed with few modifications. Non oil residue was extracted with methanol.

To determine the MH content in boiled potatoes the same analytical procedure used for the analysis of MH in raw potatoes was followed.(adapted method of Vadukul (1991) described in Chap. 2, section 2.2.1.)

3.1. Effect of frying on MH residues in potatoes

3.1.1. Introduction:

Frying of potatoes to make chips and crisps is a large scale industry in UK. Over 10% of potatoes grown in the UK goes to the crisping industry alone (anon 1994a) and these products are readily available in the market as snacks. These processed products are not always made from freshly harvested potatoes. Potatoes used for commercial processing are mostly taken from stores, where the potato tubers are treated with various sprout suppressant chemicals such as tecnazene, IPC and CIPC to prevent sprouting during storage as mentioned earlier. As these chemicals are usually applied on to the surface of the tubers they mostly remain on the peel (Dalziel and Duncan 1980). First step in the commercial crisp manufacturing process normally involves washing and peeling of potato tubers. Therefore the major part of these chemical residues present in the tubers will be removed before frying. But, since the sprout suppressant chemical MH is applied to the potato crop before harvesting and since it is evenly distributed throughout the tubers, washing and peeling will not help in removing the MH residues. Therefore even if the tubers are washed and peeled before frying they may contain almost the same amount of MH residues as they had before. Therefore the aim of this study here was to find out the effect of frying on MH residues present in potato tubers and also to develop a suitable method to analyse MH residues in fried products.

Anglin and Mahon (1958) have studied the effect of frying on MH residues in a potato tuber. They have made some modifications in the spectrophotometric method initially developed by Wood (1953) and used in the analysis. They have carried out only one determination and reported that no MH is destroyed during the cooking of potato chips(crisps). McKenzie (1989) also had studied the effect of

frying on MH residues in potatoes. But the results he presented are somewhat confusing. In his study he used three samples of MH treated potatoes for frying and the percentage of free MH residues he found in fried potato material varied from 13.6 - 32.9 compared to the amount detected in fresh potatoes. His experimental details were not very clearly explained. Therefore as there was no clear answer to the effect of frying on MH residues in potatoes it was decided to reinvestigate this aspect.

Usually when making crisps thin potato slices of 0.8 - 1.5mm thickness are deep fried in oil at 180 - 190 ^{0}C for about 90 - 120 sec. Hence the same frying procedure was followed in this study.

3.1.2. **Experimental**:

In addition to the frying of MH treated potatoes, a series of other experiments were carried out in this study, using a filter paper model to find out the effect of frying and also to improve the method of analysis of MH residues in fried potatoes. Whatman No.1, 5.5 cm diameter. filter papers were used in the model experiments. i.e. instead of potato slices filter papers were spiked with known amounts of MH and used in the investigations carried out.

When the spiked filter papers were fried, the oil in the fried filter papers and then the MH residues in the oil fraction were extracted following the procedure developed by Ritchie *et.al* (1983) in the analysis of chlorpropham residues in potato crisps. MH residues in the oil free filter papers were extracted using methanol. MH residues in both extracts were determined by HPLC, after cleaning the extracts using 'Bond Elut' SCX cartridges.

In the case of fried potato slices the extractions were carried out in much the same way as filter papers and the MH content determined using HPLC.

3.1.2(a). Experiment No.1: Investigating the effect of frying time on MH using the filter paper model

3.1.2(a).1. Materials and Method:

Materials:

Whatman No.1 filter papers (diameter 5.5 cm)

Maleic hydrazide standard solution (100 ppm)100 µg MH/ml water (prepared from 1000 ppm MH stock solution)

500 µl(0.5 ml) micro pipette with disposable tips

Sunflower cooking oil

Diethyl ether (RP Normapur AR)

Sodium chloride (Fisons analytical reagent)

Methanol, Acetonitrile and Hexane (HPLC grade, Rathburn chemicals Ltd.)

Bond Elut SCX (Varian)

Glacial acetic acid (Pronalys'AR, May & Baker Ltd.)

Tetramethylammonium hydroxide (Lancaster)

Method:

Sample preparation:

Four sets of filter papers each containing 12 filter papers were taken and two sets were spiked with MH by wetting each filter paper with 0.25 ml of 100 ppm MH standard solution (25 µg MH), using the 0.5 ml micropipette. Other two sets were wetted with pure water (each filter paper with 0.25 ml of water) and considered as blank samples. Out of the two filter paper sets spiked with MH, one set was fried in Sunflower cooking oil at 180 ^{0}C for 90 sec and the other set was just immersed (approx. 5 sec) in the hot oil at 180 ^{0}C and taken out. Two filter paper sets wetted with water were also fried separately in oil in the same manner.

A domestic deep fryer (Kenwood) was used for frying.

Extraction:

Fried filter papers were extracted as follows to determine the MH contents in the oil fraction and non oil fraction of the filter papers.

Extraction of oil from fried filter papers:

Four sets of fried filter papers were cut separately into small pieces and put into 4 bottles (each 60 ml capacity) having screw caps. 40 ml hexane was put into each bottle, the cap screwed on tightly and shaken in a wrist shaker for 15 min. The hexane extracts were decanted into four previously weighed 500 ml R.B. flasks. Filter papers were again shaken with 30 ml hexane + 2 ml diethyl ether for 15 min. and the extracts decanted into the R.B flasks. Filter papers were shaken once more with 30 ml hexane + 4 ml diethyl ether and shaken for further 10 min. and the extracts decanted to the flasks. Combined extracts in each flask were evaporated at 40 ^{0}C in a rotary evaporator. After evaporation the flasks containing oil were weighed. Evaporation was continued until a constant weight was obtained and weight of the oil extracted was calculated by the difference in weights.

Weight of the oil = (weight of the flask + oil) - weight of the empty flask.

The oil fraction and the non oil fraction were extracted and analysed separately for MH.

Extraction of MH from oil (oil fraction):

a) 5 ml hexane was added to the flask containing oil (approx. wt. 5 g) to reduce the viscosity of the oil and the solution transferred into a 100 ml separating funnel.

b) 25 ml acetonitrile extractant was added to the separating funnel containing oil + hexane and shaken vigorously for 1 min. The layers were allowed to separate.

(acetonitrile extractant was prepared by saturating 250 ml acetonitrile with hexane and adding 2.5ml 2.5% aq. sodium chloride solution. Precipitated sodium chloride was removed by filtration)

c) The acetonitrile extract obtained in (b) was vigorously shaken in sequence in a series of three separating funnels each containing 10 ml hexane.

d) The hexane layer in (b) was extracted with a further 25 ml acetonitrile extractant which was then washed in the same lots of hexane contained in the same funnels and in the same sequence as the first acetonitrile extract.

e) Two lots of acetonitrile extracts were then combined in a R.B. flask and evaporated to dryness in the rotary evaporator at 40 ^{0}C. 3 ml water was added to the residue in the flask and dissolved by simple swirling (solution A).

Extraction of MH from oil free filter papers (non oil fraction):

After removing oil by extracting with hexane and diethyl ether, 40 ml methanol was added to each bottle containing oil free filter papers and shaken for 1 h in the wrist shaker. Methanol extracts were filtered into 100 ml R.B. flasks and evaporated to dryness in the rotary evaporator at 40 ^{0}C. The residue was dissolved by adding 3 ml water and swirling gently (solution B).

Clean-up:

2 ml aliquots of the above solutions (solution A and solution B) were passed through Bond Elut SCX cartridges and eluted with 2ml water. The eluates were collected in 5ml volumetric flasks and the volumes were made up to 5 ml with water.

Detection:

10µl aliquots of the cleaned up extracts were injected into the Perkin Elmer HPLC system with UV detector operating at 313 nm.

HPLC conditions were as follows.

Analytical column (25 cm * 4.6 mm i.d.) and guard column (5 cm * 4.6 mm i.d.) self packed with Spherisorb SAX 5 µm.

Mobile phase: 0.1 mol dm^{-3} acetic acid, adjusted to pH 4.8 with tetramethylammonium hydroxide (Flow rate: 1 ml/min).

3.1.2(a).2. Results:

HPLC chromatograms of the extracts of oil fraction and non oil fraction of the blank samples (filter papers wetted with water and fried) had no interference peaks at the retention time of MH. And also no MH was detected in the extracts of oil fraction of fried filter papers spiked with MH. (limit of detection was 0.375 μg/g oil).

MH was detected only in the extracts of non oil fraction of the MH treated fried filter papers.

HPLC chromatograms of the extracts of oil fraction and non oil fraction are given in Fig.3.1.

Analytical results of the filter papers spiked with MH and then fried, are presented in Table 3.1

Table 3.1. Recovery of MH from spiked filter papers after frying

Sample description	Amount of MH added (μg) before frying	Amount of MH* recovered(μg) after frying		Average % recovery
		oil fraction	non oil fraction	
Filter papers fried for 5 sec.	300	n.d.**	184.39	61.43 ± 1.98
Filter papers fried for 90 sec.	300	n.d.**	175.24	58.41 ± 1.21

* These values are an average of 3 replicates.

**. not detectable

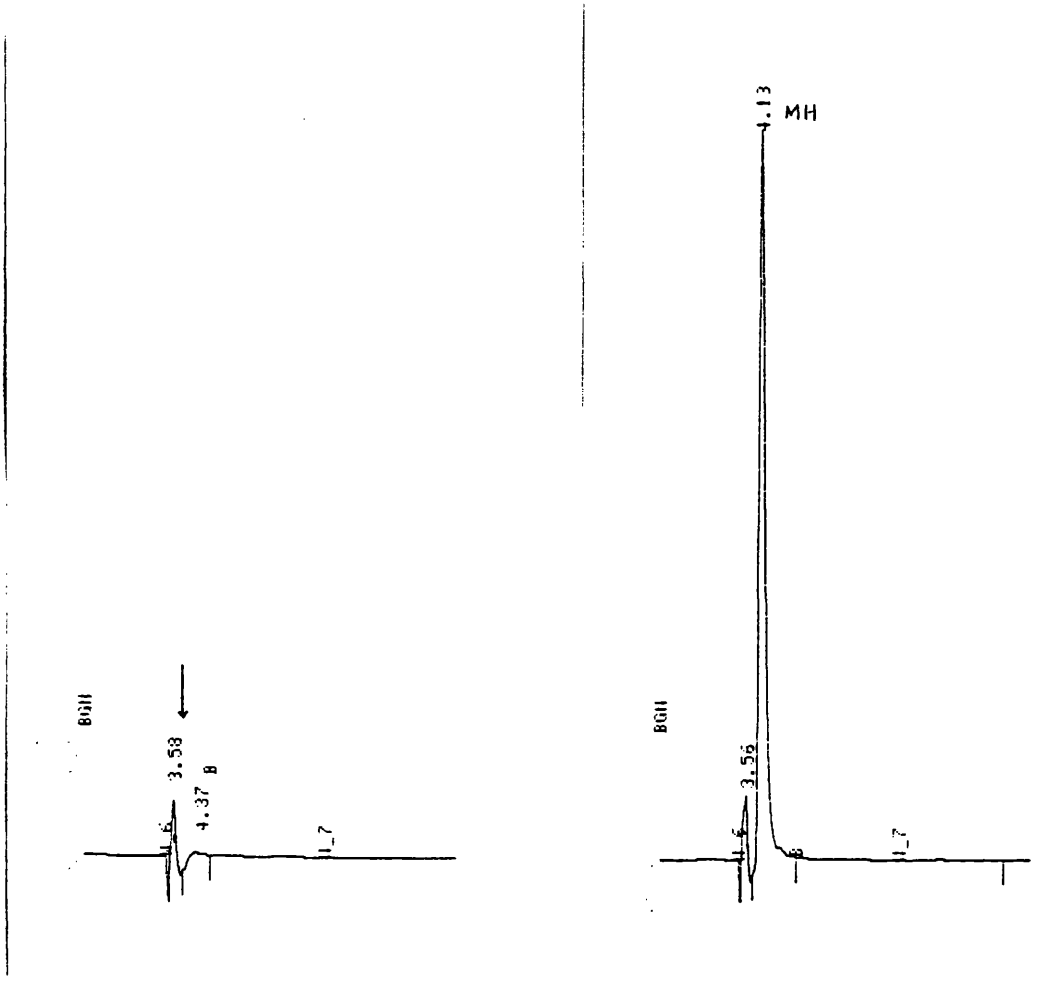
From the above results it was noted that about 60% of MH was recovered after frying and there is no obvious effect of the frying time on the recovery of MH.

And also no MH was recovered in the oil fraction. Absence of any MH in the unspiked filter papers which were fried in the same oil in which the spiked filter papers were fried showed that no MH has gone into the oil in the fryer.

Fig 3.1 HPLC chromatograms of the extracts of a) oil fraction and b) non oil fraction of the fried filter papers spiked with 300 μg MH

a) oil fraction

b) non oil fraction



HPLC conditions:

column : Spherisorb SAX 5 um (25 cm * 4.6 mm i.d.)
mobile phase: 0.1 mol dm $^{-3}$ acetic acid adjusted to pH 4.8 with tetramethylammonium hydroxide (flow rate 1 ml/min)
detector : UV spectrphotometric detector operating at 313 nm

(refer to the Section 3.1.2(a).2)

3.1.2(b). Experiment No. 2: Frying of MH treated potatoes

3.1.2(b).1. Materials and method:

Materials:

A potato tuber from a potato crop (cv. Saturna) treated with 'Fazor'(a.i. Potassium salt of MH 80%w/w) at the rate of 5 Kg/ha.

Solvents and reagents are the same as in Section 3.1.2(a).1.

Method:

Sample preparation:

The potato tuber was rinsed with water to remove adhering soil, then hand peeled and halved by cutting along the rose and heel axis. One half was cut into small pieces, mixed well and 20 g representative samples were prepared for the analysis in the raw state.

The other half was weighed and sliced using a knife to give slices of approximately 1 mm thickness and fried in oil at 180 ^{0}C for 90 sec. Fried slices (crisps) were allowed to cool and then weighed.

Extraction:

Extraction of raw potato:

20 g sample of raw potato was extracted with methanol

as shown in the flow chart below.

SAMPLE (20 g)

1. Blended with 100 ml methanol for 2 min. and filtered through a Whatman no.1 filter paper in a Buchner funnel under vacuum.
2. Rinsed the blending cup with another 20 ml methanol and transferred to the filter funnel in order to rinse the residue.
3. Rinsed the residue again with 10 ml methanol

COMBINED FILTRATE

↓

Concentrated to 4-6 ml
on the rotary evaporator at 40 ^{0}C

↓

Made the volume to 10 ml with water
in a 10 ml volumetric flask.

Extraction of fried potato(crisps):

Extraction of oil from crisps:

40 g crisps were successively extracted with 80 ml hexane, 80 ml hexane + 5 ml diethyl ether and again 80 ml hexane + 5ml diethyl ether by blending in a Waring blender for 90 sec. each time. Extracts were filtered through a Whatman No.1 filter paper on a Buchner funnel under suction. Combined filtrate was transferred to a pre weighed 500 ml R.B. flask and evaporated at 40 ^{0}C under reduced pressure using the rotary evaporator.

Oil free residue remaining on the Buchner funnel was weighed and a 5 g sample was taken for extraction.

Extraction of non oil residue:

5 g sample of non oil residue was shaken with 40 ml methanol in a screw capped bottle for 1 h using a wrist shaker. After 1 h the mixture was filtered under

suction through a Whatman No.1 filter paper. The filtrate was transferred to a 100 ml R.B. flask and evaporated to dryness at 40 ^{0}C under reduced pressure in a rotary evaporator. Residue was dissolved in 3 ml water by simple swirling.

Clean-up:

2 ml aliquots from the fresh potato extract and the fried potato extract were cleaned up by passing through Bond Elut SCX cartridges and eluting with 2 ml water. Eluates were collected in 5 ml volumetric flasks and the volume made up to 5 ml with water and filtered through 0.45 µm membrane filters before injecting into HPLC for MH determination.

Detection:

10 µl aliquots from the cleaned up extracts were injected into the HPLC under the same conditions given in section 3 .1.2(a).1. and the MH residue content determined by comparing the chromatogram with the chromatogram of the MH standard run under the same conditions.

3.1.2(b).2. Results:

Results obtained by HPLC analysis are presented in Table 3.2.

Table 3.2. Effect of frying on MH residues in potatoes (tuber No.1).

Residue content of fresh potato (µg/g)	Residue content of fried potato (µg/g)	% MH in the fried product
16.87*	1.82*	9.73

* These values are calculated on the basis of the weight of fresh potato.

These results indicate, only about 10% of MH from the original quantity, was present in the fried product. Compared to the recovery of MH from filter papers

(60%) potato slices have given a low recovery (10%). Although this is similar to the results obtained by McKenzie (1989), it is difficult to compare these results with his as his experimental details are not very clearly stated.

Anyhow, in this experiment MH residue data for fresh and fried potatoes were obtained using 2 halves of the same potato tuber, and in an earlier study it was found that there was no difference in MH content between 2 halves of a potato (Chap. 2, section 2.2.3.). Therefore the low recovery may be due to some effect of the frying process on MH residues.

This experiment was repeated with another tuber from the same lot of potatoes. But the MH residue content in the tuber before frying was very low (2-3 ug/g). In the chromatogram of the extract of fried material, although there was a peak at the retention time of MH for which peak area and peak height values were given by the integrator it was not possible to quantify accurately since the peak was too small to consider as a signal among the noise. Therefore it was not possible to get an accurate result.Those potatoes were in the store at 9-10 ^{0}C for a few months, and their skin also had become wrinkled. The few tubers analysed at this stage gave very low values for MH residue content. Therefore the frying experiment could not be repeated until a new batch of potato tubers treated with MH was obtained for analysis.

Therefore another experiment was carried out using the filter paper model to find out whether there is any obvious effect of potato components (eg. juice) on the recovery of MH on frying.

3.1.2(c). Experiment No. 3: Effect of potato juice on the recovery of MH on frying

3.1.2(c).1. Materials and method:

Materials:

All the materials were the same as described in section 3 .1.2(a).1. including potato juice extracted from an untreated (MH free) potato tuber.

Method:

Potato juice extraction:

An untreated potato tuber was washed, peeled and cut into small pieces and blended for 1 min in a small blender. Macerated potato pieces were further crushed using a mortar and pestle. The crushed potato material was squeezed through a muslin cloth to obtain the potato juice.

Treating filter papers with MH plus potato juice and frying:

2 sets of Whatman No.1 filter papers (diameter 5.5 cm), each containing 12 filter papers were taken and each set spiked with 300 ug MH (each filter paper with 0.25 ml 100 ppm MH solution). They were left for 15-20 min to dry and one set was spiked with clarified potato juice (supernatent liquid after settling down of the starch material), by wetting each filter paper with 0.25 ml juice (one dose). Both filter paper sets were fried separately in oil at 180 ^{0}C for 90 sec.

Another two sets of filter papers were also spiked with the same amounts of MH, allowed to dry and then each set wetted with one dose of unclarified potato juice (potato juice suspension). Both sets were allowed to dry and one set was again spiked with another dose of unclarified potao juice (all together two doses). Both sets were fried separately in oil at 180 ^{0}C for 90 sec. after drying.

Extraction of fried filter papers:

Removing the oil from fried filter papers, extraction of MH from oil free filter papers and the cleaning up of the extracts before injecting into the HPLC were accomplished following the same procedure as described in Section 3.1.2(a).1.

Detection:

MH content was determined by injecting 10 µl aliquots of the cleaned up extracts into the HPLC under the conditions described in section 3.1.2(a).1.

3.1.2(c).2. Results:

Results obtained by HPLC analysis are presented in Table 3.3.

Table 3.3. Effect of potato juice on the recovery of MH on frying

sample description	MH content before frying (µg)	MH content after frying (µg)	% recovery
Filter papers with out potato juice	300	166.06	55.35
Filter papers spiked with one dose of clarified potato juice	300	125.29	41.76
Filter papers spiked with one dose of unclarified potato juice	300	101.90	33.96
Filter papers spiked with two doses of unclarified potato juice	300	60.66	20.22

The above results show a reduction in the recovery of MH in the presence of potato juice. The reduction is greater in the presence of unclarified potato juice than in the presence of clarified potato juice. Therefore it can be suggested that there is some effect of the constituents particularly particulate material in the potato juice on MH during frying.

At the same time it was noted that the fried filter papers spiked with MH did not give a good recovery even in the absence of potato juice. Only 55% recovery was noted. Three reasons can be proposed for the low recovery.

1. Frying in the oil at high temperature may affect MH and change its chemical nature.

2. When MH is spiked on to filter papers it may have become bound to some materials such as ash in the filter papers.
3. Efficiency of the method of extraction of MH from fried material is low.

Another experiment was planned to find out whether the frying process has an effect on the recovery of MH.

3.1.2(d). Experiment No. 4: Effect of frying on the recovery of MH

Here again two sets of filter papers (12 in each set) were taken and each set spiked with 300 µg MH. One set was fried in oil at 180 ^{0}C for 90 sec and the other set was analysed without frying. Extraction, cleanup and determination of MH in both sets of filter papers were carried out in the same manner, following the same procedure used before in the analysis of fried filter papers.

Both sets of filter papers were extracted with hexane/diethyl ether followed by extraction with methanol as described earlier when analysing fried filter papers. Methanol extracts were cleaned up by passing through Bond Elut SCX cartridges. MH content in methanol extracts of both sets of filter papers and the MH content in the hexane/diethyl ether extract of the non fried filter papers were determined using HPLC.

Results:

Amounts of MH recovered from methanol extracts of the filter papers are tabulated in Table 3.4.

Table 3.4. Effect of frying on the recovery of MH.

sample description	Initial amount of MH (μ g)	Amount of MH recovered (μg)	% recovery
Filter papers not fried	300	159.03	53.01
Filter papers fried	300	158.26	52.75

The hexane/diethyl ether extract of the non fried filter paper contained 0.62 μg (0.2% from the total amount) MH which is negligible.

According to the results there is no obvious difference between the recoveries of MH from the two sets of filter papers. Therefore it can be inferred that frying in oil at a high temperature does not effect the recovery of MH from filter papers.

As mentioned earlier, the presence of other materials on the filter papers such as ash may have affected the recovery. It may be possible that metal ions such as Ca^{2+} present in ash could form some salts with MH which are insoluble in methanol. Therefore this could lower the recovery.

To confirm this possibility it was decided to use some ashless filter papers such as Whatman No.40 containing only 0.01% ash, instead of the normal filter papers Whatman No.1(ash content 0.06%), the type of filter papers which were used for all the investigations carrieed out so far.

3.1.2(e). Experiment No.5: Effect of ash in the filter papers on the recovery of MH.

A set of filter papers (Whatman No.40) was spiked with MH and extracted with 40 ml methanol in the same way as with Whatman No.1 filter papers and analysed for MH content in the methanol extract using HPLC.

Results:

The amounts of MH recovered are given in table 3.5.

Table 3.5. Effect of ash in the filter papers on the recovery of MH

Type of filter paper	% MH recovered
Whatman No.1	63.83
Whatman No.40	68.42

In this extraction about 68% of MH was recovered from Whatman No.40 filter papers. Therefore compared to the Whatman No.1 filter papers, Whatman No.40 filter papers gave a slightly higher recovery (about 5% more). As there were too few replicates on this occassion statiscal significance of the higher recovery was not evaluated.

A few other extractions were also being carried out with both types of filter papers using methanol/water and methanol/aqeous phosphate buffer mixture as extractants. The reason for using methanol/water was that MH is more soluble in water than in methanol. By adding water to methanol it will enhance the solubility of MH and then the mixture can remove more MH from the filter papers during extraction. When the phosphate solution was used, metal ions such as calcium(Ca^{2+}) which may be present in the filter papers can react with phosphate(PO_4^{3-}) to form calcium phosphate. This will prevent or reduce MH forming insoluble salts with Ca^{2+}. Therefore adding PO_4^{3-} also should enhance the recovery of MH from filter papers.

3.1.2(f). Experiment No.6.: Determination of the recovery of MH from spiked filter papers using methanol/water and methanol/phosphate as extractants.

Four sets of filter papers of Whatman No.1 and Whatman No.40 (two sets of each kind) were spiked separately with MH and two sets (one set of each kind)

were extracted with a mixture of 35 ml methanol and 5 ml water separately. The other two sets were extracted with a mixture of 35 ml methanol and 5 ml of 0.01M aqueous solution of potassium dihydrogen phosphate. The extracts were evaporated and the volume was made to 5 ml with water in each case and analysed for MH using HPLC.

Results:

The results are presented in Table 3.6.

Table 3.6.: Recovery of MH from treated filter papers using methanol/water and methanol/PO_4^{3-} as extractants.

	% MH recovered	
Type of filter paper	methanol/water	methanol/PO_4^{3-}
Whatman No.1	67.16	68.60
Whatman No.40	63.55	60.79

In the previous experiment, when MH spiked Whatman No.40 filter papers were extracted with methanol only, a higher recovery was noted than with Whatman No.1 filter papers (Table 3.5.). But, in the above extractions (i.e. methanol/water and methanol/PO_4^{3-}) the recoveries obtained from Whatman No.40 filter papers were if anything less compared to Whatman No.1 filter papers. Therefore by looking at the overall results it is difficult to say that the ash content in the filter papers has any effect on the recovery of MH.

Another possibility for the low recovery of MH was the low efficiency of the method of extraction. In all the experiments carried out so far using the filter paper model, the extraction of MH from the spiked filter papers was carried out by shaking with 40 ml methanol for one hour in a wrist shaker. But, that extraction may

not have removed all the MH present in the filter papers. Therefore it was decided to rinse the filter papers a few times with methanol, after the first extraction.

3.1.2(g). Experiment No.7: Investigating the efficiency of extarction of MH with methanol.

Again two filter paper sets were taken and spiked with MH. One set was extracted with 40 ml methanol as before. The other set was extracted with 40 ml methanol followed by rinsing three times successively with 2 * 20 ml methanol and 10 ml methanol and the extracts combined. The extracts of two filter paper sets were evaporated separately to dryness and the residues dissolved in 3 ml water. MH content was determined by HPLC.

Results:

Results are presented in Table 3.7.

Table 3.7. Investigating the efficiency of extraction

% MH recovered by one methanol extraction	% MH recovered by methanol extraction followed by rinsing
63.83	80.69

Therefore it is clear that rinsing has resulted in a definite increase in the recovery.

Up till now, before determining the MH content in the methanol extract of spiked filter papers, the extract was evaporated to dryness and dissolved in 3 ml water by simple swirling. But it was felt that instead of adding 3 ml water in one batch it would be more accurate if the flask containing the residue was rinsed 2-3 times with small quantities of water and transferred quantitatively into a volumetric flask and made to a constant volume (5 ml).

Therefore with these modifications included in the analytical procedure, another potato tuber containing MH was fried and analysed.

3.1.2(h). Experiment No.8.: Determination of MH content in fried potatoes with improvements in the method of extraction.

3.1.2(h).1. Materials and method:

Materials:

A potato tuber from a new batch of tubers obtained from a potato crop (cv. Saturna) treated with 'Fazor'(a.i. potassium salt of MH 80%w/w) at the rate of 4.5 Kg/ha. Other materials are the same as described in Section 3.1.2(b).1.

Method:

Sample preparation:

Same as in section 3.1.2(b).1.

Extraction:

Extraction of fresh potato and extraction of oil from the fried material was the same as given under section 3.1.2(b).1.

Extraction of non oil residue:

5 g of the non oil residue was extracted with 40 ml methanol by shaking in the wrist shaker for 1 h followed by a few more extractions as follows.

i) shaken for 10 min with 20 ml methanol

ii) shaken for another 10 min with 20 ml methanol

iii) the residue rinsed again with 10 ml methanol

Combined methanol extract was transferred to a 250 ml R.B flask and evaporated to dryness at 40 ^{0}C under reduced pressure. The residue was dissolved in water by rinsing the flask 2-3 times with small aliquots of water and the solutions transferred quantitatively into a 5 ml volumetric flask and the volume made up to 5

ml. Solution was passed through a 0.45um membrane filter before injecting in to the HPLC.

Clean up:

2 ml aliquots from the above solution and the extract of fresh potato sample were passed through Bond Elut SCX cartridges and eluted with 2 ml water. Volumes of the eluates were made to 5 ml with water in 5 ml volumetric flasks.

Detection:

MH content in the cleaned up extracts were determined by injecting 10 µl aliquots to the HPLC.

3.1.2(h).2. Results:

Results obtained are presented in Table 3.8.

Table 3.8. Residue results of frying of potatoes.(tuber No.2)

MH content in the fresh potato (µg/g)	MH content in the fried product (µg/g)	% recovery
5.423*	1.695*	31.26

* MH residue values given in this table are calculated on the basis of fresh weight of the potato.

This result shows, after improving the extraction method, the recovery of MH from fried potato slices has gone up to 31% from 10%, which was the recovery value obtained from fried potatoes before improving the method. But still it was a rather low recovery. Therefore it was decided to find out whether any substances like sugars (e.g. glucose) and amino acids (e.g. lysine) present in fresh potato material have any effect on MH during frying causing the lowering of recovery.

Therefore using the filter paper model, with glucose and lysine as additives another experiment was carried out.

3.1.2(i). Experiment No.9: Effect of additives on the recovery of MH on frying

3.1.2(i).1.

Materials and method:

Materials:

1%(w/v) aqueous solution of glucose (prepared by dissolving 0.25 g D(+)glucose (Hopkin and Williams) in 25 ml water)

1%(w/v) aqueous solution of lysine (prepared by dissolving 0.25 g DL-lysine monohydrate (Sigma chemical company) in 25 ml water)

Three sets of Whatman No.1 filter papers (10 filter papers in each set)

Other materials were the same as in Section 3.1.2(a).1.

Method:

Three sets of filter papers were spiked with MH (each set with 250 µg MH). The papers were left to dry and two sets were again spiked with 1% glucose solution (each filter paper with 0.25 ml of glucose solution). They were then dried and one set was spiked again with 1% lysine solution.(each filter paper with 0.25 ml of solution) and dried. All three sets of filter papers were fried in oil at 180 ^{0}C for 90 sec. Fried filter papers of all three treatments were extracted separately.

Extraction of oil from filter papers:

Fried filter papers were cut into small pieces and extracted with 30 ml hexane followed by another two extractions with 30 ml hexane + 2 ml diethyl ether and 30 ml hexane + 4ml diethyl ether by shaking in a screw capped bottle for 15 min. each time in a wrist shaker.

Extraction of oil free filter papers:

Non oil fractions were extracted with methanol in the same screw capped bottles in four steps as follows.

i) shaken with 40 ml methanol for 1 h.

ii) shaken with 20 ml methanol for 10 min.

iii) shaken with 20 ml methanol for 10 min.

iv) rinsed with 10 ml methanol

The combined methanol extracts of each treatment were evaporated to dryness, dissolved in 5 ml water, cleaned up using Bond Elut SCX cartridges and the MH content determined by HPLC.

3.1.2(i).2. Results:

Results obtained are presented in Table 3.9.

Table 3.9.: Effect of additives on MH on frying.

Name of additive	Amount of MH applied (µg)	Amount of MH recovered (µg)	% recovery
No additive	250	161.21	64.48
Glucose	250	136.23	54.49
Glucose + Lysine	250	140.93	56.37

These results show about 8-10% reduction in the recovery of MH from the filter papers spiked with additives. Between the two treatments of additives there is no obvious difference in recoveries. Anyhow the constituents in potato material may have some slight effects at least on MH, during frying.

At this stage, another MH treated potato tuber (tuber No.3) was fried and analysed and 48.5% MH was recovered from the fried product. But the recovery obtained from the previous potato sample was only 31%. Therefore the frying experiment was carried out with a few more potato tubers.

3.1.2(j). Experiment No.10: Frying and analysing of MH treated potatoes.

Another two potato tubers were analysed as fresh and fried for MH residues in the same way as described in Experiment 8. But at the detection step a different analytical column was used. As the back pressure of the previous column started to increase, the column had to be changed. The new analytical column used was Nucleosil 5SB which is also a strong anion exchange column. All the other HPLC conditions were same as before.

The results obtained in the analysis of two potato tubers are presented in Table 3.10.

Table 3.10. Residue results from frying of potatoes (tuber Nos. 4 & 5).

Tuber No.	MH content* (µg/g)		% MH in fried product
	Fresh potato	Fried potato	
4	4.57	3.67	80.31
5	10.32	7.32	72.98

*MH contents were calculated on the basis of fresh weight of the potatoes.

According to these results it was noted that there was a considerable increase in the recovery of MH from fried potato slices, compared to the recoveries obtained before (10%, 31% and 48%).

In addition to the improvement in the method of analysis it was also thought that the recovery may have been enhanced by an effect from the oil in which the

potato slices were fried because this oil had been used for frying for a long period of time. i.e from the beginning of all these experiments. Therefore it was thought that the oil might have changed in chemical nature (by polymerisation) and caused some effects on MH. Therefore it was decided to repeat the frying experiment using new oil.

3.1.2(k). Experiment No.11: Determination of recovery of MH from fried potatoes using new cooking oil for frying

3.1.2(k).1. Materials and method:

Materials:

Sunflower cooking oil (newly purchased)

Other materials were the same as in Section 3.1.2(h).1.

Method:

Another MH treated potato tuber from the same batch of tubers used in the previous analyses was analysed as fresh and fried following the same method as described in experiment 8.

3.1.2(k).2. Results:

Given in Table 3.11.

Table 3.11. Residue results from frying of a potato (tuber No.6) using new oil.

Tuber No.	MH content (µg/g)		% recovery from fried product
	Fresh potato	Fried potato	
6	10.00	7.72	77.20

The recovery obtained was similar to the old oil. Therefore the old oil may have had no effect on MH to enhance the recovery.

All the values given in these results were calculated using peak height values of the chromatograms. But, it was noted that after replacing the old column in the HPLC with the new column (Nuleosil 5SB) there was a considerable difference between the MH content values of fried potato material calculated using peak heights compared with peak areas (about 20-50% more with peak area values). For raw potato samples the values calculated using peak areas and peak heights were almost the same. This kind of difference was not observed with previous experiments. Therefore the reason for this difference also had to be investigated.

As the results obtained with the improved method of analysis showed an increase in the recovery of MH from fried material (crisps) it was also thought to repeat the experiment carried out earlier to see the effect of potato juice on the recovery of MH on frying using the filter paper model.

3.1.2(l). Experiment No.12: Investigating the effect of potat juice on MH on frying using improved method of analysis.

3.1.2(l).1. Materials and method:

Materials:

Same as in section 3.1.2(c).1.

Method:

Potato juice was extracted from an untreated (MH free) potato tuber as described in section 3.1.(2c).1. Two sets of filter papers (each containing 12 papers) of Whatman No.1 were used and they were spiked separately, one set with only MH and the other set with both MH and potato juice as described in section 3.1.2(c).1.

Both filter paper sets were fried separately in the oil at 180 ^{0}C for 90 sec and extracted for MH as follows:

Removing the oil from fried filter papers using hexane/di-ethyl ether and the extraction of oil free filter papers with methanol was carried out in the same way as described in section 3.1.2(a).1.

Methanol extracts of the two treatments were evaporated to dryness, dissolved in 5 ml water, cleaned up using Bond Elut SCX cartridges and the MH content determined using HPLC.

3.1.2(l).2. Results:

Results are presented in Table 3.12.

Table 3.11: Effect of potato juice on MH on frying

sample description	MH content (µg)		% recovery
	Before frying	after frying	
filter papers without potato juice	300	215.74 (214.44)	71.91 (71.48)
Filter papers with potato juice	300	189.34 (220.81)	63.11 (73.60)

Values in parentheses were calculated using the peak area readings of the chromatogram. Open values were calculated using peak height readings.

According to these results it was noted that the improvement of the method of extraction has increased the recovery of MH from fried material (filter papers as well as crisps). In the presence of potato juice there is a reduction in the recovery (about 9%), according to the values calculated with peak heights. But with peak area values no reduction is shown.

Although there is a difference between the values calculated using peak area and peak height for MH content in fried filter papers having potato juice, no difference is shown between those values in the case of fried filter papers without potato juice. The slight difference shown can be due to experimental error. In the chromatogram of the fried product having potato material (filter papers as well as potato slices.) another small adjacent peak was noted before the MH peak. Those

two peaks had separated from each other before coming to the baseline. Therefore when integrating the area of MH peak it is likely that this small adjacent peak was counted as part of the major peak as well, giving a large peak area value. In the chromatogram of the fried filter papers without potato juice, this additional peak was not present. Therefore it was obvious that this interfering peak was coming from the fried potato material.

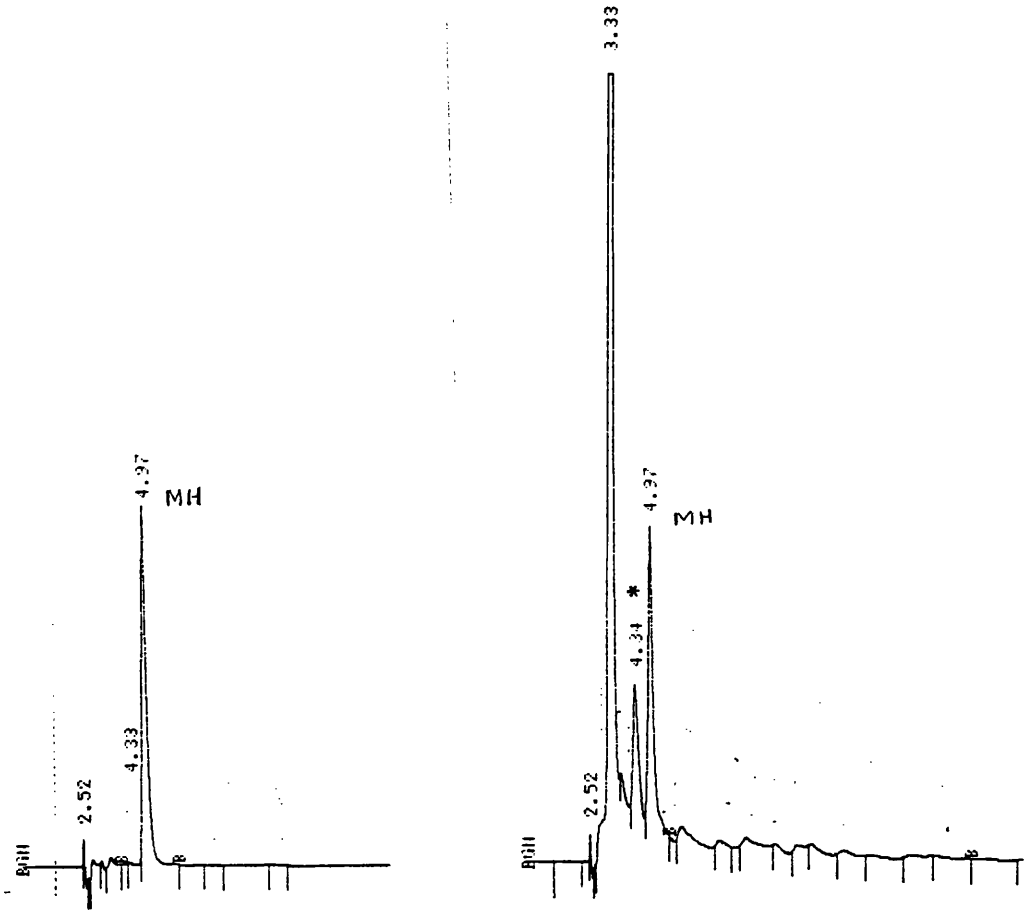
Chromatograms of an extract of fried filter papers spiked with MH and wetted and not wetted with potato juice are given in Fig.3.2.

Fig 3.2 HPLC chromatograms of fried filter papers spiked with MH and potato juice (obtained from a MH free potato tuber)

a) spiked with 300 µg MH and no potato juice

b) spiked with 300 µg MH and wetted with 3 ml potato juice

* peak coming from potato material



HPLC conditions:

column : Nucleosil 5 SB (25 cm * 4.6 mm i.d.)
mobile phase : 0.1 mol dm $^{-3}$ acetic acid adjusted to pH 4.8 with tetramethylammonium hydroxide (flow rate 1 ml/min)
detector : UV spectrophotometric detector operating at 313 nm.

Therefore it was decided to investigate whether there is a real effect of this adjacent peak on the peak area and peak height value of MH peak. Hence, another experiment was carried out to find out the possible effect of the adjacent peak caused by potato material on the peak area and peak height value of the MH peak using the filter paper model.

3.1.2(m). Experiment No.13: Investigating the effect of potato juice on the area and height values of the peak for MH in the HPLC chromatogram.

This study was carried out by spiking known quantities of MH into the methanol extract of a set of fried filter papers which were spiked with MH free potato juice extracted from an untreated potato tuber.

3.1.2(m).1. Materials and method:

Materials:

Potato juice extracted from an untreated potato tuber

MH stock solution (1000 ppm)

Other materials are the same as in section 3.1.2(a).1.

Method:.

A set of 24 filter papers (Whatman No.1) was wetted evenly with potato juice using a micro pipette (each filter paper with 0.25ml potato juice), left to dry for 15-20 min and fried in oil at 180 ^{0}C for 90 sec (12 filter papers at a time) Two lots of filter papers were extracted separately with hexane/diethyl ether to remove the oil and then the oil free filter papers were extracted with methanol as described in section 3.1.2(i).1. The methanol extracts of two lots of filter papers were combined and evaporated to dryness under reduced pressure at 40 ^{0}C. The residue was dissolved in water and the volume made to 10 ml. 3 * 2 ml aliquots of this extract

were cleaned up separately using Bond Elut SCX cartridges. Three eluates were spiked respectively with 50 µg, 100 µg and 150 µg MH using 0.05 ml, 0.10 ml and 0.15 ml of the 1000 ppm MH stock solution. Volumes of the spiked eluates were made to 5 ml with water.

10 µl aliquots from these solutions were injected into the HPLC under the same conditions as used before and the MH contents in the spiked solutions were calculated using both peak area and peak height values from the chromatograms. These were compared with the chromatogram obtained for 10 ppm standard solution which was prepared using the same stock solution of MH used for spiking, and run under the same HPLC conditions.

3.1.2(m).2. Results:

HPLC chromatographic results are presented in Table 3.13.

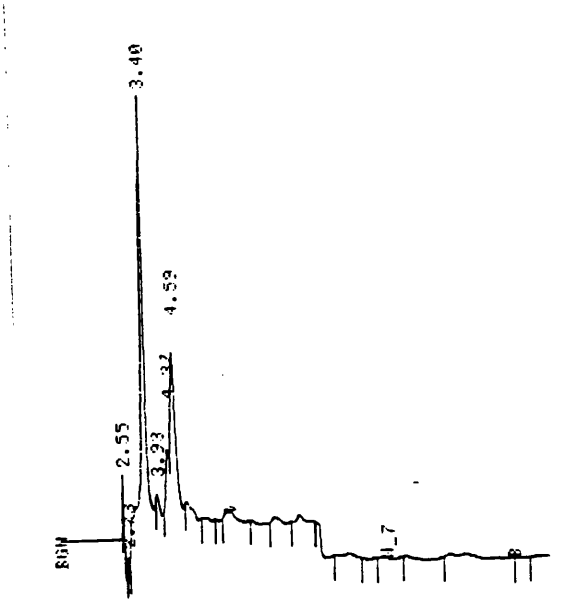
Table 3.13. MH contents in the spiked extracts calculated using peak area and peak height values in the chromatogram.

Extract No.	Amount of MH added (µg)	Amount of MH detected (µg)	
		Based on peak area	Based on peak height
1	50	56.27	51.32
2	100	110.98	105.59
3	150	153.44	147.33

Chromatograms of these 3 samples and a blank sample (extract of filter papers spiked with potato juice and no MH was added) are given in Fig.3.3.

Fig 3.3 HPLC chromatograms of MH spiked extracts of fried filter papers wetted with potato juice before frying (Refer to Section 3.1.2(m) Experiment 13)

a) blank sample (no MH added)



b) spiked with 10 µg/ml MH

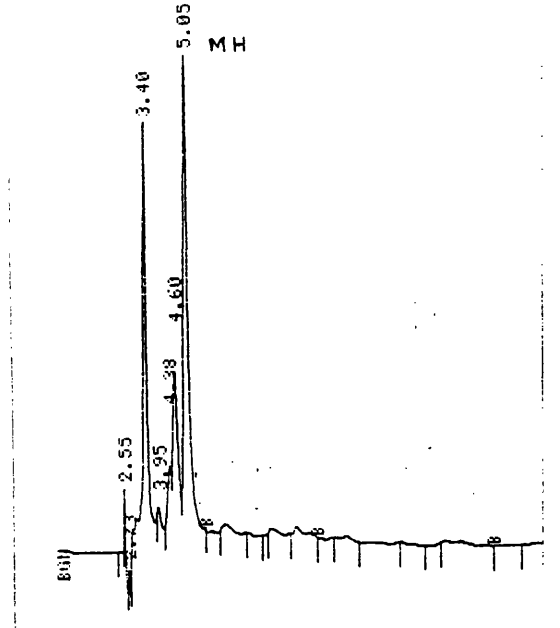
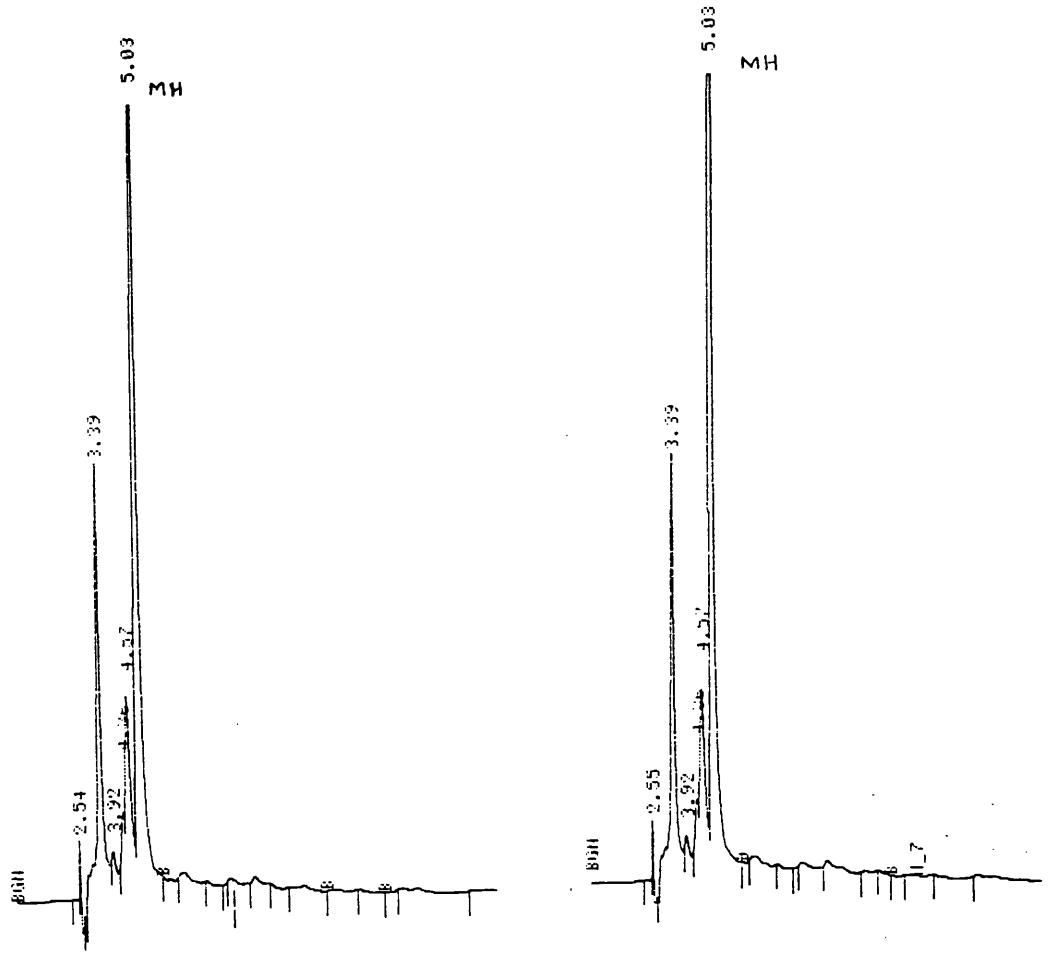


Fig 3.3 (contd...)

c) spiked with 20 µg/ml MH d) spiked with 30 µg/ml MH



HPLC conditions:

column : Nucleosil 5 SB (25 cm * 4.6 mm i.d.)
mobile phase : 0.1 mol dm $^{-3}$ acetic acid adjusted to pH 4.8 with tetramethylammonium hydroxide
detector : UV spectrophotometric detector operating at 313 nm

In all chromatograms the additional peak adjacent to MH peak was present. Therefore it was obvious that the interfering peak was coming from a constituent in the potato material.

As shown in the Table 3.13 the values for MH content calculated using peak area are higher than the values calculated using peak heights. But the differences are not very high. When the values calculated using peak areas were considered, at the lower concentration of MH (50 μg) the difference between the actual value and the calculated value is 12%. At 100 μg level it is 10% and at 150 μg level only 2%. Therefore with the increase in the amount of MH the effect from the adjacent peak was less, because when the peak for MH is large the percentage contribution of peak area from adjacent peak to the peak area of MH peak is small.

When analysing potato slices since they had only small quantities of MH there was considerable effect from this adjacent peak. That was the reason for getting higher values for the MH content in fried potatoes when they were calculated using peak area values. Therefore it was more accurate to do the calculations using peak height values. Anyhow, this interference peak in the fried potato material should be removed by improving the clean up step of the extracts during analysis. But this aspect was not looked in to further due to the lack of time available.

As the last experiment another MH treated potato tuber was fried and analysed using the improved method to confirm the effect of frying on MH residues in potatoes, and a 67% recovery of MH after frying was obtained.

3.1.2(n). Final method for the determination of free MH residues in potato crisps (chips):

Apparatus:

High speed blender fitted with leak proof glass jar and explosion proof motor.

Buchner funnel (diameter 10 cm and 6 cm)

Round filter paper (Whatman No.1, diameter 9 cm and 5.5 cm)

Filtration flask (1lit and 100 ml)

R.B. flasks with ground joint (500 ml and 250 ml)

Volumetric flask (5 ml)

Screw capped glass bottle (60 ml)

Laboratory wrist shaker

Bond Elut SCX cartridge (Varian)

0.45μm millipore HVLP membrane filter

Rotary vacuum evaporator 40 ^{0}C bath temperature

HPLC equipped with UV/VIS detector operating at 313 nm.

Reagents:

Hexane (HPLC grade, Rathburn chemicals Ltd.)

Diethyl ether (RP Nomapur AR)

Methanol (HPLC grade, Rathburn chemicals Ltd.)

Glacial acetic acid (Pronalys'AR, May and Baker Ltd.)

Tetramethylammonium hydroxide (Lancaster)

Procedure:

Homogenize 40 g crisps with 80 ml hexane for 90 sec. in the blender. Suction filter the homogenate through Whatman No.1 filter paper in a Buchner funnel. Blend the filter cake again with 80 ml hexane and 5ml diethyl ether for 90 sec and filter through the same funnel. Blend the filter cake once more with 80 ml

hexane plus 5 ml diethyl ether and filter. Weigh the filter cake and save for further analysis.

Transfer the combined filtrate into a pre weighed 500 ml R.B. flask and evaporate in the rotary evaporator at 40 ^{0}C to obtain oil fraction by removing the solvent.

Transfer 5 g of the above filter cake into the screw capped glass bottle (60 ml capacity) and extract with 40 ml methanol by shaking for 1 h in the wrist shaker. Filter under suction and extract the filter cake two more times with 20 ml portions of methanol using the wrist shaker by shaking for 15 min each time. Filter the extract through the same funnel and rinse the filter cake with another 10 ml methanol. Transfer the combined filtrate into a 250 ml R.B. flask and evaporate to dryness in the rotary evaporator at 40 ^{0}C. Dissolve the residue in small quantities of water 2-3 times and quantitatively transfer into a 5 ml volumetric flask. Make the volume to 5 ml with water.

Clean up:

Pass 2 ml aliquot from the above extract through Bond Elut SCX cartridge and elute with 2 ml water. Collect the eluate in a 5 ml volumetric flask and make the volume to 5 ml with water. Filter the solution through a 0.45 µm membrane filter.

Detection:

Inject 10 µl aliquot of the above filtered extract into the HPLC under the following conditions.

HPLC conditions:

Column : 25 cm * 4.6 mm i.d. packed with Necleosil 5SB

Mobile phase: 0.1 mol dm^{-3} acetic acid adjusted to pH 4.8

with tetramethylammonium hydroxide. (flow rate 1 ml/min.)

Detector : UV spectrophotometric detector operating at 313 nm.

3.1.3. **Results**:

The recoveries of MH obtained by analysing the fried potatoes using the above method were as follows.

Table 3.14. Recoveries of MH from potato slices after frying.

Tuber No.	% MH recovered.	% Overall recovery
4	80.31	
5	72.98	74.31± 5.87
6	77.20	
7	66.74	

3.1.4. **Discussion**:

The experiments carried out using the filter paper model showed that frying has no effect on MH, as no difference was observed between the amounts of MH recovered from the fried and non fried filter papers. And also the duration of frying did not make any difference either, in the recovery. No MH was found in oil fraction of the fried filter papers and it was found only in the non oil fraction. It was also found that no MH had gone into the fryer oil, as no contamination from MH was observed in unspiked filter papers when they were fried in the same oil in which the spiked filter papers had been fried previously. Although no MH was present either in the oil fraction of filter papers or in the fryer oil, the amount of MH recovered from the non oil material was not 100%. Initially only about 60% of MH was recovered from the non oil material of MH spiked fried filter papers. After improving the method of extraction it went only up to 72%. Therefore still there was no complete recovery.

This suggested that some materials in the filter papers may be affecting the recovery of MH when frying. The filter papers used in these model experiments were Whatman No.1 filter papers which contain 0.06% ash. As there was a doubt about

whether the metal ions in ash combine with MH to form any insoluble salts which are not soluble in methanol and hence not extractable, it was decided to measure the recovery of MH using some ashless filter papers (Whatman No.40, ash content 0.01%) spiked with MH. This exercise was carried out with out frying the filter papers. A slightly higher recovery was obtained with Whatman No.40 filter papers compared to Whatman No.1 filter papers(refer to Table 3.5). When this experiment was repeated using methanol/water and methanol/aq. phosphate as extractants the recoveries from Whatman No.40 filter papers were less than that from Whatman No.1 filter papers (refer to Table 3.6). Therefore a clear idea about the effect of ash content on the recovery of MH could not be obtained.

When the MH spiked filter papers were spiked with potato juice and fried there was about 10% reduction in the recovery, indicating that potato juice has some effect on MH during frying. When the MH spiked filter papers were spiked with some additives like glucose and lysine and then fried, then also a reduction in the recovery was observed. Therefore it can be suggested that some components in the potato material may be having some effect on MH during frying. Initially when MH treated potatoes were fried and analysed only 10% of MH from the original concentration was found in the fried product. After carrying out a series of experiments using the filter paper model and then having made some improvements in the extraction procedure 50% recovery was obtained. At this stage the column in the HPLC had to be replaced with a new column due to some malfunctioning of the old column, blockage etc. Under the new conditions four samples of fried potato material from four different tubers were analysed and a recovery ranging from 67-80% was obtained. But one drawback was observed when the extracts of fried samples were injected into the HPLC with the new column. In the HPLC chromatograms of the samples there was an additional peak adjacent to the MH peak which was not observed with the old column. This was not present in the chromatogram of the extract of raw potato sample either with old column or with the new column.. And also it was observed that the amount of MH in the fried

potato material calculated using peak area value was higher than the value calculated using peak height. Therefore the effect of this adjacent peak to the peak of MH was also examined using the filter paper model and it was found that at lower concentrations of MH there may be some effect on the peak area value of MH peak from the adjacent peak. But there was not much of an effect on peak height value. All the calculations were done using peak height values. Considering the results obtained from all these experiments it can be suggested that frying has no effect on MH. But in the presence of potato material as there was a reduction in the amount of MH recovered after frying it can be suggested that there is some effect of frying on MH residues in potatoes.

For the observed reduction in the amount of free MH residues found in fried potatoes two reasons can be suggested.

1) the method of extraction is not efficient enough to extract all the MH in fried material.
2) during frying some of the free MH becomes bound to some components in the potato material.

Anyhow the initial recovery of MH from fried potato material was only 10%. After improving the method of extraction it went up to 80%. Because there was no MH found in the oil fraction of the filter papers which were spiked with MH and fried, the oil fraction of fried potatoes were also discarded with out further analysis. It is doubtful whether some of the MH in fried material has been removed with oil when it was being extracted with hexane/diethyl ether.

Therefore further investigations had to be carried out to identify a possible reason. But due to the limitation in time further work on this aspect could not be carried out.

3.2. Effect of boiling on MH content in potatoes:

3.2.1. Introduction:

Boiling of potato tubers in water is the most common way of cooking potatoes. Other methods include steaming, roasting, baking and microwave cooking. MH residues are found in raw potatoes and this chemical has some health risks due its possible mutagenic potential (Sandhu and Waters 1980) Therefore if this chemical is present in potatoes in high amounts at the time of consumption it may be harmful to human health. Hence, in addition to frying it is also important to find out the effect of boiling on the MH residue content in potato tubers.

Experimental:

3.2.2. Materials and method:

Reagents:

Methanol (HPLC grade, Rathburn chemicals Ltd.)

Glacial acetic acid (Pronalys'AR, May and Baker Ltd.)

Tetramethylammonium hydroxide (Lancaster)

Bond Elut SCX (Varian)

Procedure:

Sample preparation:

A potato tuber was randomly selected from a batch of potato tubers obtained from a MH treated potato crop and rinsed with water to remove adhering soil. The tuber was weighed, and halved by cutting it along the rose and heel axis. One half was cut into small pieces, mixed well and 20 g representative samples were taken for the analysis without boiling. The other half of the tuber was cut into four and boiled in 200 ml of water in a beaker for about 20 min. (water level was about 1 cm above the potato pieces before boiling.) When the potato pieces were fully cooked, (checked by penetrating a fork) they were taken out and left on a clock glass

to cool down. When cold potato pieces were weighed again and cut into further small pieces, mixed well and 20 g representative samples were taken for analysis.

Water used for boiling was filtered through a Whatman No.1 filter paper in a Buchner funnel under vacuum. Volume of the filtrate was measured. This water could not be easily filtered through a normal filter funnel as it was viscous due to the dissolved starch released from the potato pieces during boiling. Therefore filtering had to be done under vacuum.

Extraction:

Extraction of raw and boiled potato samples were carried out using the same procedure as described in section 3.1.2(b).1.

Clean-up:

2 ml aliquot from the above extract was passed through a Bond Elut SCX cartridge and eluted with 2 ml water into a 5 ml volumetric flask. Volume of the eluate was made to 5 ml with water.

Water used for boiling of potatoes also was cleaned up by passing a 2 ml aliquot through Bond Elut SCX cartridge and eluting with 2 ml water. Eluate was collected in a 5 ml volumetric flask and the volume made up to 5 ml with water.

All the cleaned up extracts were filtered through 0.45 μm membrane filter before injecting to HPLC.

Detection:

10μl aliquots of above extracts were injected to HPLC under following conditions and the MH concentration determined by comparing the chromatogram with the chromatogram of MH standard run under the same conditions.

HPLC conditions:

Column : 25 cm * 4.6 mm i.d. packed with Nucleosil 5 SNB.

Mobile phase: 0.1 mol dm^{-3} acetic acid adjusted to pH 4.8 with tetramethyl ammonium hydroxide (flow rate 1 ml/min).

Detector : UV spectrophotometric detector operating at 313 nm.

3.2.3. **Results**:

Results are presented in Table 3.15

Table 3.15. MH contents in potatoes before and after boiling.

	MH content (µg/g)		
	Rep I	Rep II	Rep III
Before boiling	6.82	7.87	7.79
After boiling	8.35	9.92	9.44

Rep II and Rep III are two replicate samples from the same potato tuber. Rep I is from another potato tuber. The average amount of MH released into the water during the boiling of potatoes was 1.33 µg/g potato.

3.2.4. **Discussion**:

The results show that the concentration of MH in boiled potatoes is higher than the amount in raw potatoes. The water used for boiling of potatoes also contained MH. Therefore there was an increase in the amount of MH present in potatoes after boiling. It was thought that this observed increase in the MH content could be due to the loss of moisture and starch from the potato pieces during boiling. But the weight loss due to boiling was only 2.5%. Therefore as there was an increase of MH by about 20% the weight loss can not be solely responsible for this increase. The other possible reasons that can be suggested for this observation are as follows.

1. The recovery factor of MH in raw potato is low compared to that in boiled potatoes. Because when the potatoes are boiled all the cells get destroyed and the MH present in the potatoes may be more easily extracted with methanol.

2. If the MH in raw potatoes remains incorporated into starch material of potato the methanol may not be extracting the MH completely. When the potatoes are boiled, as all the starch looses its structure and becomes gelatinized, it is possible to release MH. This would have made an increase in the amount of MH detected.

This is not a proven fact here and it can only be put forward as a suggestion. There may be many other reasons for this observed effect which should be further investigated.

3.3. **Conclusions**:

In this chapter, the effect of two methods of processing (frying and boiling) on MH residues in potatoes was examined. According to the final results, frying made a reduction of about 20-30% of MH content in potatoes. Boiling made about 20% increase in the MH content. Reasons for these observations have been suggested but need to be further investigated.

All these experiments were carried out to find out the methanol extractable free MH contents in the potatoes both raw and processed. But there can be some forms of MH in potatoes which are not extractable with methanol due to the reason that they could possibly bound to some components in the potato material. McKenzie (1989) showed that total MH content in potatoes are higher than the methanol extractable MH contents. Therefore some of the MH present in potatoes may be in the bound form. In the next chapter the identification and the possible presence of bound residues will be discussed.

CHAPTER 4

STUDY ON BOUND OR CONJUGATED MALEIC HYDRAZIDE (MH) RESIDUES IN POTATOES

4.1. **Introduction**:

The studies discussed in previous chapters were limited only to the determination of methanol extractable free MH present in potato tubers and potato processed products. But, there is evidence that when maleic hydrazide is sprayed on crops, it can be present in the crop not only as free MH, but also as bound or conjugated metabolites. From an experiment done by Towers *et al* (1958) with young leaf segments of wheat fed with ^{14}C-MH, it was found that 15% of the MH in leaf segments was present in the form of glycoside. Haeberer *et al.* (1978) have reported that there is a decrease of about 50% of the MH content in tobacco after harvesting and curing steps. Further reduction was observed during storage (Haeberer and Chortyk 1979). This has indicated a gradual conversion of free MH into a bound form. Frear and Swanson (1978) also applied ^{14}C-MH to tobacco plants and found such a major methanol soluble metabolite as beta-glucoside of MH. Enzyme hydrolysis (reaction with beta-glucosidase for 20h in 0.05M acetate buffer at pH 4.5) and acid hydrolysis (reaction with 1M hydrochloric acid at 100 ^{0}C for 2h) of the metabolite released glucose which was identified qualitatively by TLC and quantitatively by colorimetric analysis. Domir (1978,1980) injected ^{14}C-MH into seedlings of different plants (silver maple, American sycamore and American elm) and found that it translocated to all parts of the plant within 1 day after treatment. He observed that MH was converted to a MH-sugar complex. With the passage of time, the amount of metabolite seemed to increase in proportion to that of MH. Nooden (1970, 1975) studied the metabolism and binding of ^{14}C-MH in corn and pea sedling roots and tobacco pith explants. He observed that ^{14}C-MH was taken up by plant tissues and

bound to some unknown macromolecules, probably the cell wall fragments. He reported that the binding process takes place in older parts of the plant. Nooden (1975) observed that many of the enzymes failed to release bound MH and he suggested that MH is more likely to be bound to a polysaccharide but probably not to cellulose.

Newsome (1980b) has tried to determine the level of beta-glucoside of MH occurring in potatoes after field treatment with MH. He has hydrolysed the potato extracts by incubating the extract with beta-glucosidase enzyme at 37 ^{0}C for 30 min. (Newsome 1980a). No increase in the MH content was found indicating that there was no beta glucoside present in the extracts.

McKenzie (1989) has shown the presence of beta-glucoside as a metabolite of MH in potatoes. He has observed an increase of free MH content in the juice extracts of MH treated potatoes, after enzyme hydrolysis with beta-D-glucosidase. Although he tried to synthesise the conjugated product using MH and uridine diphospho glucose(UDPG) from potato leaf protein, he was not successful. Therefore, still there is no clear-cut idea about the presence of bound or conjugated MH residues in potatoes. Hence, the aim of this study was to find out whether there was any bound or conjugated MH residues present in potato tubers resulting from the field treatment of the potato crop with MH. Two analytical methods were found in recent literature for the determination of total MH (free and bound) in tobacco. Renaud *et al.* (1992) had developed a GC method for the routine determination of free and glucosidically bound MH in tobacco by hydrolysis with hydrochloric acid. Hydrolysed product was methylated in aqueous dimethyl sulphate, transferred to an organic solvent and determined by GC with Nitrogen Phosphorus Detector (NPD). In the other method developed by Yang (1992) for the determination of total MH in tobacco and tobacco products, high performance liquid chromatography with UV-VIS photodiode array detector has been used in the detection of MH after hydrolysing the sample with hydrochloric acid. In this hydrolysis method samples were mixed with hydrochloric acid

and sonicated in a water bath at 60 ^{0}C. After sonication the solutions were neutralised with sodium hydroxide and the free MH content was determined by HPLC. He tried out other sample preparation procedures such as water extraction and enzyme hydrolysis, NaOH digestion and refluxing and NaOH digestion and microwave irradiation. He found that the sample preparation by acid hydrolysis is less time consuming and also the results using this procedure were consistent with the more rigorous NaOH reflux sample preparations. Even though these methods have not been tried out with potatoes it was thought reasonable to follow one of these methods in the study described here. The HPLC method developed by Yang (1992) was selected since HPLC was the detection technique used in all the previous analyses.

4.2. Experimental

4.2.1. **Determination of conjugated maleic hydrazide in potatoes by acid hydrolysis:**

Acid hydrolysis method described by Yang (1992) for the determination of MH in tobacco was used. In his method 0.25 g portions of ground tobacco were taken and hydrolysed with 12M hydrochloric acid by sonicating at an elevated temperature followed by neutralization with 12M sodium hydroxide. In case of potatoes since it was rather difficult to take a representative sample of such a small quantity (0.25 g) for analysis, it was decided to macerate the potato with an equal quantity of water and then take a representative sample of 0.5 g. In this method, at the MH detection step Yang has used HPLC with a photodiode array detector. But as the UV/VIS detector was used for the MH analyses carried out here and also due to the fact that there was no photodiode array detector available in the laboratory, the same UV/VIS detector was used in the experiments discussed here.

Before analysing the MH treated potatoes the method was tried out with samples of potatoes spiked with MH, to find out the recovery of MH using this method to identify whether there would be any effect of hydrolysis on MH itself.

4.2.1.1. **Investigating the effect of acid treatment on MH**

4.2.1.1.1. Materials and method:

4.2.1.1.1.1. Reagents:

Hydrochloric acid (R.P. Nomapur AR, Rhone pulenc Ltd.)

Sodium hydroxide (Analar BDH Chemical Ltd.)

Cetyltrimethylammonium bromide(CTAB) (Genlab Koch-Light Ltd.)

Maleic hydrazide (1000 ppm stock solution prepared by dissolving 100 mg of maleic hydrazide (obtained from Chem. Services Inc.) in 100 ml water)

Sodium dihydrogen phosphate (Analar, Hopkin and Williams)

Disodium hydrogen orthophosphate (Analar, Hopkin and Wiiliams)

4.2.1.1.1.2. Method:

50 g of a MH free potato sample was blended with 50 ml water for 2 min. Two 0.5 g portions from the blended potato sample were transferred into 2 universal vials of 30 ml capacity. Samples were then spiked with 37.5 μg and 43.2 μg of MH by weighing 37.5 mg and 43.2 mg of MH stock solution into the 2 vials separately. Samples were left to stand for one hour. After 1h, 2 ml 12M hydrochloric acid was added to each spiked sample. The vials were capped and placed in an ultrasonic bath containing hot water at 60 ^{0}C and sonicated for 40 min. The samples were then cooled in a cold water bath and two, 1ml aliquots of 12M NaOH were sequentially added to the samples while in the cold water bath. After the neutralisation process the samples were filtered through a 0.45 μm membrane filter and 10 μl aliquots were injected in to the Perkin Elmer HPLC equipped with UV spectrophotometric detector, under the following conditions. MH

concentrations were determined by comparing the chromatograms with the chromatogram of MH standard run under the same conditions.

HPLC conditions:

A:

Analytical column: Hypersil ODS 5 μm (25 cm * 4.6 mm i.d.)

Mobile phase: consisted of two solutions

Solution A: 2.5 mM CTAB in 40 mM phosphate buffer* (pH 7.0)

Solution B: 7.5 mM CTAB in 40 mM phosphate buffer* (pH 7.0)

Solvent gradient used was as follows

30%B + 70%A - 4 min.

B raised to 90% in 0.5 min.

90%B + 10%A - 4 min.

30%B + 70%A - 11.5 min.

Total run time was 20 min.

Detector: UV spectrophotometric detector operating at 330 nm

(* phosphate buffer was made using sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$) and disodium hydrogen orthophosphate ($Na_2HPO_4.H_2O$). 0.5M aq. solutions were prepared from both salts. By mixing the 0.5M NaH_2PO_4 solution with 0.5M Na_2HPO_4 solution in 4:6 ratio a 0.5M (500 mM) phosphate buffer of pH 7.0 was obtained. This solution was diluted with deionized water to obtain a 40 mM solution).

B:

Analytical column: Spherisorb SAX 5 μm (25 cm * 4.6 mm i.d.)

Mobile phase : 0.1M acetic acid adjusted to pH 4.8 with tetramethyl ammonium hydroxide (flow rate 1 ml/min.) Isocratic condition was used.

Detector : UV spectrophotometric detector operating at 330 nm

4.2.1.1.2. Results and discussion:

An accurate result could not be obtained due to some experimental problems. A few difficulties were experienced during HPLC analysis under the condition A. When the MH standard was injected in to the HPLC the retention time of the MH peak changed with each injection. With time retention time of the peak increased.

When the sample was injected, for each injection it gave a different chromatogram. At the same time back pressure also increased.

Then it was decided to use isocratic conditions rather than solvent gradient. A few injections of MH standard (10 μl aliquots from 10 ppm MH standard solution) was made in to the HPLC under isocratic conditions with the solvent ratio 30%B + 70%A. But, no improvement was observed. Still the retention time was increasing.

Therefore it was decided to carry out the HPLC analysis under the same conditions as used for the determination of MH in the studies discussed in previous chapters. Hypersil ODS 5 μm column was replaced with the Spherisorb SAX 5 μm column and the mobile phase also was changed. Therefore samples were injected under the conditions B. Under these conditions a few injections of standard MH were made into the HPLC and it was found that the retention time for MH was quite constant. Therefore further analyses were carried out under the condition B.

During the acid hydrolysis process one out of the two vials which contained samples was broken while sonicating. Therefore only one sample which had been spiked

with 43.2 μg of MH was left. After neutralisation process before injecting into the HPLC the pH of the sample was checked and found to be quite acidic . Therefore it had to be neutralised again with few microlitres of NaOH before injecting into the HPLC. According to HPLC analytical results the amount of MH found in that sample after acid hydrolysis was 63.7 μg. That was an increase of about 47% from the original amount which did not seem to be an accurate value. Therefore this experiment was repeated.

Repeated experiment was carried out with a few alterations. This time it was decided to prepare the potato sample for analysis by macerating the potato material without adding water, because it was felt that the sample taken after homogenising with water was not really representative. HPLC condition B was used for analysis.

4.2.1(R). Acid hydrolysis of potato samples spiked with MH (repeated experiment)

4.2.1(R).1. Method:

25 g of a MH free potato sample was macerated in a small blender without adding water. Three 0.5 g portions of this blended potato were transferred in to 3 universal vials of 30 ml capacity. Two of the samples were spiked respectively with 50 μl and 100 μl of MH standard solution containing 1000 μg MH/ml and left to stand for one hour. After 1h, 2 ml aliquots of 12M hydrochloric acid were added to all 3 vials. Vials were capped and placed in an ultrasonic bath containing hot water at 60 ^{0}C and sonicated for 40 min. After 40 min samples were taken out and cooled in a cold water bath. (unfortunately the sample spiked with 50 μl MH was broken while sonicating. Therefore only two samples were left.

While the samples were in the cold water bath 2 * 1 ml aliquots of 12M NaOH were added sequentially to each vial. After the neutralisation process the samples were filtered through 0.45 μm membrane filter and 10ul aliquots were injected into the HPLC

under condition B. Amount of MH was determined by comparing the chromatograms with the standard chromatogram run under the same conditions.

4.2.1(R).2. Results and discussion:

By HPLC analysis it was found that the recovery of MH from the sample spiked with 100 μg of MH was 104%.

The chromatograms obtained for standard MH, and acid hydrolysed unspiked sample and spiked sample were given in Fig 4.1.

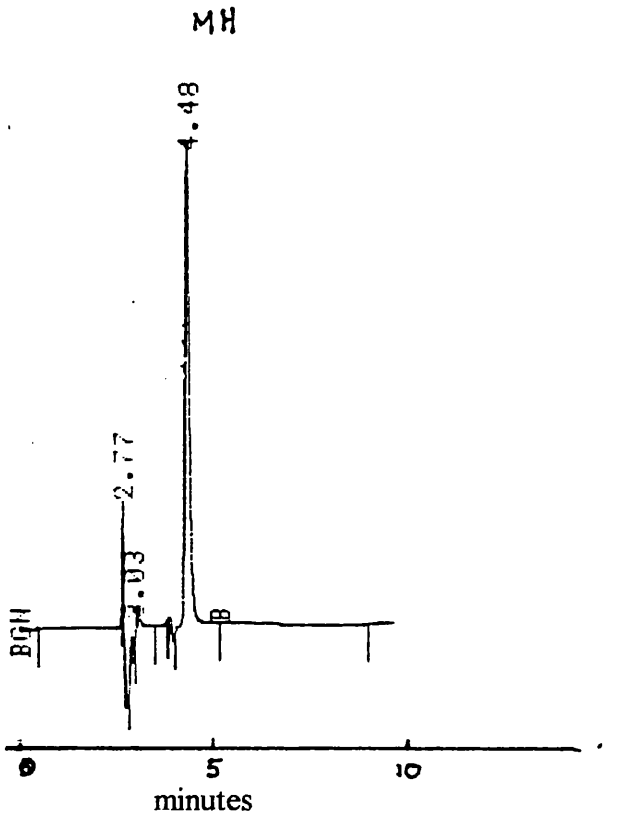
In the chromatogram of the spiked sample there was another peak very close to the MH peak. These two peaks appeared as one peak split into two. But as this additional peak was present in the unspiked sample as well it was clear that this extra peak was due to an interfering material present in the sample. Therefore it was decided to clean the spiked sample by passing through Bond Elut SCX cartridge. After the clean up the extra peak disappeared and the peak resembling MH remained. Chromatogram of the spiked sample before and after clean up is given in Fig 4.2.

This experiment also had the same problem as experienced in the previous experiment i.e. after neutralisation with NaOH the solution was not completely neutral. As it was slightly alkaline this time it was acidified with little HCl to make it neutral before injecting in to the HPLC.

The high recovery (150%) obtained from the analysis of the previous spiked sample (sample spiked with 43.2 μg MH) could be due to the fact that the peak which appeared in the chromatogram at the retention time of MH was a combination of two peaks, one of which was an interfering compound in the potato sample or alternatively may be due to the experimental errors caused by the many steps involved in the analysis. From the result of the repeated analysis which gave 104% recovery it can be concluded that acid hydrolysis has no destructive effect on the compound MH.

Fig 4.1 HPLC chromatograms of
a) standard MH
b) Acid hydrolysed potato sample (unspiked)
and
c) Acid hydrolysed potato sample (spiked with 100 µg MH)

a) Standard MH (10 mm^3 injection of 20 µg/ml standard solution)



HPLC conditions:

Column : Spherisorb SAX 5 µm (25 cm * 4.6 mm i.d.)

Mobile phase: 0.1M acetic acid adjusted to pH 4.8 with tetramethylammonium hydroxide (flow rate 1 ml/min)

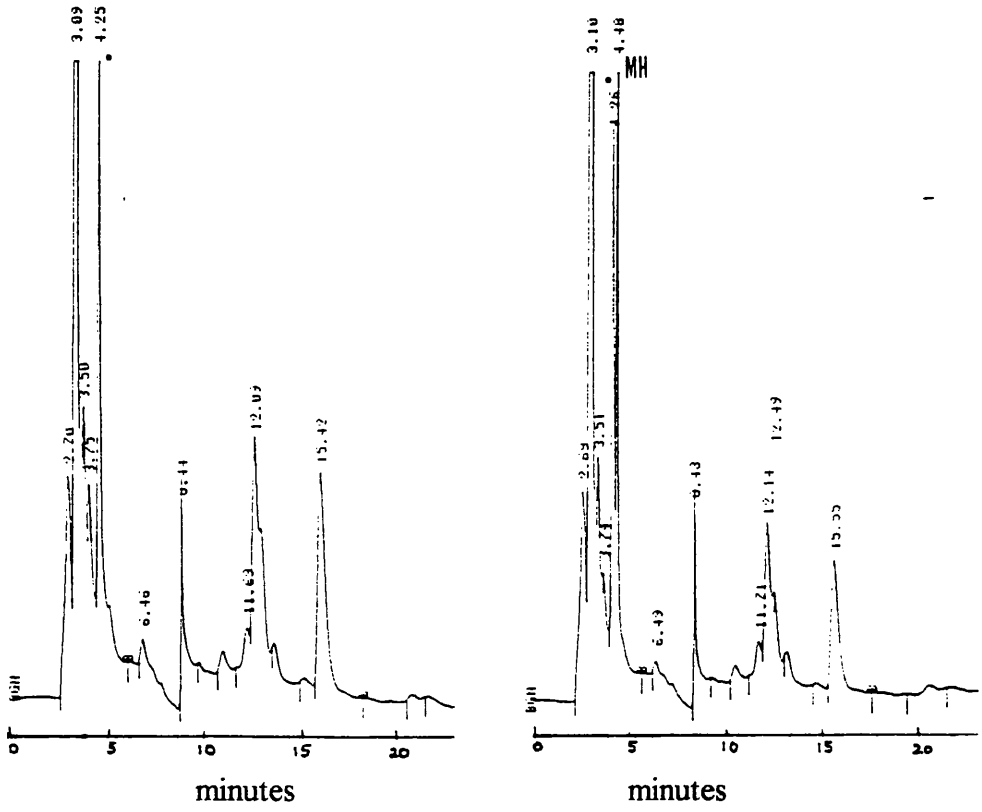
Detector : UV spectrophotometric detector operating at 330 nm

Fig 4.1 (contd..)

b) acid hydrolysed potato sample (unspiked)

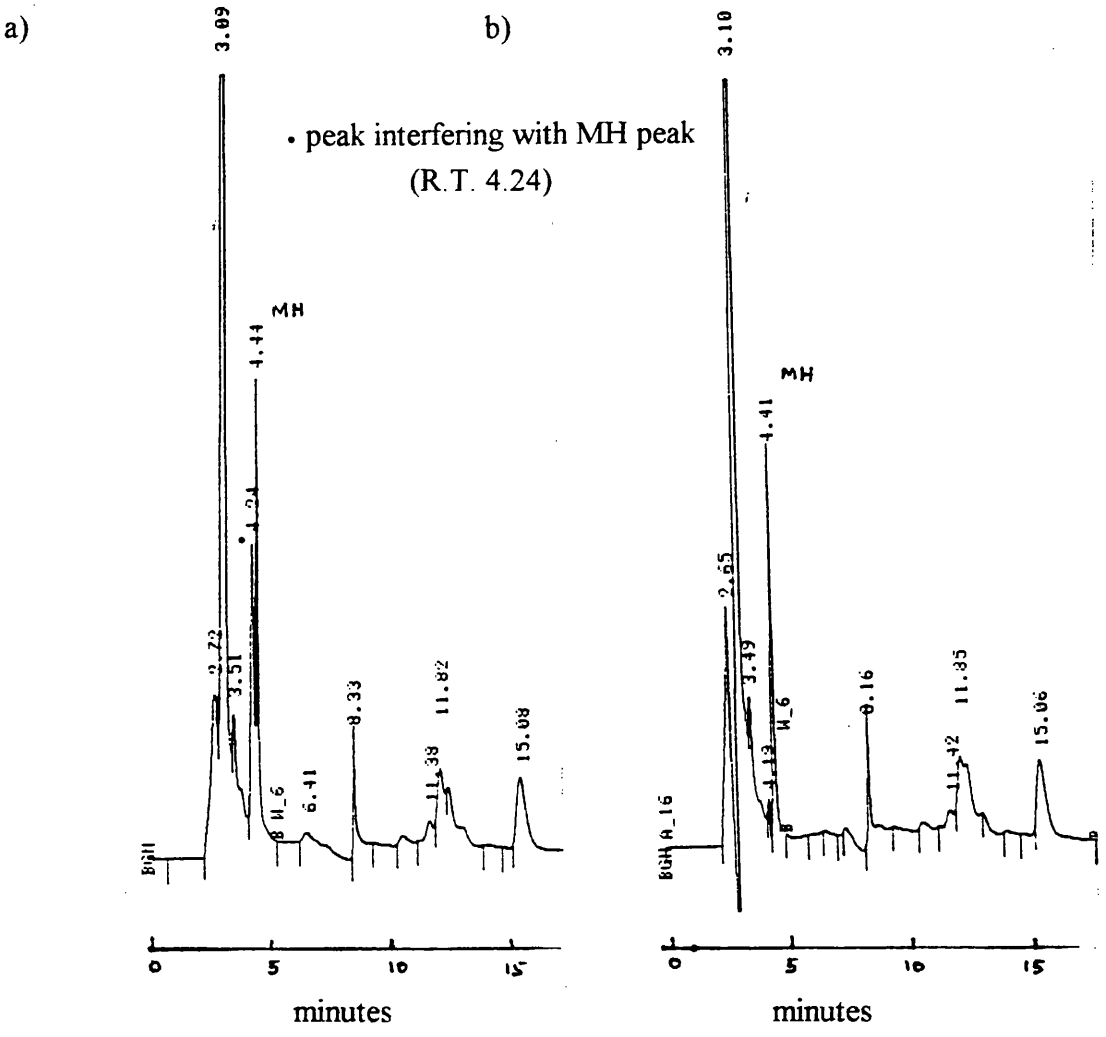
c) acid hydrolysed potato sample (spiked with 100 ug MH)

• peak interfering with MH peak



HPLC conditions are the same as in previous page

Fig 4.2 HPLC chromatogram of acid hydrolysed potato sample spiked with MH
a) before clean up
b) after clean up



HPLC conditions:

Column : Spherisorb SAX 5 μm (25 cm * 4.6 mm i.d.)
Moboile phase : 0.1M acetic acid adjusted to pH 4.8 with tetramethylammonium hydroxide (flow rate 1 ml/min)
Detector : UV spectrophotometric detector operating at 330 nm

4.2.1.2. **Determination of conjugated MH in field treated potatoes by acid hydrolysis.**

Methanol extract of MH treated potatoes and the residue remaining after methanol extraction were used in acid hydrolysis.

Two lots of potatoes were used in this experiment. First lot was held in the cold store at 10 ^{0}C for nearly 5 ½ months.(old potatoes) and the other lot was freshly dug potatoes. (new potatoes)

4.2.1.2.1. **Acid hydrolysis of old potatoes:**

4.2.1.2.1.1. Materials and method:

4.2.1.2.1.1.1. Reagents:

Methanol (HPLC grade, Rathburn chemicals Ltd.)

Glacial acetic acid (Pronalys'AR, May & Baker Ltd.)

Tetramethylammonium hydroxide (Lancaster)

Bond Elut SCX (Varian)

4.2.1.2.1.1.2. Method:

Sample preparation:

A potato tuber was taken, washed with tap water, dried, cut into small pieces and mixed well. A 20 g representative sample was taken and blended with 100 ml methanol in a blender for 2 min. The homogenised mixture was filtered through a Whatman No.1 filter paper in a Buchner funnel using vacuum (extraction was done as described in Chap. 2, section 2.2.2.1.)

Filtrate was concentrated to 4-6 ml on the rotary evaporator at 40 ^{0}C bath temperature and the volume made to 10 ml with water. The residue remaining on the filter paper was weighed and kept for analysis.

Acid hydrolysis:

2 * 1ml aliquots from the methanol extract were transferred into 2 universal vials, while a 0.25 g portion from the methanol insoluble residue was transferred into a another vial. 2 ml aliquots of 12M hydrochloric acid were added to all 3 vials, which were capped, placed in a ultrasonic bath containing hot water at 60 ^{0}C and sonicated for 40 min. After 40 min samples were taken out and cooled in a cold water bath. (one of the vials which contained 1 ml of potato extract had broken while sonicating. (only two vials remained). 2 * 1 ml aliquots of 12M sodium hydroxide were sequentially added to each of the two vials while they were in the cold water bath. The caps of the vials were kept slightly presse during the neutralisation process. When adding NaOH several minutes were allowed for heat dissipation after addition of each 1 ml aliquot. After the neutralisation process samples were cleaned up as follows, before injecting into the HPLC.

Clean up:

2 ml aliquots from the neutralised samples were passed through two Bond-Elut SCX cartridges and eluted with 2 ml water. The eluates were collected in 5 ml graduated test tubes and the volumes were made to 5 ml with water. At the same time a 2 ml aliquot from the original methanol extract (before hydrolysis) also was cleaned up following the same procedure. Cleaned up extracts were filtered through 0.45 μm membrane filter before injecting in-to the HPLC.

Detection:

10 μl aliquots of the cleaned up samples were injected into the HPLC under the condition B given in section 4.2.1.1.MH concentration was determined by comparing the chromatogram with that of MH standard run under the same conditions.

4.2.1.2.1.2. Results:

Given in Table 4.1.

Table 4.1. The amount of free MH detected in the methanol extract, before and after acid hydrolysis (old potatoes)

	Before hydrolysis	After hydrolysis
MH content (μg/g potato)	3.45	15.57

The MH content found in the methanol insoluble residue was 31.58 μg/g potato on fresh weight basis.

4.2.1.2.2. **Acid hydrolysis of new potatoes:**

In this experiment only the methanol extract of potato was used due to the experimental difficulties with the methanol insoluble residue which will be explained in the discussion.

4.2.1.2.2.1. Materials and method:

Same as in the acid hydrolysis of old potatoes (Section 4.1.2.1.1.)

4.2.1.2.2.2. Results:

Given in Table 4.2.

Table 4.2. The amount of free MH detected in the methanol extract, before and after acid hydrolysis (new potatoes)

	before hydrolysis	after hydrolysis
MH content (μg/g potato)	7.45	12.74 (13.07,12.41)

(Values in the parentheses are the values obtained for two replicates).

4.2.1.3. Discussion:

According to the results, in the case of old potatoes free MH content in the methanol extract increased by about 4 ½ times by the acid hydrolysis process. Acid hydrolysis of the methanol insoluble potato residue also gave quite a large amount of free MH. The potatoes from which a tuber was taken for this acid hydrolysis experiment were stored in the cold room at 10 ^{0}C for nearly 5 ½ months, and they had started to sprout also. At the beginning of the storage period some of these potatoes were analysed and the amount of free MH found was 20 μg/ml methanol extract giving an average of 10 μg/g potato. The present analytical results showed that the free MH content in the potato sample before acid hydrolysis was 3.45 μg/g of potato (6.89 μg/ml methanol extract). After the acid hydrolysis it has gone up to 15.57 μg/g of potato (31.15 μg/ml methanol extract).

These results showed that during the storage, the free MH content in the potatoes has decreased. But, by acid hydrolysis again the MH content increased. From this observation it can be suggested that during storage there may be a conversion of free MH into a bound form, which releases MH again when treated with acid. Since there was a considerable amount of free MH found in the methanol insoluble potato residue after acid hydrolysis, it can be suggested that there is a conjugated form of MH which is

not soluble in methanol and can release MH by acid hydrolysis. As the methanol insoluble residue gave a very sticky solution after the acid hydrolysis, difficulties were experienced in filtering it through membrane filter. Therefore for the experiment with new potatoes only the methanol extract was used.

Compared to the old potatoes the increase in MH content in the methanol extract of new potatoes was much less. Therefore it can be suggested that the amount of bound or conjugated metabolites of MH present in new potatoes is less compared to the old potatoes. With time there may be a conversion of free MH into a bound form. The old potatoes had started sprouting at the time they were taken for this experiment. Decrease in free MH content with time might have caused the start of sprouting. Another difficulty faced during the acid hydrolysis process was that it was impossible to use 3 vials together for ultrasonicating , because when three sample vials were kept together in the ultrasonicator bath always one broke.

4.2.2. **Determination of conjugated MH in field treated potatoes by enzyme hydrolysis:**

The work done in the past (Frear & Swanson 1978; Towers *et al.*1958) on conjugated metabolites of MH in plants treated with MH has shown that the major methanol soluble metabolite was beta-D-glucoside. Therefore if this metabolite was present in MH treated potatoes as well, by reacting with beta-D-glucosidase under proper conditions it should be able to release MH. McKenzie (1989) has found an increase of free MH content in potato juice extracts, by reacting with beta glucosidase, while Newsome (1980b) did not. Therefore this study was undertaken to find out the exact situation in potatoes.

Enzyme hydrolysis method described by Yang (1992) was followed in this experiment. Instead of water extract, methanol extract of the potato, concentrated to an

aqueous extract was used. The HPLC conditions used in the detection step were different from Yang's.

4.2.2.1. Enzyme hydrolysis of potaoes

MH treated potatoes from the newly stored lot was used in this in experiment.

4.2.2.1.1. Materials and Method:

4.2.2.1.1.1. Reagents:

Methanol (HPLC grade, Rathburn chemicals Ltd.)

beta-glucosidase (Sigma Chemical Co.,)

4.2.2.1.1.2. Procedure:

4.2.2.1.1.1.1. Sample preparation:

4 * 20 g representative samples from a potato tuber were extracted with methanol in the same way as described in Section 4.2.1.2.1.1.2.

4.2.2.1.1.1.2. Enzyme hydrolysis:

2 ml aliquots from the 4 methanol extracts were transferred to 4 test tubes of 5 ml capacity. 10 mg of beta-D-glucosidase was added to each test tube and incubated at 37 ^{0}C, by immersing in a water bath for 4 h with occasional shaking by hand. After 4 h reaction mixtures were taken out from the water bath and cleaned up as follows.

4.2.2.1.1.1.3. Clean up:

2 ml aliquot from each reaction mixture was passed through Bond Elut SCX cartridge and eluted with 2 ml water. Volume of the eluate was made to 5 ml with water and filtered through 0.45 μm membrane filter.

At the same time 2 ml aliquots from the original methanol extracts (before hydrolysis) also were cleaned up following the same procedure.

4.2.2.1.1.1.4. Detection:

10 µl aliquots from all cleaned up samples were injected into HPLC under the following conditions and the MH content was determined by comparing the chromatograms with the chromatogram of MH standard run under the same conditions.

HPLC conditions:

Analytical column : 25 cm * 4.6 mm i.d. Nucleosil 5 SB

mobile phase : 0.1 mol dm^{-3} acetic acid, adjusted to pH 4.8 with tetramethylammonium hydroxide (flow rate 1 ml/min).

Detector : UV detector operating at 313 nm.

4.2.2.1.2. Results:

Given in Table 4.3

Table 4.3. Amount of free MH detected in the methanol extract before and after enzyme hydrolysis

Replicate No.	before hydrolysis (µg/g potato)	after hydrolysis (µg/g potato)
1	4.98	5.07
2	4.93	4.73
3	4.96	5.23
4	5.28	5.21
Average	5.04	5.06

4.2.2.1.3. Discussion:

According to the results there was no significant increase of free MH content by enzyme hydrolysis. Although the extract taken for enzyme hydrolysis was a methanol extract of potatoes, it hardly contained any methanol (which can greatly reduce the activity of the enzyme) because after extracting the potatoes with methanol it was concentrated by evaporating in the rotary evaporator at 40 ^{0}C. At this stage almost all the methanol evaporated off and only the aqueous residue remained. Then it was diluted to 10 ml with water. Therefore it was almost a water extract. Therefore it was thought that as no increase in MH content was observed, this may be due to one of the following reasons.

1. The enzyme was not active.
2. pH of the extract was not the correct pH at which the enzyme gives maximum activity.
3. No beta glucoside was present in the potato extract.

Hence, it was decided to look in to these aspects as well.

4.2.1.2. **Assessing the activity of the enyme**:

The pH at which the enzyme gives maximum activity was known to be pH 5.0.

2 ml of beta-glucosidase (0.5 mg/ml 0.1M sodium acetate of pH 5.0) was mixed with 2 ml of 2 mM p-nitrophenyl beta-D-glucopyranoside made up in 0.1M sodium acetate pH 5.0 in test tube and incubated at 37 ^{0}C for 20 min by immersing in a water bath. After 20 min the solution in the test tube gave a slight yellow colour due to the release of p-nitrophenol. When few drops of 0.5M NaOH was added the yellow colour became very intense.

This experiment showed that the enzyme was active.

Since there was a doubt about the pH of the potato extract, the pH of the extract also was measured and found to be pH 5.4. Therefore there was not much difference between the pH of the potato extract and the pH value at which the enzyme gives maximum activity. Thus, it was clear that pH of the medium has not affected the activity of the enzyme. The only possibility for the enzyme not to be active can be the presence of some compounds in the potato extract which inhibit the activity of the enzyme. Therefore the enzyme activity was measured in the presence of potato juice.

Assessing the enzyme activity in the presence of potato juice:

Two reagents were made as follows.

Reagent 1: Dissolved 5 mg of beta-D-glucosidase in 1 ml of 0.1M sodium acetate pH 5.0.

Reagent 2: Dissolved 10 mg of p nitrophenyl beta-D-glucopyranoside in 1 ml 0.1M sodium acetate pH 5.0

Potato extract and the above reagents were transferred to three test tubes as follows.

1. 1ml potato extract + 0.2 ml Reagent 1 + 0.2 ml Reagent 2
2. 1ml potato extract + 0.2 ml Reagent 2 + 0.2 ml acetate buffer
3. 1ml potato extract + 0.4 ml acetate buffer

All 3 test tubes were incubated at 37 ^{0}C for 20 min. After incubation 5 drops of 0.5M NaOH were added to each test tube. The contents in the test tube No. 1 which had both the substrate and the enzyme gave a dark yellow colour compared to the other two. The colour of the contents in test tubes no. 2 & 3 were similar. This demonstrated that even in the presence of potato extract the enzyme was active. This indicated that potato extract does not contain any enzyme inhibiting compounds.

As this experiment was carried out in the presence of acetate buffer, it was thought wise to repeat the enzyme hydrolysis process of potato extract in the presence of acetate buffer. i.e. instead of adding the solid beta-D-glucosidase the enzyme (5 mg beta-D-glucosidase) was dissolved in acetate buffer (pH 5.0) and added to the potato extract. The sample was incubated at 37 ^{0}C for 20 min.

The free MH content in the potato extract before enzyme hydrolysis was 6.55 μg/ml. After enzyme hydrolysis process the free MH content was 6.89 μg/ml. No significant increase in the free MH content after enzyme hydrolysis was observed. According to these results it can be suggested that MH was not present in potato as the beta-glycoside metabolite.

4.3. **Conclusions**:

Since there was a substantial increase in free MH content in potatoes by the acid hydrolysis it was clear that there were some conjugated forms of MH present in potatoes. These conjugated products were present in both the methanol soluble and insoluble fractions. As the beta- glucosidase enzyme hydrolysis did not result in an increase in the MH content in potatoes, it suggests that the conjugated form is not a beta-glucoside. Enzyme hydrolysis process was carried out only with new potatoes. Nevertheless it is doubtful whether the old potatoes would contain any beta-glucoside. As the increase in MH content in old potatoes by acid hydrolysis was more compared to the new potatoes it can be suggested that old potatoes contain more of the conjugated product. i.e. It is possible that when the potatoes are stored for a long period of time the free MH present may be getting converted to a conjugated form from which MH can be released again by acid hydrolysis.

As the acid hydrolysis can release MH without any change it is clear that binding process has not made any structural changes to MH. i.e. no irreversible chemical change has been taken place between MH and the binding component in potato. Therefore

further investigation has to be carried out to identify this bound form and its possible biological significance.

CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Pesticides are extensively used by growers, in order to maintain a high quality in food crops. As a result these pesticides or their biologically active transformation products may remain on or in vegetables and fruits as residues, thereby gaining entry into our food supply. If this happens it will be deleterious to consumers health either acutely or in long term. Therefore the aim of this thesis was to study the fate of pesticides in food both raw and processed resulting from the application of pesticides to agricultural crops. The example chosen for this study was MH, which is used regularly in USA as a growth regulant and recently introduced in to the UK on a staple crop potato, as a sprout inhibiting agent.

As MH is a very stable compound (Rakitin *et al.*(1971) and also due to its potential toxic effects (Sandhu *et al.*1980) the introduction of this chemical for use on potatoes in the UK has led to several questions regarding its residues in both raw and processed potatoes. Apart from the work done by Anglin and Mahon (1958), Hoffman and Carson (1962a), Newsome (1980b) and McKenzie (1989), no work has been carried out on the residues of MH in potatoes. Most of the work carried out on MH in connection with potatoes were on the efficiency of absorption of this chemical by potato tubers (Franklin and Lougheed (1964), its effectiveness as a sprout suppressant (Gul et al. 1990; Kumar and Mukerjee 1989; Matlob 1979) or of its biological effects on potatoes such as on quality and yield (Baijal et al. 1978; Weis et al. 1980; Yada et al 1991). Hence, this study was initiated to study the residues of MH in potatoes resulting from the field application of MH to the potato crop. In the previous studies carried out on MH residues, except Newsome other authors have measured the amount of total MH which included both free and bound residues of MH present in potatoes. McKenzie (1989) also had carried out a few determinations of free MH. As there was not much information on MH residues as

free and bound separately, the objectives of this study was to determine the residues of free MH in potatoes both raw and processed and also to search for the presence of possible bound residues.

In general, bound residues mean or can be defined as "chemical species originating from pesticides used according to good agricultural practice, that are unextracted by methods which do not significantly change the chemical nature of these residues"(Roberts 1984). Determination of these bound residues in plants are important if they are biologically available to man or animals and if they are located within the edible parts of the plant. Otherwise they can be considered as insignificant residues.

In Chapter 1 some background knowledge about the chemical MH was presented.

Numerous methods were available in the literature for analysing MH residues in various commodities including potatoes, and were reviewed in Chapter 2. Since MH is being used in many countries in the world as an inhibitor of sucker growth on tobacco plants, most of the methods developed were for the analysis of MH residues in tobacco and tobacco products. Officially recommended spectrophotometric method (Anon 1990) for the analysis of MH residues gives a measure of total MH (free plus bound) present in the sample. A few analytical methods which involve GC analysis (Haeberer and Chortyk 1979; King 1983 and Renaud et al 1992) and HPLC analysis (Newsome 1980a; Victor et al. 1984; Cessana 1991 and Vadukul 1991) for determination of free MH in potatoes and in some other commodities were available in the literature. As HPLC methods seemed to be simple and quick and also due to the fact that there was an HPLC available in the laboratory the method of choice for this study was HPLC. The HPLC method described by Vadukul (1991) for the determination of MH residues in potatoes and onions was used in the analyses carried out here to determine the MH residues in potatoes. This method involved the extraction of the sample with methanol, clean up of the extract using solid phase extraction (SPE) cartridge and determination of

MH by HPLC with UV detection at 313 nm. Compared to other clean up methods use of SPE cartridges reduces the clean up time as well as the use of and exposure to larger quantities of hazardous solvents used in traditional clean up methods such as column chromatography and solvent partitioning. And also when these bonded phase silica sorbents are used for the cleaning up there are usually fewer chromatographic interferences during analysis compared to the situation when polymeric resins such as XAD (as in the Newsome 1980a method) is being used (Johnson *et al.* 1991). There are many merits of SPE cartridges for pesticide analysis. In the analysis of water soluble pesticides SPE cartridges have been found to be very useful. With the extensive use of pesticides in agriculture the drinking water sources can be get contaminated with water soluble pesticides. As the concentrations of pesticides in water are too low to be analysed directly by solvent extractions, preconcentration of these pesticides using SPE cartridges having a suitably chosen sobent are selected. Here the preconcentration is being combined with purification as well (Tatarkovicova and Machac 1992). And also the difficulties occur during solvent extraction procedures such as the hazard due to formation of emulsions is avoided in SPE. In addition to all these, SPE cartridges can be used also at instances where the chemical is not extractable in organic solvents.

In the Vadukul method strong cation exchange (SCX) cartridges were used in the cleaning up of extracts.

Due to all these facts the anion exchange HPLC method with UV detection described by Vadukul (1991) was selected for the analysis of MH residues in potatoes.

Before analysing the MH treated potatoes using this method, different steps of the method were tested out. The HPLC analytical column used in his method was Nucleosil 5 SNB. Therefore the performance of MH in HPLC with the analytical column Spherisorb SAX 5 μm which was available in the laboratory was checked. MH gave the peak at a retention time of 5 min.

Linear response of the UV detector was also checked and observed that it was linear in the range of 10 ng - 1000 ng. The response of the detector below the level of 10 ng and above the level of 1000 ng was not checked because of the reason that the MH residue levels that will be working with would be definitely in the region of 10 ng to 1000 ng.

Efficiency of the SCX cartridges in cleaning up of extracts was also tested out and it was found that the cleaned up extract did not give any interfering peaks at the retention time of MH. And also there was no loss of MH during the cleaning up step.

Efficiency of this method was assessed by carrying out recovery studies with potatoes spiked with known concentrations of MH. Potatoes were spiked with 3 levels of MH. i.e 10 ppm, 25 ppm and 50 ppm. The recoveries obtained were in the range of 85-93%. Mean recovery was 88.65% with a standard deviation of 4.41%. Therefore as it was found to be a suitable method to analyse methanol extractable MH residues in potatoes, MH treated potatoes were analysed using this method. The analyses were carried out to find out the amount of methanol extractable free MH residues in total potato tuber, amounts in various sized tubers and also in various parts of the tuber to identify the distribution. The results obtained showed that the MH is evenly distributed through out the tubers (Table 2.2). There was no uniformity in the MH contents among different tubers obtained from the same potato crop (Table 2.3). It ranged from 2 - 10 ppm with an average value of 7.56 ppm in the 14 tubers analysed at various experimental stages. Bishop and Schweers (1961) also had observed a wide range of variation in the amount of MH residues found in the tubers of the same crop at harvest. Therefore it could be suggested that the variation in the concentration of MH in potato tubers can be due to either uneven application of MH to the potato crop or uneven distribution of MH from leaves into the tubers after application.

In this study it was found that MH residues are evenly distributed within the tuber. Hoffman (1962) reported that MH levels in the potato peelings tended to

be higher, than in the remainder of the potato. McKenzie (1989) also studied the MH content in various parts of the tubers and reported that the outer layer of the potato tuber including skin has the higher concentration of MH compared to the inner portion. Anglin and Mahon (1958) reported that MH is distributed uniformly within the potato tuber. All these inferences were based on the amount of total MH levels measured by the spectrophotometric method. As the analyses carried out here were for the determination of only the methanol extractable free MH levels, the present results can not be directly compared with their previous results. Although it was felt that the total MH determination should also have been carried out along with the free MH analyses the time limitation precluded that. Therefore it would be worthwhile to carry out further investigations on this aspect with more replicates. According to present observations it can be suggested that when MH is applied to the potato crop it does not get evenly distributed among the tubers. But, once MH goes into a particular tuber it gets evenly distributed throughout the tuber. In this study it was also found that there was no relationship between the amount of MH and the tuber size, because large tubers had high quantities as well as low quantities of chemical present.

In Chapter 3 effect of processing on MH residues in potatoes has been studied. Normally potatoes are consumed after subjecting them to some kind of processing such as frying and boiling. Therefore to assess the risks to consumers from intake of MH residues, the effects of processing on MH also was studied. The two methods of processing considered here were frying and boiling. To see the effect of frying on MH residues in potatoes, slices from a MH treated potato were fried in sunflower oil at 180 ^{0}C for 90 sec. and analysed for MH residues using HPLC. The amount of MH found in the fried crisps was only 10% of the amount at raw state. Because of this low recovery it was decided to carry out some model experiments using filter papers to study the effect of frying on MH. i.e. instead of potato slices, filter papers (Whatman No.1, diameter 5.5 cm) spiked with known amounts of MH were used to simulate potato slices containing MH and to simplify

the system under test. In these experiments the spiked filter papers were fried in sunflower cooking oil at 180 ^{0}C for 90 sec. By these filter paper model experiments, it was found that frying has no effect on MH because both fried and non fried filter papers which were previously spiked with known amounts of MH gave similar recoveries. The effect of the duration of frying also was assessed and it was found that duration of frying has no effect on the MH content.

And also it was observed that after frying no MH was present in the oil fraction of fried filter papers. It was also observed that no MH has gone to the fryer oil either, as no MH was detected in filter papers which were not spiked with MH but fried in the same oil in which MH spiked filter papers were fried. When MH spiked filter papers were fried and analysed, although no MH was found in either oil fraction of the filter papers or in the fryer oil the amount of MH recovered in non oil fraction was not 100%. It was only about 60%. Therefore a series of experiments were carried out using this filter paper model to find out possible reasons for the low recovery and to improve the recovery from filter papers. The effect of ash content in the filter papers on the recovery of MH from filter papers also was examined and found that there was no considerable effect. The increase in recovery was obtained by increasing the efficiency of extraction. By exhaustive extraction with methanol the recovery from fried filter papers went up to 72%. But, still there was about 25% fraction which was not recoverable. The effect of potato material on MH during frying was studied using the filter paper model. When MH spiked filter papers were wetted with potato juice from an untreated potato and fried and analysed it was found that there was a reduction of about 10% in the recovery of MH compared to the amount recovered from filter papers without potato juice. When the filter papers were wetted with unclarified potato juice there was a further reduction in the recovery.

Effect of additives like glucose and lysine also was examined and found some reducing effect on the recovery of MH on frying. Therefore it can be

suggested that some components in potatoes may be having some effects on MH on frying.

Using the improved method of extraction when the fried MH treated potatoes were analysed a recovery of 67 - 80% (mean 74.31±4.41%) of MH was obtained. Before improving the method the recovery was only about 10%. After improving the method of extraction still there is a fraction (25% of MH) which is not accountable. It can be suggested that the reduction in recovery could be either due to a loss in efficiency in the method of extraction or may be due to some kind of binding of MH with some components in the potato material or with oil during frying. Clements (1962) and King (1983) reported that MH can get oxidized to a product which can easily form a Diels-Alder adduct with dienes. This has made a basis of a method for determining MH residues by GC with electron capture detection (King 1983) as well. Conjugated dienes could be present in oil fraction or they could be formed due to the dehydration of sugar in potato material during frying at a high temperature such as 180 ^{0}C Therefore during frying if the free MH could get oxidised someway, there is a possibility for this oxidised product to bind to these conjugated dienes. i.e. either to a component in the potato material (sugars) or to the oil, forming Diels-Alder adducts. When analysing potato slices the oil fraction was discarded with out further analysis, as it was found that the oil fraction of the fried filter papers did not contain any free MH. Therefore it is doubtful whether any MH has gone into the oil fraction by binding to oil. Although the occurrence of above type of reaction was not demonstrated it is worth to carry out further investigations on this aspect to find out the actual reason for the reduction of this recovery due to frying.

Boiling of potatoes made about 20% consistent increase in the amount of MH content found in the raw potatoes. Some of the MH had been released into the water used for boiling of potatoes as well. McKenzie (1989) analysed boiled potatoes and observed that there is a small reduction in the content of MH when the potatoes are boiled. He had not determined the amount of MH which goes into the

water during boiling. It was thought that this observed increase on boiling could be due to the loss of moisture and starch from the potato pieces during boiling. As the weight loss observed due to boiling was only 2.5% it could not be solely responsible for this increase. Other possible reasons that could be suggested for this present observation, as stated under the discussion in Chapter 3. Section 3.2.4. are as follows.

1. The recovery factor in boiled potatoes is more than that in raw potatoes. Because when the potatoes are boiled all the cells get destroyed and the MH present in the potatoes may be more easily extracted with methanol.
2. The MH residues which were being incorporated into potato materials such as starch have been released during boiling. i.e. if the MH in raw potatoes remains incorporated into starch material of potato, methanol may not be extracting the MH completely. When the potatoes are boiled, as all the starch looses its structure and becomes gelatinised, it is possible to release MH. This would have made an increase in the amount of MH in boiled potatoes.

This is not a proven fact here and it can only be put forward as a suggestion. There may be many other reasons for this observed effect. Therefore further investigations would need to be carried out to find out the exact reason.

Chapter 4 deals with the study on the possible presence of bound or conjugated MH residues in MH treated potatoes. Acid hydrolysis with hydrochloric acid and enzyme hydrolysis with beta-glucosidase enzyme was carried out to release any bound or conjugated forms of MH present in potatoes. Acid hydrolysis was carried out using methanol extracts of both old and new potatoes and an increase in the MH content in the methanol extracts was observed. The increase found in old potato extracts was greater compared to that in new potatoes. For the enzyme hydrolysis process only new potatoes were used and it did not result in any increase in the MH content indicating that no beta-glucosides were present in new potatoes. But it is doubtful whether the old potatoes would contain any beta-glucosides either.

McKenzie (1989) observed an increase in MH content by enzyme hydrolysis of the methanol extracts of MH treated potatoes. But Newsome (1980b) did not. According to the results of the present experiments it can be suggested that no beta-glucoside of MH is present in new potatoes. But, there is some conjugated form of MH present in potatoes, which can release MH when treated with acid. The amount of this conjugated form is more in old potatoes than in new potatoes.

When following the acid hydrolysis process many problems were experienced. The acid hydrolysis was done following the procedure described by Yang (1992) in the analysis of MH in tobacco. He had used only 0.25 g samples of the powdered tobacco in the acid hydrolysis process. But, in the case of potatoes it was difficult to use such small samples. Therefore the sample size was increased to 0.5 g which was also found to be too small later. And also, the reaction mixture obtained after acid hydrolysis process had to be cleaned up by passing through a SCX Bond Elut cartridge before injecting into the HPLC, as it gave an interfering peak very close to the peak of MH in the HPLC trace when injected with out the clean up. The HPLC conditions used in the analysis were different from Yang's method. The use of the conditions given in his method was not successful. But the wavelength (330 nm) used in the UV detector of the HPLC was the same as in his method. When the analysis was carried out using lower wave length (313 nm) at which all the previous determinations of MH were carried out, many interfering peaks in the HPLC chromatogram of the acid hydrolysed were produced. The sample size used (0.5 g for solid and 1 ml for liquid) was too small, because in the final extract the MH content was too low to be measured. Therefore this method should have been tried out with a larger sample size or with a more concentrated extract.

A few suggestions could be made from this study. The HPLC method described by Vadukul (1991) which was used in this study for the determination of free MH residues in potatoes worked successfully. The improved method for the analysis of free MH residues in fried potatoes which was also based on Vadukul's

method gave a recovery of about 74% of MH compared to the initial recovery of 10% from the fried material. Therefore this method was found to be a suitable method for the analysis of free MH residues. But, still there was about 25% MH which was not recovered from the fried potatoes using this method. A possible reason for this could be the binding of some free MH either to oil or to potato material, although it was not demonstrated. Even though it was felt that it is necessary to carry out further studies on this aspect time limitation precluded it. Therefore it will be worthy of further studying on this line in future.

The work carried out on bound or conjugated residues in raw potatoes showed the presence of conjugated MH which is acid labile and no beta-glucoside was found as a conjugated product of MH. And also it was found that MH treated potatoes which are stored for a long period of time contained more conjugated product compared to the new potatoes. Therefore it can be suggested that the potatoes may have a mechanism to convert free MH to a conjugated form possibly to overcome MH induced dormancy. This conjugating process is important in nutritional point of view as well. As MH get converted to a conjugated form with age it may be safer to consume old potatoes rather than new potatoes which are being treated with MH. But, as the biological significance of this bound form to man or other animals has not been demonstrated it is not definite whether the conjugated form is safer to consume or not compared with free MH. Fischnich *et al* 1958, (cited by Ponnampalam *et al.* 1983) reported decreased fertility in rats fed potatoes that had been treated with MH prior to harvest versus those treated after lifting. This suggested that some metabolite(s) of MH produced in plant tissues was more toxic to animals than MH itself. As this metabolite(s) has not been identified yet, it is not possible to say whether this metabolite and the conjugated product noted in this study is the same. Therefore further studies on identification of the metabolite(s) should be carried out and the biological availability of this metabolite or/and conjugated product to animals should be investigated.

The fact that boiling in water releases MH (see boiled potato data) implies that adsorption onto plant components rather than co-valent bonding in involved on this occasion.

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