Studies on Oxamyl: Analytical Method Development and Investigation of Fate in Peach Seedlings and Corn Seeds

by

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To Lois,

who has been patient and encouraging
in the student life which has composed
our first three years of marriage.

ABSTRACT

A high performance liquid chromatographic method employing two columns connected in series and separated by a switching valve has been developed for the analysis of the insecticide/nematicide oxamyl (methyl-N',N'-dimethyl-N-[(methylcarbamoyl)oxy]-1-thiooxamimidate) and two of its metabolites. A variation of this method involving two reverse phase columns was employed to monitor the persistence and translocation of oxamyl in treated peach seedlings. It was possible to simultaneously analyse for oxamyl and its corresponding oxime (methyl-N',N'-dimethyl-N-hydroxy-1-thiooxamimidate), a major metabolite of oxamyl in plants, without prior cleanup of the samples. The method allowed detection of 0.058 µg oxamyl and 0.035 µg oxime. On treated peach leaves oxamyl was found to dissipate rapidly during the first two-week period, followed by a period of slow decomposition. Movement of oxamyl or its oxime did not occur in detectable quantities to untreated leaves or to the root or soil.

A second variation of the method which employed a size exclusion column as the first column and a reverse phase column as the second was used to monitor the degradation of oxamyl in treated, planted corn seeds and was suitable for simultaneous analysis of oxamyl, its oxime and dimethylcyanoformamide (DMCF), a metabolite of oxamyl. The method allowed detection of 0.02 µg oxamyl, 0.02 µg oxime and 0.005 µg DMCF. Oxamyl was found to persist for a period of 5 - 6 weeks, which is long enough to permit oxamyl seed-treatment to be considered as a potential means of protecting young corn plants from nematode attack. Decomposition was

found to be more rapid in unsterilized soil than in sterilized soil.

DMCF was found to have a nematostatic effect at high concentrations ($\geqslant$2,000 ppm), but at lower concentrations no effect on nematode mobility was observed. Oxamyl, on the other hand, was found to reduce the mobility of nematodes at concentrations down to 4 ppm.

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INTRODUCTION

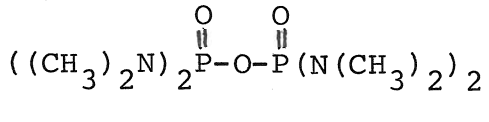
Brief History of Pesticide Development

The history of pesticide use goes back to the sixteenth century when Chinese farmers used arsenic sulfide as an insecticide[1]. The first use of a natural organic insecticide occurred in 1763 when nicotine, as a tea of tobacco leaves, was used to control aphids[2]. About 1800 pyrethrums from chrysanthemum flowers began to be used in Asia as a flea and louse powder[3]. Rotenone, obtained from derris roots, was first used as an insecticide against leaf-eating caterpillars in 1848[4]. In 1865 came the development of Paris green (copper acetoarsenate) for the control of the Colorado potato beetle[2].

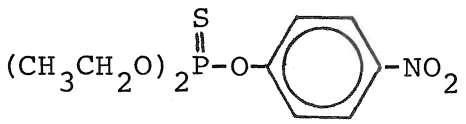
The first synthetic organic pesticide, potassium dinitro-o-cresylate, was introduced in 1892[2]. This compound was effective as an insecticide and as a herbicide, but is very toxic to mammals by today's standards. Cryolite(Na_3AlF_6), an inorganic pesticide with a very low mammalian toxicity, was used in sprays or as dusts from 1929[5].

The most significant event in the recent history of pesticide use was the discovery of the insecticidal properties of the organochlorine compound DDT (for dichlorodiphenyltrichloroethane) by Paul Müller in Switzerland in 1939. The control of typhus and malaria by DDT was demonstrated so dramatically during World War II that before the end of the war U.S. production exceeded 2,000,000 lbs/month[6]. Also during World War II the herbicides 2,4-D (2,4-dichlorophenoxyacetic acid) and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) were discovered, and the first organophosphorus pesticides were developed[7].

Examples of early organophosphorus insecticides are schradan (1941) and parathion (1944). Parathion is very toxic to



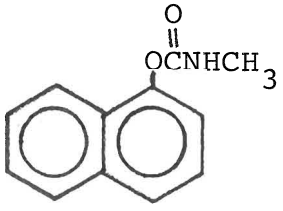
Schradan



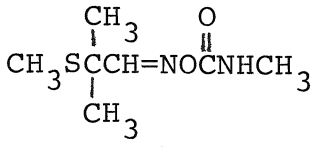
Parathion

mammals as well as to insects. Its discovery led to the development, by slight structural modifications, of several less toxic insecticides[8].

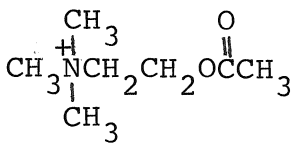
These discoveries during World War II initiated the era of synthetic organic pesticides, and revolutionized agriculture. In the last 35 years a multitude of synthetic organic pesticides have been introduced. In the early 1950's a new class of pesticides, derivatives of carbamic acid, was introduced commercially. One of the most successful early carbamates, carbaryl, was introduced as an insecticide in 1957. Carbaryl has an aryl N-methylcarbamate structure which has become the basis of most commercial carbamate insecticides. In 1966 the first oxime carbamate pesticide, aldicarb, was registered.



Carbaryl



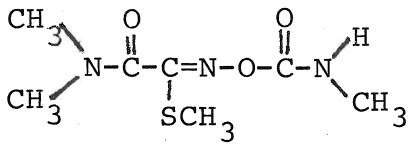
Aldicarb



Acetylcholine

This was also an N-methylcarbamate, and while possessing some of the electronic characteristics of an aryl carbamate, the oxime structure bore a closer spatial resemblance to the nerve cholinesterase substrate acetylcholine[9]. The oxime carbamates tend to be nematicidal and/or miticidal, as well as insecticidal.

In 1968 a patent was granted to E.I. duPont de Nemours and Company for a pesticide which was given the designation DPX-1410. This pesticide has since been registered in the United States and elsewhere for use on a number of crops, both agricultural and ornamental. It has been given the trade name Vydate, the active ingredient of which is the compound oxamyl.



Oxamyl

Oxamyl has been found to have insecticidal, miticidal and nematicidal qualities and is the pesticide with which this thesis is concerned.

Mode of Action of Insecticides and Nematicides

Pesticides in general may be classified according to their mode of entry into the target pest as well as by their biological mode of action against the pest.

A pesticide which must be ingested by the pest before it is effective is said to be a stomach poison. Examples of this type of pesticide include the inorganic arsenicals such as Paris green, inorganic fluorine compounds such as cryolite, and compounds of mercury, boron, antimony, thallium and phosphorus[10]. An advantage of this type of pesticide is that it will affect only those species which actually feed upon the treated plants. Therefore non-target beneficial insects will not be harmed.

A second classification of pesticides is a large group

of compounds known as contact poisons. The most generally held view of the mode of entry of a contact insecticide is penetration through the cuticle into the hemolymph, which rapidly transports it throughout the insect body, and from which it spreads to all kinds of tissues[11]. While stomach poisons largely include inorganic compounds, the contact poisons include most of the synthetic organic pesticides developed since World War II. For example, the solubility of DDT in waxy materials allows it to readily penetrate the insect cuticle[12]. The chlorinated hydrocarbons, organophosphorus compounds, carbamates, synthetic pyrethroids (the newest class of pesticides) and the natural organic pesticides all fall into the general classification of contact poisons. An advantage of the contact mode of entry into the pest is that treated plants do not have to be eaten before protection is achieved, but mere contact with treated plants will allow the pesticide to become effective. A disadvantage is that both pests and beneficial insects such as bees are susceptible to contact with the pesticide, resulting in a general decrease in insect populations.

Some contact poisons are also systemic pesticides, that is they have the ability to penetrate the tissues of the plant and be translocated in the transport system to parts remote from the site of application[13]. This property is of value in combatting pests such as certain nematodes which spend all or part of their life within the plant, where only a pesticide which penetrates the plant tissues can be effective. Systemic action also facilitates complete protection of the plant, even if the whole surface has not been treated. This can

result in a reduction of spray volume required.

A third general classification of pesticides is the fumigants. These are gases which kill pests upon entering the respiratory system, and are generally used in enclosed areas. Examples include hydrogen cyanide (released from calcium cyanide by moisture), methyl bromide, ethylene dibromide and methylisothiocyanate. Fumigants are used to kill pests of stored produce and against insects, nematodes and fungi in soil.

The term "biological mode of action" refers to the physiological mechanism which causes the death of the pest once the pesticide has entered. For our purposes a brief consideration of the mode of action of contact insecticides and nematicides will suffice.

The precise location of the site of action of DDT and other organochlorine insecticides is uncertain[12], although based on the symptoms of DDT poisoning it is apparent that the nervous system is attacked. DDT poisoning produces hyperactivity and convulsion resulting in paralysis and death which are thought to occur from metabolic exhaustion or from enhancement of a naturally occurring neurotoxin[14].

The organophosphorus and carbamate pesticides, on the other hand, act according to a relatively well-understood mechanism, that of acetylcholinesterase inhibition. Acetylcholine is the mediator of nerve impulses across cholinergic synapses in the nervous system of insects and mammals. When a nerve impulse has been conducted across a synapse, it is necessary to inactivate the transmitter substance so that the synapse is ready for a subsequent impulse[15].

This inactivation of acetylcholine is catalysed by the enzyme acetylcholinesterase (AChE) and is accomplished by the decomposition of acetylcholine to acetic acid and choline by a three-step process[16], illustrated by Figure 1-1.

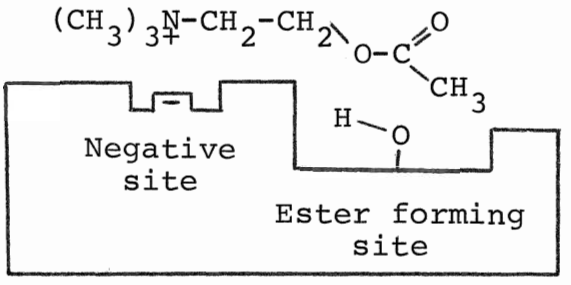
Organophosphorus and carbamate pesticides may be cleaved in the same way by AChE. In the case of an organophosphorus compound, the ester-forming site of the enzyme becomes phosphorylated. The O-P bond of the phosphorylated enzyme corresponds to Bond A in Figure 1-1 but is more stable with respect to hydrolysis by a factor of at least 10^7 [17]. This means that hydrolysis and resulting reactivation of the enzyme are very slow and in fact the enzyme virtually ceases to function.

In the case of a carbamate (eg. oxamyl), the ester-forming site of the enzyme becomes carbamylated. The O-C bond of the carbamylated enzyme is also more stable to hydrolysis than the corresponding Bond A, but is less stable than the O-P bond. The half-life of hydrolysis of the carbamate group and reactivation of AChE has been calculated to be about 40 minutes[18]. Thus the inhibition of AChE by a carbamate pesticide is largely due to competition with acetylcholine for the active site of the enzyme.

The result of inhibition of AChE is that the nerve impulse transmitter acetylcholine is not inactivated, causing prolonged stimulation of and eventual failure of the nerve or effector tissues. The difference in hydrolysis rates between carbamate and organophosphorus pesticides by AChE has led to their description by the terms "reversible" and "irreversible" AChE inhibitors, respectively. While not entirely correct, the terms reflect the added margin of safety of carbamates over

(a)

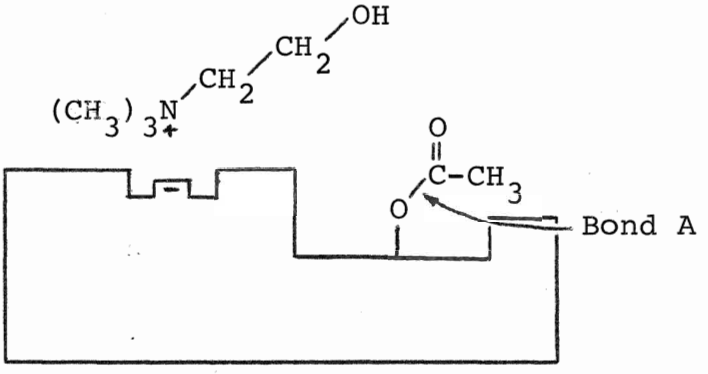
Acetylcholine



Cholinesterase

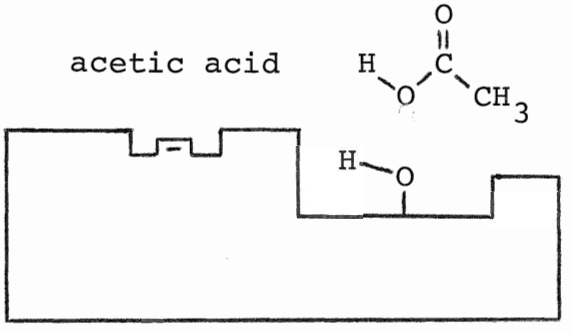
(b)

Choline



Acetylated cholinesterase

(c)



Free enzyme

Figure 1-1 Inactivation of acetylcholine
(a) the enzyme combines with acetylcholine to form an intermediate complex; (b) the esterified enzyme is formed with the release of free choline; (c) the esterified enzyme is hydrolysed at Bond A to regenerate the free enzyme and liberate acetic acid.

organophosphorus pesticides[15].

Symptoms shown by insects poisoned with carbamate pesticides include hyperactivity, incoordination, convulsions, paralysis and death. The actual cause of death is not certain, but may be due to exhaustion following general failure of critical areas within the central nervous system[19].

Cholinesterases have been found in many nematode species as well and it is presumed that organophosphorus and carbamate nematicides inhibit the cholinesterase of nematodes[20]. Bunt[21] demonstrated the difference in activity between an organophosforus nematicide, fenamiphos, and oxamyl. Nematodes placed in fenamiphos solution were killed within 1 - 2 weeks, whereas nematodes treated with oxamyl for up to three weeks were able to recover when transferred to water, illustrating the reversible nature of carbamate poisoning.

The Environmental Impact of Pesticides

All pesticides, when applied to plants or soil, eventually decompose to other compounds due to the action of light, air, water and microorganisms. These compounds along with any undecomposed compounds constitute residues, and may be found in plants, soil, water and air. From plants and soil, pesticide residues may be evaporated or carried by the wind, washed into waterways, or picked up by animals or other plants.

Many organochlorine pesticides have been found to be very persistent in the environment, that is they are very stable in the presence of light, air and water. Thus they do not rapidly decompose to non-toxic compounds, but persist in the environment for long periods of time, up to several years.

This can result in long-term contamination of soil and waterways and hazards to animals and man.

Another characteristic of organochlorine pesticides is that they are generally broad-spectrum pesticides, and not highly selective in their activity. This means that the pesticide may kill not only the particular pest for which it was intended, but also beneficial insects such as bees and insect predators of the pest. The present trend in pesticide development is toward pesticides which are selective, as far as possible, for the target pests.[22]

Thus prolonged persistence and non-selectivity can be causes of much damage. Because of this the use of DDT and other organochlorine pesticides such as aldrin and dieldrin was either banned or severely restricted in many developed countries in the early 1970's.

However, a degree of persistence must be recognized as a necessity if any pesticidal action is to be obtained from the pesticide. For example, a problem with some of the early synthetic pyrethroids was that they degraded so rapidly that their use as pesticides was ineffective[5]. Therefore a desirable characteristic of a pesticide is that it persist for a limited period of time after application, several days or a few weeks, depending on the situation, and degrade to non-toxic forms. In general this is true for the organophosphorus and carbamate pesticides.

It is not the case, however, that all pesticides degrade to compounds less toxic than themselves. For example, heptachlor (an organochlorine) and dimethoate and malathion (organophosphorus insecticides) degrade to residues which are

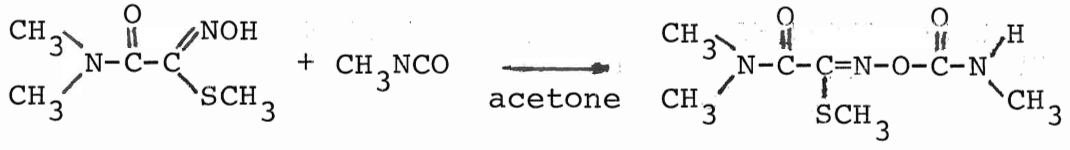
more toxic than themselves. An example of a variation of this problem is provided by DDT. The most common breakdown product of DDT is DDE (2,2-bis-(p-chlorophenyl)-1,1-dichloroethylene) which is non-toxic to insects. However, DDE has been found to be largely responsible for eggshell thinning and cracking resulting in the decline of several species of birds in Europe and North America[23]. Therefore an important consideration regarding the environmental impact of a pesticide is the toxicology of its residues.

The environmental impact of pesticide use may be minimized by several measures. Improvement of application methods which increases the percentage of pesticide which is deposited on the plant surface will result in a decrease in the overall volume of pesticide applied. An example of a highly efficient application method is seed treatment, especially with systemic insecticides, which provides minimal dosages and high ecological selectivity[24]. Careful timing of application to coincide with emergence of larvae or adult insects will reduce the number of sprays necessary in a season and may result in less damage to beneficial species. The combination of pesticide use with biological and cultural control methods may reduce the volume and frequency of pesticide applications necessary to provide crop protection.

These and other measures which minimize the quantity of pesticides applied to the environment, while providing adequate crop protection, are positive steps toward sound management of environmental quality.

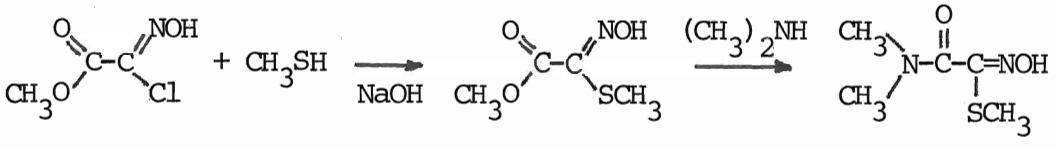
The Chemistry of Oxamyl

The common name oxamyl has been given to the oxime carbamate pesticide with the chemical name methyl-N',N'-dimethyl-N-[(methylcarbamoyl)oxy]-1-thiooxamimidate. This compound may be synthesized by the reaction of the oxime (methyl-N',N'-dimethyl-N-hydroxy-1-thiooxamimidate) with methylisocyanate in a solvent such as acetone.



Oxime | Methyl-isocyanate | Oxamyl

The oxime starting material may be synthesized by reacting methoxycarbonylformhydroxamyl chloride with methylmercaptan in the presence of a base, and then reacting the product with dimethylamine.



Oxime

Oxamyl is a crystalline solid with a slight sulfurous odor. It melts at 100 - 102°C, changing to a different crystalline form which melts at 109 - 110°C. The vapour pressure of oxamyl is very low, 2.3×10^{-4} mm Hg at 25°C and 7.6×10^{-3} mm Hg at 70°C.

The solubility of oxamyl in various solvents at 25°C is summarized in Table 1-1[26]. The parameter p' is a solvent

Table 1-1

Approximate Solubility of Oxamyl at 25°C

Solvent	g/100g solvent	p'
Water	28	(9)
Methanol	144	6.6
DMF	108	6.4
Acetone	67	5.4
Ethanol	33	5.2
Cyclohexanone	29	4.5
Isopropanol	11	4.3
Toluene	1	2.3

Table 1-2

Stability of Oxamyl in Aqueous Solution

Time in Soultion	% Oxamyl loss (16 oz./100 gal.)		
	pH 4.7	pH 6.9	pH 9.1
0 h	0	0	0
6	0	0	30
24	0	3	45
48	0	9	47
72	0	16	60
96	0	26	66

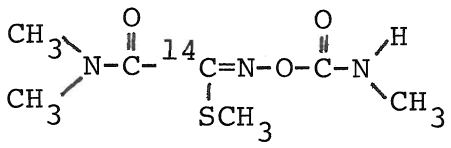
polarity parameter[27]. It may be noted from the table that oxamyl is moderately soluble in water, soluble in polar organic solvents and virtually insoluble in non-polar organic solvents.

The stability of oxamyl in solution under acidic, neutral and alkaline conditions is summarized in Table 1-2[26]. These data were obtained at room temperature in 0.1M aqueous buffer solutions. These results indicate that oxamyl is stable under slightly acidic conditions. In fact after 11 days in solution at pH 4.7 there was still no decomposition of oxamyl. Slow hydrolysis to the oxime occurs in neutral solution. Under alkaline conditions rapid hydrolysis occurred, with 30% conversion in the first 6 hours. During this period the hydrolysis reaction neutralized the sodium bicarbonate buffer, and the subsequent rate of hydrolysis was approximately equivalent to that in neutral solution[28].

Fate of Oxamyl in Plants

The most comprehensive study to date on the fate of oxamyl in plants was published by Harvey et al. in 1978[29]. These researchers studied the metabolic fate of oxamyl in tobacco, alfalfa, peanuts, potatoes, apples, oranges, and tomatoes using a combination of thin-layer chromatography (TLC), liquid scintillation counting of radiolabelled residues, high performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC-MS).

Fifteen days after foliar treatment of a tobacco plant with [^{14}C]oxamyl, they found that 50% of the original treatment (O.T.) was still a surface residue. Analysis of this surface residue indicated that it was 96% oxamyl and



[^{14}C] Oxamyl

3% oxime. Radioactivity was distributed throughout the plant with 37% O.T. in the treated leaves, 1.3% in the new growth and 0.1% in the root. The leaf residue was found to be 56% oxamyl, 5% oxime and 39% a polar fraction which was shown by GC-MS to be a glucose conjugate of the oxime (93%) and N,N-dimethyloxamic acid (7%). The identity of the root radioactivity was not determined.

Seventeen days after the last of three treatments with [^{14}C]oxamyl, alfalfa hay analysis indicated that about 90% of the extracted radioactivity was in the form of a glucose conjugate of the oxime with just traces of oxamyl (0.8%) and oxime (0.8%).

Young peanut plants were given a foliar spray of [^{14}C]-oxamyl, and 4 weeks later, no trace of oxamyl or oxime was found. The extracted radioactivity was found to be about 99% glucose conjugates. In mature peanut foliage, 7 weeks after treatment with [^{14}C]oxamyl, no oxamyl was found but 1% of the radioactivity was found to be the oxime. Most of the extracted radioactivity (99%) was in the form of very polar conjugates, probably polysaccharide conjugates of the oxime.

The interior portion of potato tubers, after 5 foliar treatments with [^{14}C]oxamyl, was found to contain radioactivity probably in the form of insoluble polysaccharide conjugates, with little or no free oxamyl or oxime.

The fruit of apple and orange trees and a tomato plant was treated directly with [^{14}C]oxamyl. The apples and oranges were harvested 6 weeks later and the tomatoes 2 weeks later. The radioactive residues were found to include oxamyl, oxime, glucose conjugates and some polysaccharide conjugates, and a metabolite positively identified as N,N-dimethylcyanoformamide (DMCF). The percentages of total ^{14}C residues are summarized as follows:

	oxamyl	oxime	DMCF	polar fraction
apple	16%	42%	17%	23%
orange	9	6	20	65
tomato	59	13	4	24

These results led the authors to conclude that oxamyl in plants initially decomposes to the oxime, which then further reacts with glucose to form conjugates which may combine with further hexose units to give polysaccharide conjugates. It was found that some of the radiolabelled oxamyl had been converted to DMCF, but no direct evidence was obtained that oxamyl or the oxime is translocated to the roots after foliar application of oxamyl.

Several other workers have reported studies on the movement of oxamyl or its metabolites in plants using radiolabelled oxamyl. By way of introduction to these studies it may be profitable to point out that translocation in plants occurs in each of two distinct systems, the apoplast and the symplast. The apoplast includes the interconnecting cell walls and intercellular spaces, including the water-filled xylem elements[30]. In this system occurs the acropetal or upward movement of substances in the plant. The symplast consists of the protoplasts of the cells,

which constitute an interconnected system through which dissolved materials may move, and includes the phloem. Movement in the symplast may be both basipetal and acropetal.

Many systemic pesticides show an apoplastic pattern of transport, that is they have been found to be translocated upward in the plant. A material which may be translocated in both the apoplast and the symplast is termed ambimobile.

In a study reported by Peterson et al.[31] mature leaves of potato plants were treated with [^{14}C]oxamyl. As soon as one day later, radiolabel could be extracted from all areas of the plants. Until the 12th day, from 77 - 90% of the translocated radioactivity was recovered from the seed piece and root. This represented from 4 - 22% of the total radioactivity per plant. On the 6th and 12th days the root extract was analysed by TLC using scintillation counting and the radioactivity was found to be 90% and 84% oxamyl on the respective days. This indicates that oxamyl was translocated largely intact downward through the phloem. Also, a small but increasing proportion of the label was translocated toward the plant apex. After 12 days 72% of the radiolabel remained in or on the treated leaflets and 90% of this was found to be oxamyl by TLC. The conclusion drawn from this study was that oxamyl is an ambimobile systemic in that it can be translocated in both phloem and xylem.

In a similar study by Edgington et al.[32] leaves of cucumber plants were treated with [^{14}C]oxamyl. One week later the leaves were washed to remove surface residues, and autoradiographs of the plants were obtained. A count

of radioactivity in the surface wash indicated that 75 - 90% of the oxamyl had penetrated the leaf. The autoradiographs indicated that the accumulation of radioactivity was mainly in the leaf margins and veins and sometimes in the buds. This led the authors to conclude that oxamyl is ambimobile with some downward transport in the phloem. However, the radioactivity was not analysed to determine whether oxamyl or one of its metabolites, also carrying the ^{14}C label, had translocated downward.

In another study Wright el al.[33] found that application of 12 µg of [^{14}C]oxamyl to the leaves of cucumber seedlings resulted in a root oxamyl concentration of 3 ng/g from 1 - 21 days after treatment. Oxime was also present in the root in increasing concentrations from 3 ng/g to 8 ng/g. The sand in which the plants were grown was found to contain less than 0.2 ng oxamyl/mL of sand water, but up to 12.8 ng oxime/mL sand water.

Bunt and Noordink[34] studied the translocation of oxamyl in bean plants. When the second leaf from the bottom of a plant was treated with[^{14}C]oxamyl, autoradiographs two weeks later indicated that the ^{14}C label was present in all parts of the plant above and below the treated leaf. Even the top leaves and the roots showed a high radioactivity. However, the radioactivity levels were not quantitated. In another experiment the second leaf from the top of both young and mature plants was treated with [^{14}C]oxamyl. Again, 2 weeks later, autoradiographs showed that radioactivity had been distributed to all parts of the plant. Even leaves formed after application showed a relatively high radioactivity.

It was found that when the leaves were kept in darkness immediately after treatment, translocation was highly inhibited. In order to study the ^{14}C activity of the root exudate, the sand in which the treated plants had grown was washed with water. This solution was developed on a TLC plate along with a [^{14}C]oxamyl standard. An autoradiograph of the plate showed that in the root exudate mainly oxime and only traces of oxamyl were present. According to these authors, "oxamyl is the first nematicide for which sufficient evidence has accumulated that translocation in the plant is two-way: acropetal and basipetal".

Fate of Oxamyl in the Environment

The rate of decomposition of oxamyl in river water and distilled water under UV light and in the dark was studied by Harvey and Han[28]. They found that at two concentrations of oxamyl (1 ppm and 1,000 ppm) decomposition was much faster and much more extensive under UV light in both distilled and river water. In both types of water kept in darkness there was little (2 - 16%) decomposition after 10 days, and the only metabolite produced was the oxime. In the samples exposed to UV light, on the other hand, extensive decomposition (39 - 98%) occurred after 7 days to both the oxime and other more polar metabolites.

A difference in the rate of decomposition was also observed between the distilled and river water samples. At both concentrations greater decomposition occurred in the river water. This was most evident in the 1 ppm samples under the UV light, in which after 7 days the distilled water showed 39% decomposition of oxamyl, while the river

water showed 98% decomposition. This suggests that natural salts and microorganisms may play a role in the breakdown reactions.

Decomposition of oxamyl in river water exposed to sunlight outdoors was even more extensive. In the initial 16 hour period (overnight), 3% of the oxamyl hydrolysed to oxime. However during exposure to sunlight the next day, complete hydrolysis occurred. The oxime was found to decompose to N,N-dimethyloxamic acid and other more polar metabolites which were not characterized.

In the same paper the authors reported on the decomposition of oxamyl in soil. They found that under aerobic conditions in a silt loam soil (pH 4.7), 51% of the [^{14}C]-oxamyl had decomposed to $^{14}CO_2$ after 42 days. Four per cent of the oxamyl, a trace of the oxime and a polar fraction accounting for 11% of the radioactivity remained after the same period. The remainder of the radioactivity was found to be an integral part of soil components. Under anaerobic conditions, 8% of the oxamyl remained after 42 days, but only 3% had decomposed to $^{14}CO_2$. The oxime accounted for 41% of the radioactivity and a polar fraction for 42%.

Under field conditions using three types of soil, radioactivity was lost rapidly from the soil by volatilization, presumably as $^{14}CO_2$, and less than 5% of the oxamyl remained 1 month after treatment. The oxime was detected in the early stages, but this also decomposed rapidly. The half-life of oxamyl in all 3 soils was about 1 week or less.

In comparison with these results, Bromilow[35] found that in fallow soil in pots kept outdoors in late summer, oxamyl

had a half-life of about 2 weeks. In another study by Bromilow et al.[36] using soils of varying organic matter content, it was found that adsorption of oxamyl onto soil was weak, being strongest on soils containing a high proportion of organic matter. A rate study using these soils showed that the breakdown of oxamyl closely followed first-order kinetics. The rate of decomposition was found to increase with increasing temperature. Increasing the moisture content from 5% to 10% increased the rate of decomposition, but an increase from 10% to 15% had little effect. At 15°C the half-life of oxamyl under controlled laboratory conditions ranged from 6.2 days to 21.5 days in 5 types of soil. The rate of degradation was not obviously correlated with any single soil property.

It is evident from the results of these studies that oxamyl is relatively non-persistent in the environment, with a soil half-life of between 1 and 3 weeks. Decomposition in natural water is even more rapid. It would appear therefore that use of oxamyl as a pesticide has little environmental impact and does not result in long term contamination of soil or water.

Applications of Oxamyl as a Pesticide

Registrations have been obtained in the United States for use of oxamyl on non-bearing apples, tobacco and selected ornamentals. Special local need registrations exist for bearing apples, celery, citrus, potatoes and tomatoes. Further registrations have been obtained in other countries for use of oxamyl on onions, beets, bananas, garlic, pineapples, cotton, beans and coffee.

Oxamyl has been found to be effective as a foliar spray against mites, cotton leafhopper, green peach aphid, Colorado potato beetle, thrips, flea beetles, weevils, scales, mealybug and whitefly. As a soil application, oxamyl is effective against flea beetles, Colorado potato beetle, thrips[37] and at least 18 varieties of nematodes. In addition, a large volume of evidence has accumulated which indicates that oxamyl is effective as a foliar spray against soil nematodes[38, 39].

Registration of oxamyl has not been obtained in Canada due to a lack of Canadian data regarding effectiveness of the product and residue levels on saleable produce.

Analytical Methods Used for Oxamyl Determination

A variety of analytical methods including titrimetric, spectrophotometric, GC and HPLC have been used in the past for the determination of oxamyl concentrations.

Singhal et al. have reported spectrophotometric[40] and titrimetric[41] methods for determining oxamyl residues in crops and soils. In both methods oxamyl was refluxed with a 10% potassium hydroxide solution. This resulted in hydrolysis of oxamyl with methylamine and dimethylamine as products, from the carbamate and amide ends of the molecule, respectively. These were shaken with Cu^{2+} and CS_2 resulting in the formation of N-methyl and N,N-dimethyl copper dithiocarbamates. In the spectrophotometric method, these copper complexes were determined at 435 nm. In the titrimetric method the Cu^{2+} remaining unreacted was titrated with EDTA and the concentration of the complexes determined by difference.

Gas chromatographic methods have been reported by a number of workers. The method reported by Holt and Pease[42] involves mild base hydrolysis of oxamyl to the more volatile oxime, and determination of the oxime by GC with a flame photometric detector in the S-mode. Bromilow and Lord[43] and Bromilow[44] used a method in which oxamyl was reacted on the GC column with trimethylphenylammonium hydroxide to give the methoxime derivative, which was determined using a flame photometric detector in the S-mode.

Chapman and Harris[45] developed a method in which the tetramethylsilyl ether of the oxime was determined by GC. By subjecting plant extracts to liquid-solid chromatography on aluminum oxide, preliminary separation of oxamyl and the oxime was obtained. The oxamyl obtained in this way was then hydrolysed and derivatized separately from the oxime, so it was possible to analyse for both oxamyl and oxime in the extracts.

In another GC method, Lee[46] reacted dinitrofluorobenzene with the dimethylamine and methylamine resulting from base hydrolysis of oxamyl. This resulted in two derivatives, dinitrophenyldimethylamine and dinitrophenylmethylamine. The former will result from hydrolysis and derivatization of both oxamyl and oxime, while the latter will result from oxamyl only. Since the two derivatives were resolvable using GC, oxamyl was determined by analysing dinitrophenylmethylamine with an electron capture detector. This provided an analysis of oxamyl separate from the oxime, but did not allow simultaneous analysis of oxime, since the yield of the oxime derivative was not reproducible.

Several workers have also reported HPLC methods for the analysis of oxamyl. Aten and Bourke[47] determined the sensitivities (at 254 nm) and retention volumes of oxamyl using reverse phase HPLC with 6 mobile phases, but made no attempt to determine oxamyl residues in plant material.

Thean et al.[48] reported a reverse phase HPLC method for oxamyl which involved extensive cleanup of the extract before analysis. This method was applied to extracts of several varieties of fruits and vegetables.

Davis et al.[49] used reverse phase HPLC to analyse oxamyl and the oxime simultaneously on the leaves of citrus trees. They did not find any interference from chlorophyll or other plant materials, however this may have been due to the fact that the levels of oxamyl residues were very high (460 µg/leaf, giving about 18 µg oxamyl/mL extract).

Purpose and Justification of Project

Even though there is much evidence for protection from nematode attack following foliar application of oxamyl, the actual mechanism by which protection is provided is not clear. It is not yet known whether oxamyl itself, translocated to the roots, or a metabolite of oxamyl or even a compound produced in response to the presence of oxamyl provides protection from nematode attack. It has been postulated[50] that oxamyl works by repelling nematodes before root penetration or hindering development or reproduction after entry. Taylor and Alphey[39] have suggested that a nematicidal exudate is produced in the soil following foliar applications of oxamyl. Wright et al.[33] proposed that oxamyl in very low concentrations at the growing surface of the root

or in the soil acts to disrupt the relatively complex behavioural sequences necessary for nematode invasion.

To shed light on this problem, a method is needed to analyse for oxamyl and its oxime and allow the detection of other metabolites in leaves, roots and soil in order to determine whether any of these compounds is translocated to the root or soil after foliar application of oxamyl.

The analytical methods previously described were not deemed suitable for several reasons. The spectrophotometric and titrimetric methods do not give the concentration of oxamyl alone, but the combined concentration of oxamyl and the oxime. This is because base hydrolysis of the oxime yields dimethylamine which will form copper complexes when reacted with Cu^{2+} and CS_2. This will impart a positive error to the oxamyl determination. The magnitude of this error could be very great, since the oxime is a major degradation product of oxamyl in plants. These methods also do not allow for a search for other metabolites in plant extracts.

Similarly, GC methods which determine oxamyl as the oxime or derivative thereof also give the combined concentrations of oxime and oxamyl. Chapman and Harris[45] and Lee[46] overcame this problem, the former by separating the oxamyl and oxime before derivatization, and the latter by analysing the derivative obtained only from oxamyl. However, separation by liquid-solid chromatography and subsequent derivatization involves the inherent possibility of loss of sample. Lee's derivatization procedure alone gave percentage yields of only 44.5 - 80.6% in terms of the expected weight of derivative. Also, these procedures are not readily adaptable to a search for other metabolites.

High performance liquid chromatography was chosen as a very versatile technique suited for the proposed analyses. Its mild operating conditions permit the analysis of thermally labile compounds and the problems associated with the analysis of non-volatile compounds are eliminated.

However, the HPLC methods previously used for oxamyl analysis suffer from several shortcomings. The method of Davis et al.[49] was not applied at the residue level expected in roots and soil. A sensitive method is required since the level of residues in roots and soil is very low. They were not able to detect the oxime in their samples 1, 2 or 3 weeks after treatment with oxamyl even though its presence is expected, based on the results of other workers. The method of Thean et al.[48] was applied at the residue level, but requires an extensive cleanup procedure, again with the inherent possibility of loss of sample. In fact their recovery of oxamyl from spiked vegetable extracts was quite poor (61 - 77%). They made no attempt to analyse for oxime or any other metabolites. Neither of these HPLC methods was applied to root or soil samples, but only to leaves or fruit.

The purpose of this project therefore was firstly to develop a convenient HPLC method for the simultaneous determination of oxamyl and oxime at the residue level in leaf, root and soil samples, with minimum cleanup. This method, once developed, was to be applied to the study of the movement of oxamyl and its metabolites by the analysis of leaf, root and soil extracts of plants following foliar treatment with oxamyl. Persistence of oxamyl on leaves and seeds was also monitored.

EXPERIMENTAL

Mass Spectrometry and Nuclear Magnetic Resonance

The mass spectra of oxamyl (supplied by DuPont) and dimethylcyanoformamide were obtained using the solid and AGHIS probes of an AEI/Kratos MS30 double beam mass spectrometer equipped with a DS-55 mass spectrometry data system. An electron impact ion source at ionization potentials of 70 eV and 20 eV was used with a source temperature of 180°C and a probe temperature of between 50° and 100°C. The accelerating voltage was 4 kV.

The proton nuclear magnetic resonance (NMR) spectrum of oxamyl was obtained in deuterochloroform solution using tetramethylsilane as an internal reference. Spectra were obtained using a Bruker WP-60 NMR spectrometer at probe temperatures of 300K, 273K, 253K and 233K. Proton NMR spectra of oxamyl were also obtained in deuterated dimethylsulfoxide (DMSO-d_6) solution at the following temperatures: 302K, 343K, 363K, 365K, 366K, 368K, 373K and 393K. All of these spectra were obtained using 16 scans with a pulse width of 1.3 µsec, the duration of a 90° pulse being 3.8 µsec. The spectrum width was 750 Hz/cm with an offset of 2625 Hz and the time required for each scan was 5.46 s. Deuterated solvents were supplied by Merck Sharp and Dohme Canada Ltd.

Preliminary Studies

A preliminary study using peach seedlings was performed. Commercial Vydate (DuPont) in the amount of 16.61 g was dissolved in 4 L of water. Since commercial Vydate contains 24% oxamyl, this gave a solution with an oxamyl concentration of approximately 1,000 µg/mL. The soil of each of eight young

peach seedlings (cv. Bailey, approximately 25 cm tall) in 12 cm clay pots was covered with aluminum foil. Seven of these plants were dipped in the oxamyl solution for 30 s each with some agitation. One plant was dipped in water as a control. After the leaves had dried, the aluminum foil was removed and the plants were placed in the Brock greenhouse for soil watering only.

One plant was extracted on each of the following days after treatment with oxamyl: 2, 6, 12, 16, 21, 26 and 30 days. The control plant was extracted on the same day as the 2-day plant. The leaves of each plant were cut into small pieces into a 125 mL Erlenmyer flask and were shaken together with technical grade methanol on a Burrell wrist action shaker for 50 min. The volume of methanol used for each extraction was either 70 mL or 40 mL and each sample was extracted from two to four times in an attempt to determine the most efficient extraction procedure.

The HPLC used in this and all experiments was a Perkin-Elmer Series 3 LC which has a two-solvent capacity using two dual-piston pumps. The chromatograph is microprocessor-controlled, permitting positive solvent gradients and four program stages. The detector used was a Coleman Model 55 variable wavelength UV detector. The wavelength used for detection was 240 nm.

The column used in this experiment was an Altex RP-18 Ultrasil ODS column, 25 cm x 4.6 mm i.d. with a packing particle size of 10 μm in diameter. This is a reverse phase column in which the packing material is silica gel with octadecylsylil (C_{18}) chains bonded to the surface. This column

was protected from contamination by a 3 cm x 4.6 mm i.d. Brownlee C_{18} guard column with a 10 µm particle size.

The methanol extracts were prepared for HPLC analysis by evaporating an aliquot to dryness in a 55°C water bath under a stream of nitrogen, redissolving in 5% methanol 95% water, and filtering through a 10 µm pore size Millipore filter. The samples were injected onto the HPLC column in the following manner. A 175 µL sample loop was filled by flushing with about 300 - 400 µL of the sample. This was then injected onto the column with no interruption of flow. The same technique was used for samples and standards.

The leaf extracts were analysed for oxamyl on the HPLC using a mobile phase of 10% HPLC-grade methanol (supplied by Caledon Laboratories Ltd.) and 90% distilled water, and a flow rate of 4 mL/min, followed by a purge of 70% methanol for 1 min. The column was allowed to equilibrate for 4 min following the purge. Each sample was run twice and the average of the two runs was obtained to give the concentration of oxamyl. Standards of appropriate concentration were also run twice, on average between each sample.

A thin-layer chromatography (TLC) cleanup procedure was attempted according to the following method. A 5 mL aliquot of a methanol leaf extract was evaporated to dryness and redissolved in a small volume of acetone. The entire amount was streaked on a silica gel TLC plate (Eastman) treated with a fluorescent indicator and developed, along with oxime and oxamyl standards, with 30% Acetone, 70% ethyl acetate. The oxime and oxamyl layers were scraped off and dissolved in methanol which was then filtered, evaporated to dryness

and redissolved in 2 mL of the HPLC mobile phase (10% methanol, 90% water).

Since the analysis of oxime was not possible on the reverse phase column due to interference by leaf coextractives, further method development was undertaken, including normal phase and two-column reverse phase systems. This development process will be summarized in the Results and Discussion section.

First Peach Plant Experiment

Seven peach plants were treated with oxamyl as in the preliminary study. Since young peach seedlings were unavailable for use, older peach plants which had been cut back and allowed to grow a new stem were used instead. These were about the same size as the plants used in the preliminary study. One plant was extracted on each of the following days after treatment with oxamyl: 2, 7, 12, 16, 21, 26 and 30 days. Again, a control plant was extracted on the same day as the 2-day plant. The methanol used for extraction contained 0.5% H_3PO_4 by volume.

The leaves of each plant were extracted three times with 70 mL of methanol for about 50 min each time. The three extractions were combined in a 250 mL volumetric flask and made up to 250 mL with methanol. The roots were extracted once overnight with 70 mL methanol, followed by shaking for about an hour. The extract was added to a 100 mL volumetric flask. The soil was extracted twice with 200-225 mL methanol by tumbling for 4 hours each time on a Fisher-Kendall Mixer. The two extractions were combined in a 500 mL volumetric flask. All samples were weighed before extraction.

The leaf samples were concentrated for HPLC analysis as follows. Five millilitres of the methanol extract and 2 mL of water were added to a graduated sample vial. The methanol was evaporated under a stream of nitrogen or air in a 55°C water bath. The volume was adjusted to 2 mL with water and the sample was filtered through a 1 μm pore size Millipore filter. This effected a 2.5x concentration of the extract.

The leaf samples were analysed on the HPLC using a two-column reverse phase system. Preliminary separation was achieved on the first column, with the oxime fraction being collected onto the second column via a switching valve. The oxime and oxamyl were analysed on the second column. The Perkin-Elmer Series 3 LC allows four program stages with either linear or curved solvent gradients. A linear solvent gradient is designated by the number 1 and curved gradients may be selected by their particular numbers. A curve of 0 indicates an abrupt change to a new mobile phase composition, without a gradient. Use of the curve 0 allows a decrease in mobile phase strength during a program, while use of any other gradient curve allows only an increase in mobile phase strength.

The mobile phase was composed of methanol and water, methanol being the stronger solvent. The program used for analysis of leaf samples is given in Table 2-1. The two-column system is illustrated schematically in Figure 2-1. The first column was a 25 cm x 4.6 mm i.d. Altex RP-18 Ultrasil ODS column packed with 10 μm particles. This column was protected by a 3 cm x 4.6 mm i.d. Brownlee C_{18}

Table 2-1

HPLC program used to analyse peach leaves
(First Peach Plant Experiment)

Program Stage	1	2	3	4	Purge	Equilibration
Duration (min)	22	10	17	18	5	8
% methanol in H_2O	0.1	10	3	10	70	0.1
Curve	1	0	0	0	0	
Flow rate (mL/min)	2	2	2	2	2	2

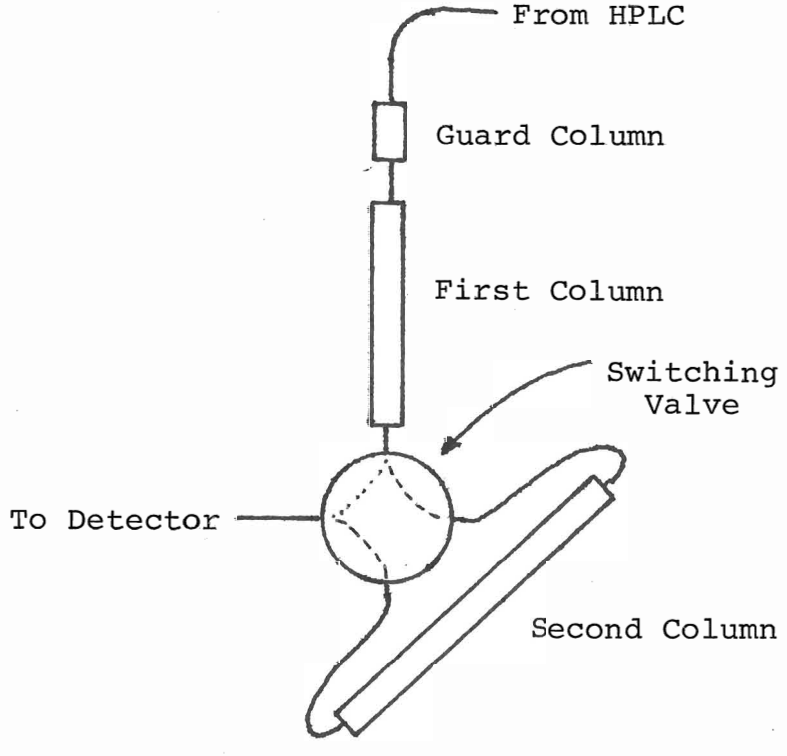


Figure 2-1 Schematic diagram of Two-Column HPLC System

···· flow directed through first column only

-·-· flow directed through both columns

guard column also containing 10 µm particles. The second column was a 25 cm x 4.6 mm i.d. Brownlee C_{18} column with 10 µm particle size.

The time during which the oxime eluted was determined using an oxime standard. During the first program stage this fraction of the sample was collected on the second column using the switching valve. During the second program stage, anything eluting between the oxime and oxamyl was allowed to pass directly to the detector. At 36.0 min into the program, just before the elution of oxamyl, the valve was again switched to direct the flow onto the second column. During the third program segment the oxime was analysed on the second column, and during the fourth stage oxamyl was analysed, also on the second column.

Each sample was run once, since each run required 80 min. Combined standards containing both oxamyl and its oxime were run on average once for every 2.7 samples.

Second Peach Plant Experiment

In this experiment, the stems of young peach seedlings planted in 8 cm clay pots were subdivided into three regions; top, centre and bottom. The leaves on the top section of the stem were wrapped with paper and the bottom section of leaves was covered with aluminum foil. This isolated the centre section of leaves for treatment with oxamyl.

A solution of Vydate was prepared by diluting 10.0 mL of the commercial material to 1 L with water. This gives a concentration in terms of oxamyl of about 2670 µg/mL. The centre section of leaves of 18 plants was dipped into this solution, care being taken not to allow the solution to

contact the top or bottom leaf sections.

The treated centre section of leaves of two plants was extracted on the day of treatment to determine the concentration of oxamyl initially applied. The next day two more plants were extracted, the top, centre and bottom leaf portions being extracted separately. The rest of the plants were placed in the Brock greenhouse for daily soil watering. The paper wrapping was removed from the top leaf section and the border between the top and centre sections was indicated by a piece of string tied around the stem. Upper leaves which contacted the centre leaves were removed. The aluminum foil separating the centre and bottom leaf sections was removed and replaced by a 15 cm x 15 cm piece of anodized aluminum screen in order to allow light to reach the bottom section of leaves and yet prevent contact of the bottom leaves with the centre leaves. A similar screen was placed over the soil to prevent contact with the leaves.

Two plants were extracted on each of the following days after treatment: 4, 8, 14, 21 and 30 days. Each leaf section was extracted separately. In an attempt to maintain consistent levels of leaf coextractives in each methanol extract, the size of leaf samples was maintained at approximately 1 g. Each leaf sample was cut into a 50 mL Erlenmyer flask and extracted three times with 30 mL of methanol (without H_3PO_4) by shaking for 1 h each time on a Burrell wrist action shaker. The extracts were combined in a 100 mL volumetric flask and made up to 100 mL with methanol. The centre leaf portions of the 1-day plants were extracted four times with 30 mL methanol, each extraction being added

to a separate 50 mL volumetric flask in order to test the efficiency of the extraction method.

Each root sample was separated from the soil and cut up into a 50 mL Erlenmyer flask. It was extracted three times with 30 mL methanol and 1 h shaking each time. The third extraction was allowed to sit overnight. Each extraction was vacuum-filtered through a sintered glass funnel, and the combined extracts were added to a 100 mL volumetric flask.

Each soil sample was separated from the root and mixed thoroughly. A small (eg 2 - 4 g) aliquot was added to a beaker and weighed before and after several days of air drying in order to determine the water content of the soil. The remainder of the soil was immediately added to a 250 mL erlenmyer flask and tumbled with 70 mL methanol for 1 h. This was repeated and then followed by a third overnight extraction. Each of the three extractions was vacuum-filtered through number 1 Whatman filter paper and the combined extracts were added to a 250 mL volumetric flask and made up to 250 mL with methanol. All leaf, root and soil samples were weighed before extraction.

The methanol extracts were prepared for HPLC analysis by adding 10 mL of extract and 2 mL of water to a graduated sample vial. The methanol was evaporated under a stream of air in a 55°C water bath and the volume was adjusted to 2 mL with water. This resulted in a 5x concentration of the sample. Instead of filtering each sample as a final step, each one was centrifuged for several minutes to settle out any particulate matter. This was found to be more convenient and resulted in no loss of sample.

The leaf and root samples were analysed using a two-column HPLC system as illustrated in Figure 2-1. The only difference from the first experiment was that the first column was a 12 cm x 4.6 mm i.d. C_{18} column with 5 µm particle size packed by Darrell Veres at the Agriculture Canada Research Station at Vineland Station. The total number of theoretical plates offered by the two-column system was 3130.

The program used in the analysis of the leaf and root samples is summarized in Table 2-2. The mobile phase again was methanol and water. The purpose of each program segment was the same as that in the program used in the first experiment. The switch to the second column to analyse the oxime and oxamyl occurred 30 min into the program.

Some of the soil samples could be analysed for oxime and oxamyl on the first column alone. Others were clean enough to allow analysis of the oxime on the first column, but required the second column for the analysis of oxamyl. In the latter case an HPLC program with two program segments sufficed and is summarized in Table 2-3. During the first program segment, the oxime was analysed on the first column. During the second, anything eluting between oxime and oxamyl was allowed to go directly to the detector. Just before the elution of oxamyl occurred, the eluent flow was switched to the second column, where analysis of oxamyl took place.

On average, one combined oxime and oxamyl standard was run for every 2.7 samples.

Table 2-2

HPLC program used to analyse peach leaf and root samples
(Second Peach Plant Experiment)

Program Stage	1	2	3	4	Purge	Equilibration
Duration (min)	19	7	21	28	12	8
% methanol in H_2O	0.1	8	3	8	99.9	0.1
Curve	1	0	0	0		
Flow rate (mL/min)	2	2	2	2	3	2

Table 2-3

HPLC program used to analyse soil samples
(Second Peach Plant Experiment)

Program Stage	1	2	Purge	Equilibration
Duration (min)	16	45	8	8
% methanol in H_2O	0.1	8	99.9	0.1
Curve	1	0		
Flow rate (mL/min)	2	2	2	2

Corn Seed Experiment

In this experiment, 1,000 corn seeds were allowed to sit for 16 h in a 16,000 ppm solution of oxamyl (source, Vydate). Another 600 seeds were allowed to sit for the same period of time in distilled water. The former seeds will be referred to as "treated" and the latter as "untreated".

The treated and untreated seeds were planted in low flats containing sandy loam soil. Half of the flats contained soil which had been sterilized for about 1 h at 15 lbs. pressure; the other half contained natural, unsterilized soil. The resulting combinations were: untreated seeds in natural soil, untreated seeds in sterilized soil, treated seeds in natural soil and treated seeds in sterilized soil. Five flats of each combination were planted, giving a total of 20 flats. Each flat was planted with 6 rows of 9 seeds each, giving 54 seeds per flat, a total of 1,080 seeds planted.

The flats were placed in plastic bags closed with cotton batting plugs to prevent the contamination of the soil by air-born bacteria and fungal spores. They were misted with water which had been filtered through an 8 μm pore size Millipore filter to remove bacteria and fungal spores. Growth of the corn plants necessitated removal of the bags after 4 days from the natural soil flats and after 5 days from the sterilized soil flats. Use of filtered water for the sterilized soil flats was continued. The flats were watered as often as necessary.

At one-week intervals for the six weeks following planting one row of each flat was removed for analysis

giving 45 plants per sample. A sample of 45 treated, unplanted seeds was analysed to obtain the initial concentration of oxamyl in the treated seeds.

Sampling was performed as follows. The stem was cut off just above the ring of prop roots and the stem and leaf portions placed in a jar. A one-inch soil core was taken around each seed and the seed and root were removed by sifting. The root and below-ground stem portions were cut from the seeds and the seeds kept in a jar. Any seeds which had not germinated were not included in samples so as not to impart a positive error to the results. The root was sifted out of the remaining soil and kept with the root obtained from the soil core. The core soil was stored separately from the remaining soil. All jars were weighed before and after addition of samples.

The unplanted treated seeds were extracted four times with 70 mL of technical grade methanol each time. The seeds were allowed to sit in methanol for long periods of time, 9 - 17 h for each extraction. Each extraction was added to a separate 100 mL volumetric flask and analysed separately in order to determine the extraction efficiency. The 1-week and 2-week seed samples were extracted three times (for 12, 9.5 and 13 h) with 70 mL of methanol each time, the extractions being combined in a 250 mL volumetric flask.

As time went on, the planted seeds became softer and the seed coats began to loosen from the core of the seed, making extended extractions unnecessary. The extraction times for the remaining weeks are summarized in Table 2-4.

Table 2-4

Extraction times for Corn Seed Samples

Sample	Extraction					
	1	2	3	4	5	6
unplanted seeds	17h	9h	15h	10h		
1-week and 2-week*	12	9.5	13			
3-week tr. ster.	21	2	1	24		
rest of 3-week and 4-week*	21	2	1			
5-week tr. ster.	1	1	1	3	22.5	19.3
5-week tr. nat.	3	2.8	17.5	25		
6-week tr. ster.	4	3.5	15.7	27		
rest of 5-week and 6-week*	3.5	3.5	15			

* 3 extractions combined in 250 mL volumetric flask
tr. = treated seed
nat. = natural soil
ster. = sterilized soil

Table 2-5

HPLC Program for Corn Seed Analysis

Program Stage	1	2	3	4	Purge	Equilibration
Duration (min)	5	15	5	11	3	8
% methanol in H_2O	8	15	99.9	8	99.9	8
Curve	1	0	0	0		
Flow rate (mL/min)	2	2	2	2	2	2

Each extraction was performed with 70 mL of methanol. Unless otherwise indicated, the extractions were kept in separate 100 mL volumetric flasks in order to determine extraction efficiencies.

The unplanted, 1-week and 2-week seed extracts were prepared for HPLC analysis as in the previous experiment, with concentration factors according to need. The rest of the samples were cleaned up prior to HPLC analysis using small, disposable reverse phase columns known by the trade-name "SEP-PAK cartridges", supplied by Waters Associates. Ten millilitres of the methanol extract and 2 mL of water were evaporated down to 2 mL in a 55°C water bath under a stream of air. The aqueous residue was eluted through a conditioned SEP-PAK cartridge with two 1 mL water rinses of the sample vial. Two millilitres of a 67% water, 33% methanol mobile phase were then eluted through the SEP-PAK cartridge and collected in a graduated sample vial. About 0.7 mL water was added and the mixture was evaporated down to exactly 2 mL.

The unplanted, 1-week and 2-week seed samples were analysed on the first column of the HPLC system used in the previous experiment. The oxime was analysed using a mobile phase of 8% methanol, 92% water during the first program segment which lasted 9 min. The oxamyl was then analysed during a second program segment of 7.5 min using a mobile phase of 20% methanol, 80% water. This was followed by a 5 min purge with 99.9% methanol. The flow rate was 2 mL/min.

The remaining samples (3-week to 6-week) were analysed

using a two column system, the first column of which was a 25 cm x 6.2 mm i.d. DuPont Zorbax PSM 60 high performance size exclusion column protected by a 3 cm x 4.5 mm i.d. Brownlee C_{18} guard column. The second column was a 25 cm x 4.6 mm i.d. Brownlee C_{18} column packed with 10 µm particles. The DuPont size exclusion column plus guard provided about 4,000 theoretical plates, and the Brownlee C_{18} column offered 1,700.

The HPLC program used to analyse samples on the two-column system is summarized in Table 2-5. During the first program stage dimethylcyanoformamide (DMCF), a metabolite of oxamyl, was analysed on the first column, and the oxime fraction was collected on the second column by means of the switching valve. During the second program stage, oxamyl was analysed on the first column. The third program stage consisted of a methanol purge of the first column. Oxime was analysed on the second column during the fourth stage of the program. This was followed by a purge of the first and second columns, and equilibration at 8% methanol, 92% water.

Combined standards containing DMCF, the oxime and oxamyl were run on average once for every 1.6 samples.

Synthesis of DMCF

DMCF was synthesized by the reaction of sodium cyanide with dimethylcarbamoyl chloride (DMCC) using acetone as a solvent.

$$\underset{}{NaCN} + \underset{\text{DMCC}}{(CH_3)_2NC(=O)Cl} \xrightarrow[\text{Acetone}]{} \underset{\text{DMCF}}{(CH_3)_2NC(=O)CN} + NaCl$$

Acetone was dried by shaking with activated K_2CO_3, decanting into a round-bottom flask and distilling into the reaction flask. DMCC (21.9 g, 0.20 mole) was added to 10.0 g oven-dried NaCN (0.20 mole) in 200 mL of acetone. This was left on a stirring plate, tightly capped, for 3 days. The acetone was evaporated off and 100 mL of water was added to hydrolyse any unreacted DMCC. The water solution was filtered through number 1 Whatman filter paper to remove a small floating precipitate and then was extracted five times with 50 mL diethylether for 30 min each time. The extractions were carried out in an Erlenmyer flask in an ice bath with stirring. The ether layers were passed through a bed of Na_2SO_4 (anhydrous) in a filter funnel, and the ether was evaporated off. The final three extractions were kept separate from the first two. The product was characterized using mass spectroscopy by comparison with the mass spectrum of a small sample of the pure compound obtained from DuPont.

Toxicological Studies

The toxicology of DMCF, the oxime and oxamyl was studied in terms of the effect of various concentrations of the chemicals on the mobility of nematodes and the effect of various lengths of time of exposure to the chemicals on their ability to recover. The nematodes used for the study were Meloidogyne incognita larvae, freshly hatched within 12 h prior to use.

The effect of various concentrations of DMCF, the oxime and oxamyl was studied by hand-picking 10 larvae into 0.1 mL of an aqueous solution of the chemical in a hanging-drop

microscope slide. The nematodes were observed under the microscope for any change in their normal sine-wave pattern of motion. Twelve concentrations of oxamyl ranging from 8,000 ppm to 4 ppm were studied in this way. Each solution was half the concentration of the one previous to it. Similarly DMCF was studied at five concentrations ranging from 8,000 ppm down to 500 ppm. The effect of the oxime was studied at 8,000 ppm.

The effect of time of exposure of nematodes to solutions of DMCF and oxamyl on their ability to recover normal motion was studied using a 4,000 ppm solution of each compound. Into 0.1 mL distilled water in a BPI watchglass 110 M. incognita larvae were transferred and 0.1 mL 8,000 ppm oxamyl was added. This gave an oxamyl concentration of 4,000 ppm. At 10 min intervals 10 larvae were removed to 0.1 mL distilled water in a hanging-drop microscope slide and observed under the microscope for recovery of normal motion. Ten larvae were left in the 4,000 ppm oxamyl solution for the duration of the experiment.

This procedure was repeated with 50 larvae using DMCF. At 4, 8, 12 and 24 h after the addition of DMCF, 10 larvae were removed to 0.1 mL distilled water and observed under the microscope. Again, 10 larvae were left in the 4,000 ppm DMCF solution.

In the above experiments the nematodes were observed over long periods of time. In order to minimize evaporation of the solutions in which the nematodes were being observed, the microscope slides were kept in humidity chambers. These chambers consisted of covered petri dishes containing a layer of water and supports on which to rest the microscope slides.

RESULTS AND DISCUSSION

Mass Spectrometry and Nuclear Magnetic Resonance

The mass and nuclear magnetic resonance spectra of oxamyl were investigated with a view to future characterization of HPLC fractions. Before the DS-55 data system was operational, mass spectra of oxamyl were obtained at ionization potentials of 70 eV and 20 eV. At 70 eV no molecular ion peak at m/e 219 was observable. At 20 eV a small peak was visible at m/e 219, but this was of no greater intensity than other small peaks at higher m/e values. With the use of the data system the molecular ion peak was not distinguishable.

This is due to the fact that oxamyl is a highly thermally labile compound and will decompose not only in the mass spectrometer, but also in the gas chromatograph[46]. Reiser and Harvey[51] were able to observe a very weak molecular ion peak using an all-glass system and found that decomposition occurred on exposure to metal.

The mass spectrum of oxamyl at an ionization potential of 70 eV may be found in the appendix. According to Reiser and Harvey[51] the oxime peak at m/e 162 is due to loss of methylisocyanate. The identity of the peak at m/e 72 was shown by the above authors to be $(CH_3)_2NCO$ using high-resolution mass measurements.

The proton nuclear magnetic resonance (NMR) spectrum of oxamyl at ambient temperature in $CDCL_3$ solution is given in Figure 3-1. The singlet at 2.34 ppm represents protons on the SCH_3 group. The doublet at 2.90 ppm arises from the methyl protons in the $NHCH_3$ group, split into a doublet due to coupling

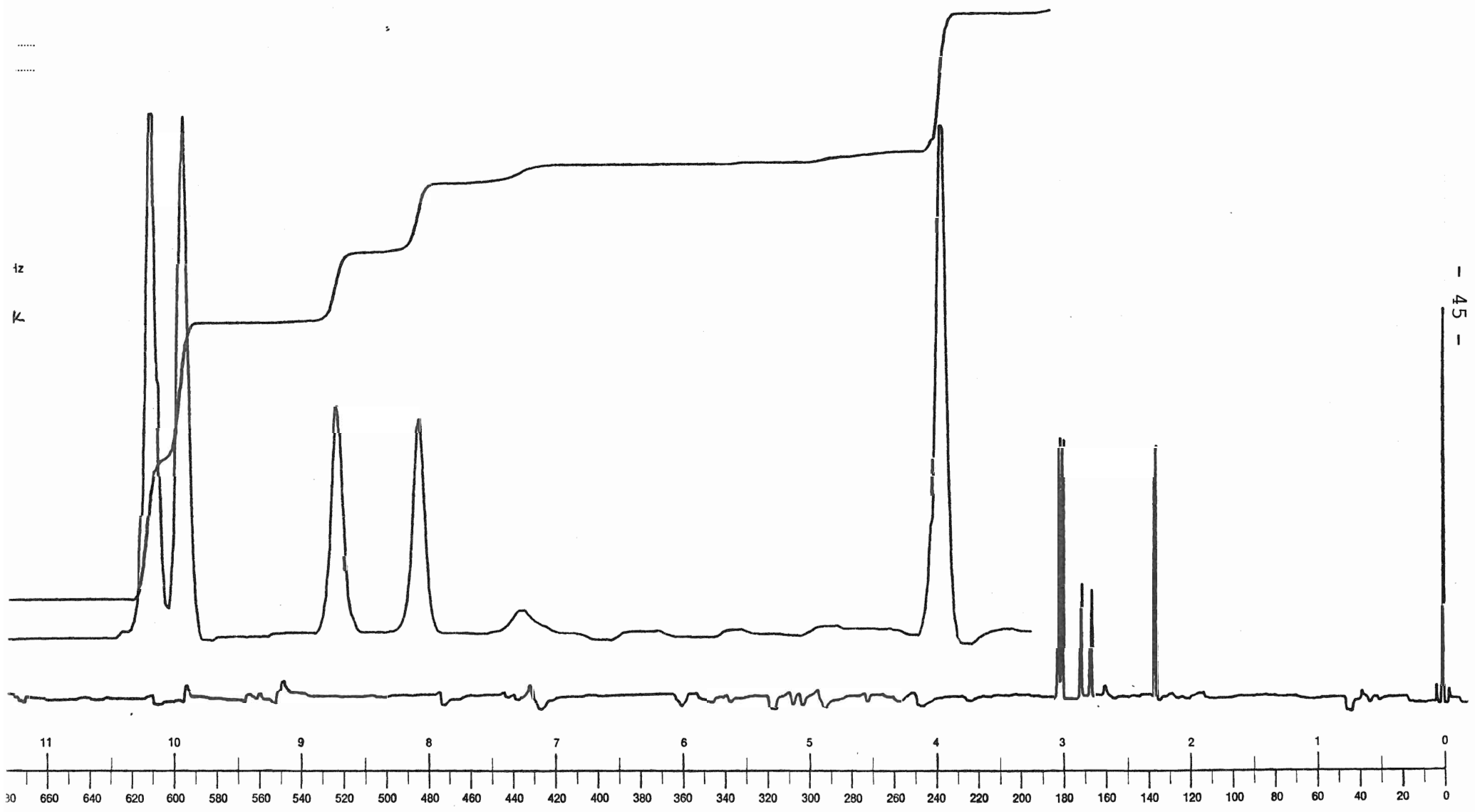
60 MHz ^{1}H Spectrum

OXAMYL

(D)

Spec. No. .....
No. of scans 16
SW 750 Hz/cm
Points 8 k / 4 k
PW 1.3 μ sec. Rep. 5.96 sec.

Figure 3-1 Proton NMR spectrum of oxamyl in $CDCl_3$ at ambient temperature



to the proton on the nitrogen. A weak quartet due to this proton was observable in the spectrum obtained at 273K (see appendix). The coupling constant $J_{H-N-C-H}$ was found to be 4.95 ± 0.36 Hz in the doublet and the quartet.

The pair of peaks at 3.07 and 3.10 ppm represent the two methyl groups in the $(CH_3)_2NCO$ group. The two peaks arise due to hindered rotation about the C-N bond. This bond exhibits partial double-bond character due to the contribution of resonance structure Ib to the ground state[52].

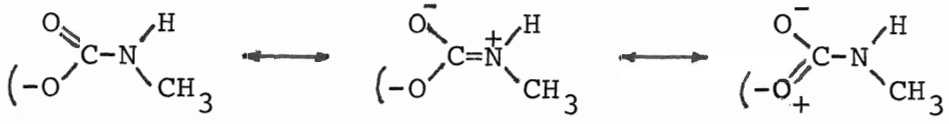
Ia Ib

Thus two separate peaks arise, since in the slow-exchange limit the two methyl groups are not chemically equivalent. According to LaPlanche and Rogers[53] the signal of the methyl protons cis to the carbonyl occurs to high field of the signal of those trans to the carbonyl. As the temperature is raised the energy barrier to rotation may be overcome, resulting in the coalescence of the two peaks into one as chemical equivalence is attained.

This was observed to happen in proton spectra of oxamyl obtained in DMSO-d_6 at several elevated temperatures (see appendix). As the temperature was increased, the two peaks were observed to broaden and move closer together. The coalescence temperature (the temperature at which the two peaks become one) was found to be 366K. As the temperature was increased further, the single peak was observed to sharpen and attain the same peak width as the other peaks in the spec-

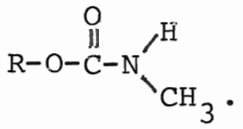
trum. Some degradation appears to have occurred at 393K as evidenced by the appearance of extra peaks at 2.19, 2.41, 2.57 and 2.96 ppm.

In an attempt to observe whether hindered rotation would occur in the carbamate end of the oxamyl molecule, proton spectra were obtained in $CDCl_3$ down to a temperature of 233K. No hindered rotation was evident although the quality of the spectra decreased as the temperature was lowered due to broadening of all the peaks. It is apparent therefore that the energy barrier to rotation in the carbamate group of oxamyl is much lower than that in the amide group. This is reasonable since the ground state of the carbamate group consists of contributions from three resonance structures (II a-c)[54]. This reduces the double-bond character of the C-N bond.



IIa IIb IIc

These results correlate well with those of Keith and Alford[55] who studied the proton NMR spectra of 16 carbamate pesticides with the general structure



Hindered rotation was not observed at ambient temperature in 15 of these compounds. The pesticide in which hindered rotation was observed at ambient temperature contained two t-butyl groups each only three bonds away from the carbamate

group. These bulky t-butyl groups apparently provide a steric hindrance to rotation. No hindered rotation due to electronic effects was observed. They found $J_{H-N-C-H}$ to be 5 Hz for all 16 pesticides, which compares closely with that obtained for oxamyl.

Preliminary Studies

In field studies involving cherry, apple, pear and peach trees it has been found that foliar applications of oxamyl result in reduced populations of nematodes in the roots.[56] In another study[57] involving apple, apricot, cherry, peach, pear and plum trees similar results were obtained. Foliar application of oxamyl was found to give protection from nematode attack that was at least as good as or better than that provided by soil applications of carbofuran and aldicarb (also carbamates). Damage to young peach replants by nematode attack has been shown to be of considerable importance in the failure of peach replants in Ontario[58]. On the basis of the demonstrated effectiveness of foliar applications of oxamyl in the protection of young peach plants from nematode attack it was decided to use young peach plants in these experiments.

The UV detection wavelength for HPLC analysis was chosen based on the UV spectrum of oxamyl (Figure 3-2) which exhibits a local maximum at 240 nm. In order to maximize the sensitivity of the method, this wavelength was selected for the determination of oxamyl. At lower wavelengths absorption due to the mobile phase solvent methanol becomes significant.

Since oxamyl is moderately soluble in water, it was possible to use reverse phase HPLC. In this mode a non-

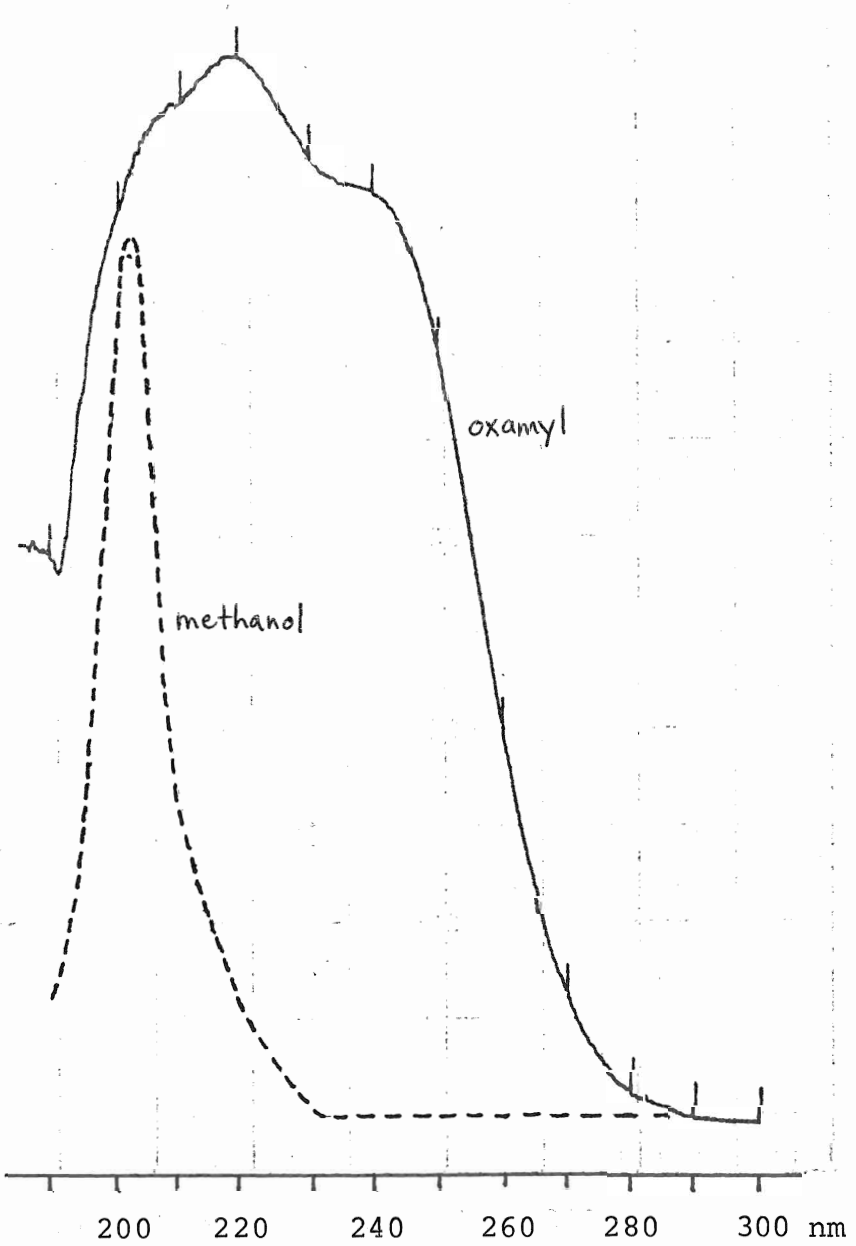


Figure 3-2 UV spectra of oxamyl (approx. 270 ppm in H_2O) and methanol (neat)

polar stationary phase is used with a polar mobile phase. Reverse phase is more convenient to use than normal phase since it is easier to equilibrate reverse phase columns and to maintain a constant level of activity of the non-polar stationary phase, resulting in easily reproducible retention times. Reverse phase is also cheaper and often safer to use than normal phase since water is a major component of the mobile phase, and it is not necessary to use expensive and often toxic non-polar organic solvents.

It was found that it was possible to analyse for oxamyl on a C_{18} column using a mobile phase of 10% methanol and 90% water and a flow rate of 4 mL/min. However, it was not possible to analyse for the oxime in leaf extracts due to interference from leaf coextractives, even though several mobile phases and flow rates were attempted. Two cleanup procedures were tried in an attempt to reduce interference from leaf coextractives. Both techniques involved the evaporation of a methanol leaf extract to an aqueous residue. Water was added and this aqueous layer was washed with diethylether in the first method and chloroform in the second. In neither case did washing with a non-polar organic solvent improve the analysis of the oxime or oxamyl.

Good reproducibility of the injection technique was demonstrated when four consecutive injections of 20 ppm oxime gave peak heights of 164.8 mm, 165.0 mm, 165.5 mm and 167.5 mm. This gives an average of 165.7 mm with a standard deviation of only 1.2 mm.

The minimum detectable concentration of a compound is defined as that concentration which gives a peak the height

of which is twice the average noise level of the chromatogram[59]. The sensitivity of this method at 0.01 absorbance units full scale (AUFS) allowed the detection of 0.4 ppm oxamyl (peak height 12 mm) with an average noise level of 6 mm. Since the volume of injection was 175 µL this gives a minimum detectable quantity of 0.07 µg oxamyl. When the baseline was smooth, it was possible to detect concentrations lower than the defined minimum detectable concentration, but these peaks were subject to a greater degree of error in peak height determination than were higher peaks. A calibration curve for oxamyl was found to be linear through the origin in the range of concentrations encountered in the leaf samples (up to 20 ppm).

In the preliminary study the efficiency of the extraction procedures was determined. The results of extraction efficiency studies performed on the 2-day, 16-day, 21-day and 26-day leaves are given in Table 3-1. It may be seen from the table that the third extraction of the 2-day leaves recovered only 0.8% of the total oxamyl extracted in three extractions. Use of a smaller volume of methanol (40 mL) on the 16-day and 21-day leaves in an attempt to decrease the total volume of extracting solvent was not as efficient, with 2.3% and 5.3% respectively of the total oxamyl being extracted in the third extraction. It was decided that three 70 mL extractions, combined in a total volume of 250 mL methanol, were necessary for efficient extraction of oxamyl from leaves. A fourth 70 mL extraction of the 26-day leaves was performed in order to check the completeness of extraction. It was found that the first three

Table 3-1

Extraction Efficiency of Oxamyl from Peach Leaves

(Preliminary Study)

2-Day Leaves

extraction (70 mL each)	1	2	3	total
µg oxamyl extracted	1300	100	10	1410
% of total	92.2	7.1	0.8	

16-Day Leaves

extraction (40 mL each)	1,2*	3	total
µg oxamyl extracted	860	20	880
% of total	97.7	2.3	

21-Day Leaves

extraction (40 mL each)	1,2*	3	total
µg oxamyl extracted	180	10	190
% of total	94.7	5.3	

26-Day Leaves

extraction (70 mL each)	1,2,3*	4	total
µg oxamyl extracted	210	10	220
% of total	95.5	4.5	

* extractions were combined

extractions recovered 95.5% of the total oxamyl extracted in four extractions. However, the accuracy of the oxamyl determination in the fourth extraction is not certain, since it was below the defined minimum detectable concentration (4 mm peak height). It was judged therefore that three 70 mL extractions achieve an adequate extraction of oxamyl. The lower extraction efficiency of the 26-day plant as compared with that of the 2-day plant is due to absorption of oxamyl into the leaf tissue, making it more difficult to extract.

Use of this type of extraction efficiency test was felt to be more realistic than the spiking of a leaf sample with a known amount of oxamyl followed immediately by extraction. Although it gives better recovery values, the latter method does not allow penetration of the leaf tissue by the chemical and is not an adequate test of extraction efficiency for leaves which have been penetrated by the chemical.

Since analysis of the oxime in leaf extracts was not possible on the reverse phase column due to interference from leaf coextractives, it was necessary to develop a better analytical method. A normal phase method was therefore tried using a mobile phase of water-saturated chloroform mixed with 1 - 2% of either methanol or 95% ethanol. It was necessary to saturate the chloroform with water in order to maintain a constant level of activity of the silica gel stationary phase. A dry mobile phase will gradually dry out the stationary phase, resulting in a steadily changing retention time for the compound being determined.

Since the mobile phase consisted of 98 - 99% water-saturated chloroform, it was necessary to transfer the extracts from methanol to chloroform solution. A problem was encountered in this transfer in that it was not possible to quantitatively transfer the oxime. Several methods were attempted in order to effect this transfer. Evaporation of a methanol extract spiked with oxime to its aqueous residue and redissolving in chloroform resulted in 60% recovery of the oxime. Evaporation of the spiked methanol extract followed by three 7-mL chloroform extractions of the aqueous residue resulted in recovery of 62% of the oxime. Even when the aqueous residue was spiked with oxime following the evaporation of methanol and then diluted with chloroform, the resulting oxime peak was only 77 - 84% of the height of a corresponding standard peak. To avoid evaporation to dryness, cyclohexanol was added to the spiked methanol extract before evaporation. The cyclohexanol residue was then redissolved in chloroform and dried with Na_2SO_4. This procedure gave between 82% and 100% recovery of oxime.

By this time, however, it was found that the reproducibility of the normal phase HPLC method was very poor. Four consecutive runs of the same 2 ppm oxime standard in chloroform solution gave peaks ranging between 49 and 60 mm in height. The reason for this lack of reproducibility was not clear. It was also discovered that some components of leaf coextractives were unstable in chloroform and the appearance of the chromatogram changed with time. A further problem was encountered when it was found to be impossible to transfer a root extract from methanol to

chloroform due to the formation of a thick precipitate.

At this point it was decided that normal phase HPLC was not a convenient technique for the analysis of oxamyl and its oxime in leaf and root extracts. It was decided to attempt a new technique, that of preliminary cleanup of the extract using TLC for reverse phase HPLC analysis.

This technique was developed to the point that good TLC separation between oxamyl and the oxime (R_f 0.26 and 0.40 respectively) was achieved using a mobile phase of 30% acetone, 70% ethyl acetate. When the TLC cleanup procedure previously described was applied to a control leaf sample spiked with oxamyl and oxime, it was found that when the sample was run on the HPLC not only was the recovery of oxamyl poor ($\sim$23%), but there was still serious interference in the region of the oxime peak.

At this point the realization came that it would not be possible to effect a more efficient preliminary cleanup by any other means than HPLC itself. Therefore, rather than try to perfect the TLC cleanup procedure, which was very tedious and time consuming and provided many opportunities for loss of sample, it was decided to attempt a two-column HPLC method. In this method preliminary separation of oxamyl and the oxime from coextractives would occur on the first column, and analysis of the oxime and oxamyl fractions would occur on the second column.

The method which was developed has been described in the experimental section dealing with the first peach plant experiment. In this method the first column performs a cleanup of the sample, eliminating plant coextractives

which elute before and after the oxime, and before oxamyl. Figure 3-3(a) illustrates a typical chromatogram of an oxime standard run on the first column alone. It may be observed that the oxime eluted from the column between 20.0 and 24.5 min after commencement of the run. Figure 3-3(b) illustrates the chromatogram of a 2.7 ppm oxime, 13 ppm oxamyl standard run on both columns using the HPLC program summarized in Table 2-1. The oxime was collected onto the top of the second column, by use of the switching valve, between 19.8 and 24.6 minutes after commencement of the run. Therefore anything eluting before and after the oxime was allowed to run directly to the detector, and only the oxime and a small volume of other eluting material was collected on the second column.

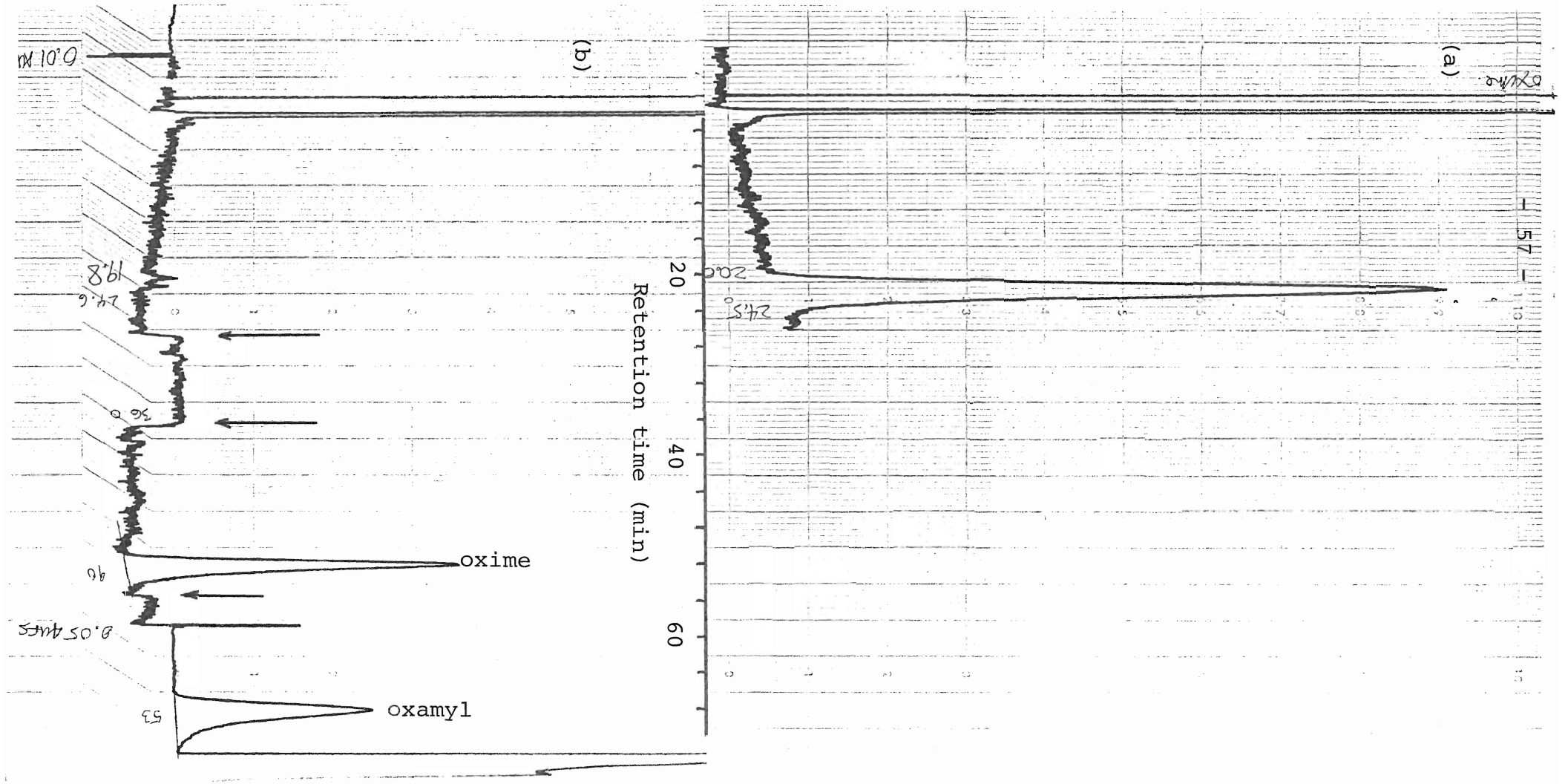
After the oxime was collected the shift in the base-line observed in Figure 3-3(b) indicates that the mobile phase had changed in composition from 0.1% to 10% methanol. This allowed oxamyl to elute more quickly. There was a delay of 4 min between the change in composition of the mobile phase being delivered by the pumps and the arrival of the new mobile phase at the detector. Therefore, although the pumps began delivering 10% methanol at minute 22 of the program, it was not observed at the detector until minute 26.

Just before oxamyl eluted to the detector, the switching valve was directed again to the second column. The first step after switching to the second column was the analysis of the oxime using a mobile phase of 3% methanol, 97% water. It was therefore necessary that this

Figure 3-3 (a) Chromatogram of oxime standard on first column alone

(b) Chromatogram of 2.7 ppm oxime, 13 ppm oxamyl standard on first and second columns

(change in mobile phase indicated with ↓)



mobile phase be eluting from the first column when the switch was made. Therefore, 4 min before the expected elution of oxamyl, the mobile phase was changed to 3% methanol at 32.0 min and the switching valve was directed to the second column at 36.0 min.

After the analysis of oxime on the second column the mobile phase was changed again to 10% methanol for the analysis of oxamyl. This was followed by a 5 min purge of both columns with 70% methanol to bring off any strongly adsorbed leaf coextractives, and an 8 min equilibration period with the starting mobile phase of 0.1% methanol, 99.9% water.

This two-column system provides an efficient preliminary cleanup of the sample with no possibility of loss of oxamyl or the oxime. The use of two columns results in long retention times and therefore some sacrifice of sensitivity. However, it is a convenient method since it eliminates pre-analysis cleanup and allows the simultaneous determination of oxamyl and its oxime.

First Peach Plant Experiment

The purpose of this experiment was to monitor the degradation of oxamyl and the formation of the oxime in peach leaves, and to look for movement of oxamyl or oxime to the roots or soil by using the HPLC analytical method developed in the preliminary study. In order to accomplish this seven peach plants were treated with oxamyl by dipping in a 1,000 ppm oxamyl solution. The degradation of oxamyl and formation of the oxime were monitored by extracting the plants at intervals, and analysing for the oxime and

oxamyl using the two-column HPLC method.

The peach plants used in this experiment were older plants which had been cut back and allowed to grow new stems. It was feared that since the plants were older, difficulties might be encountered in extracting and analysing the root, which was larger than that of the younger plants. However, since no young peach seedlings were available at the time of this study, it was necessary to use the older plants.

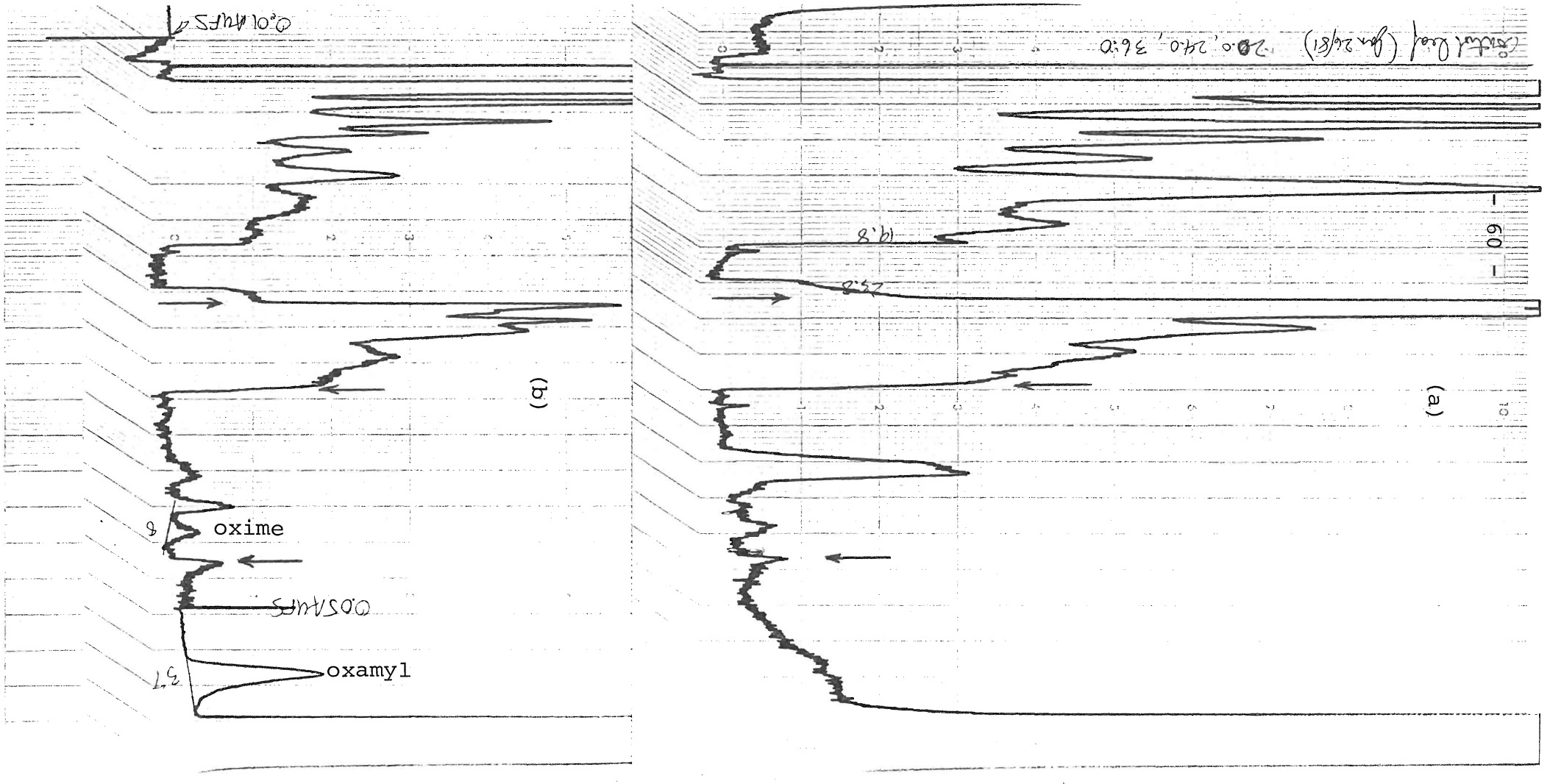
Another difference between this experiment and the preliminary study is that it was decided to stabilize the methanol plant extracts with 0.5% H_3PO_4, in order to prevent the decomposition of oxamyl. The standards also were stabilized with 0.5% H_3PO_4 based on the observation of Harvey and Han[28] that oxamyl was stable in slightly acidic solution.

The HPLC method was found to be very effective in the analysis of leaf samples for oxamyl and its oxime. Figure 3-4 illustrates the chromatograms of the control leaf and 12-day leaf extracts. It may be observed that there is a small degree of interference in the oxime region of the chromatogram of the control leaf. However, the 12-day leaf chromatogram indicates that this did not interfere with the oxime determination. There was no interference in the oxamyl region of either chromatogram. Because of the difference in concentration between oxamyl and the oxime, it was necessary to reduce the sensitivity of the detector following the determination of the oxime. The concentrations found in the 12-day leaf extract were 0.10 µg/mL oxime and

Figure 3-4 (a) Chromatogram of control peach leaf sample
(b) Chromatogram of 12-day peach leaf sample

(First Peach Plant Experiment)

(change in mobile phase indicated with ↓)



3.6 µg/mL oxamyl. These translate into on-leaf concentrations of 5 µg oxime/g leaf and 200 µg oxamyl/g leaf.

The sensitivity of the method at 0.01 AUFS allowed detection of 0.29 ppm oxime and 0.42 ppm oxamyl, giving minimum detectable quantities of 0.051 µg oxime and 0.074 µg oxamyl. A 2.5x concentration of the extracts therefore allowed determination of 0.12 µg/mL oxime and 0.17 µg/mL oxamyl in the methanol extracts.

An indication of the reproducibility of the HPLC method was provided by two consecutive runs of the 21-day sample which gave oxamyl peak heights of 62 mm and 68 mm respectively. This gives an average peak height of 65 mm with a coefficient of variation[60] of 6.5%. The degree of reproducibility depends to a certain extent on the height of the peaks obtained, and decreases as the height of the peak decreases. Therefore, since the concentrations of oxime were much lower than the concentrations of oxamyl, the reproducibility of the method for oxamyl was better than that for the oxime.

Quantitative transfer of the oxime and oxamyl from methanol to water, by the method described in the experimental section, was confirmed using oxime and oxamyl standards. This transfer of leaf extracts from methanol solution to water before HPLC analysis was necessary since peak shape is negatively affected when the sample solvent does not closely match the mobile phase compostion. It has been found that for a water/methanol mobile phase the height of a sample peak is decreased if the percentage of methanol in the sample solvent exceeds the percentage

of methanol in the mobile phase[61].

The results of the HPLC analyses of the peach leaf extracts are summarized in Table 3-2 and illustrated graphically in Figure 3-5. It may be observed from Figure 3-5 that the concentration of oxamyl in the leaf samples decreased steadily until the sixteenth day, after which it remained relatively constant, though still decreasing slightly. Oxime, on the other hand, exhibits an initial increase in concentration during the period of rapid degradation of oxamyl, followed by a steady decrease in concentration with the exception of the 26-day leaf sample, which exhibited a relatively high oxime concentration. It should be noted that oxime was not detected in the 30-day sample and the concentrations in the 12-day, 16-day, and 21-day samples were below the defined minimum detectable concentration.

The degradation of oxamyl may be monitored quite easily since it is simply being decomposed and no new oxamyl is being added to the system. The fate of the oxime is more difficult to interpret, since not only is it being formed through the decomposition of oxamyl, it is itself being dissipated through degradation, conjugation and mechanical loss.

When an attempt was made to transfer a root extract from methanol solution to water, it was found that a thick brown precipitate formed, similar to that which appeared in chloroform as mentioned previously. A hexane wash was performed on the aqueous residue of an evaporated spiked root extract, but the precipitate was not soluble in hexane

Table 3-2

Oxamyl and Oxime Concentrations on Peach Leaves
(First Peach Plant Experiment)

Sampling Day	Leaf Weight	Oxamyl Conc.		Oxime Conc.	
		In Extract	On Leaves	In Extract	On Leaves
2	2.6277 g	7.6 µg/mL	720 µg/g	0.12 µg/mL	11 µg/g
7	4.3375	5.6	320	0.35	20
12	4.5004	3.6	200	0.10	5
16	4.7653	1.6	84	0.06	3
21	4.4772	1.4	78	0.04	2
26	4.0654	1.3	79	0.19	12
30	4.7724	1.4	76	ND	

ND - not detected

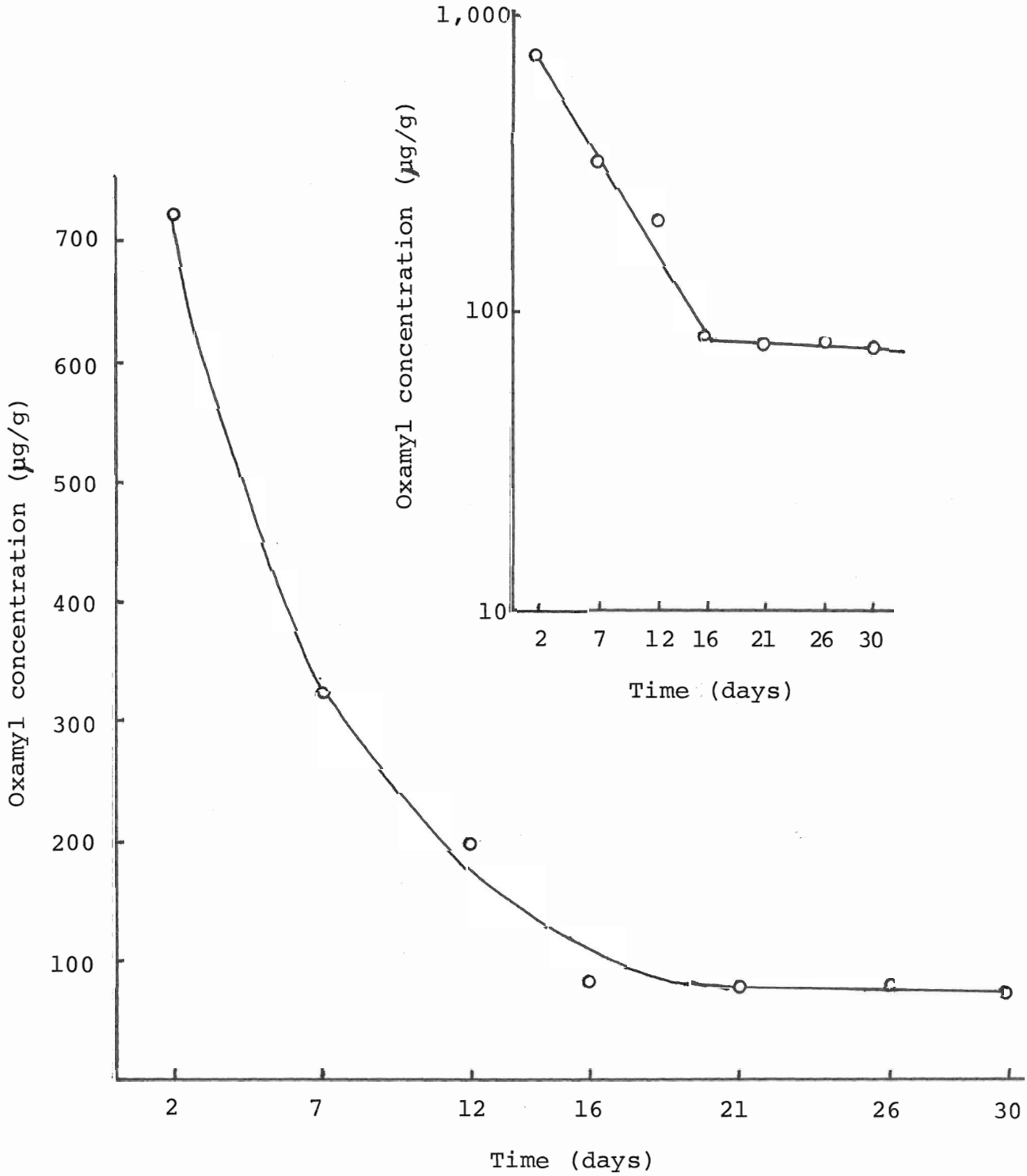


Figure 3-5 Concentration of oxamyl in treated peach leaves vs. time (first peach plant experiment)

and the recovery of oxime after the cleanup was only 76%. Also there was no noticeable improvement in the chromatogram after cleanup. It was felt that the problem of precipitate formation may have resulted from the age and weight of the root. Another problem stemming from the amount of root present was that it was difficult to achieve good separation between the root and soil before extraction.

Since young peach seedlings were shortly to become available it was decided to repeat the experiment rather than to try to overcome the problems of transferring extracts to water.

Second Peach Plant Experiment

The first peach plant experiment was repeated on a broader scale. The scope of the experiment was expanded to include not only a search for movement of oxamyl or its oxime from leaves to roots or soil, but also from treated middle leaves to untreated upper or lower leaves. The latter type of movement was observed by Peterson et al.[31] and Bunt and Noordink[34]. In order to investigate this type of movement the leaves of the middle third of peach seedling stems were dipped in oxamyl solution. Since the surface area treated with oxamyl was reduced, the concentration of the oxamyl solution was increased by a factor of about 2.5.

Plants were again extracted at intervals in order to monitor the degradation of oxamyl and look for movement of oxamyl or the oxime from treated leaves to other plant parts. In order to reduce the size of the soil sample to a more manageable level, smaller (8 cm) clay pots were used in this experiment. Since the efficiency of extraction

of pesticides from sandy loam soils has been shown to be improved by the presence of soil moisture[62], the soil samples were not dried before extraction. In order to obtain the dry weight of the soil, an aliquot of the soil sample was taken and weighed before and after air drying. In this way the water content of the soil was determined and the dry weight of the extracted soil sample could be calculated.

Phosphoric acid was not used to stabilize the methanol extracts of this experiment. It was noticed during the first peach plant experiment that the presence of H_3PO_4 in the methanol used for extraction imparted a dull gray-green colour to the leaf extract. When methanol without H_3PO_4 was used to extract a leaf sample, the extract had a bright lime-green colour. It was apparent therefore that H_3PO_4 caused a change in the extract itself. In the preliminary study it had been possible to analyse for oxamyl in leaf extracts not stabilized with H_3PO_4 using one reverse phase column, but in the first peach plant experiment two columns were required for oxamyl analysis. The impression was given that the presence of H_3PO_4 in the extracts may complicate the HPLC analysis. It was therefore decided to extract the plant samples with methanol not stabilized with H_3PO_4 and to analyse the samples as soon as possible after extraction.

The smaller leaf sample size ($\sim$1 g) permitted the use of 30 mL of methanol for each extraction. The efficiency of the extraction procedure used was determined by analysing separately each extraction of each of two leaf samples

extracted on the first day after treatment. The results are summarized in Table 3-3. It may be observed that in both cases no oxime or oxamyl were extracted in the fourth extraction. As a result it was concluded that three 30-mL extractions constitute an efficient extraction procedure for oxamyl and oxime in peach leaves.

A two-column reverse phase HPLC method substantially the same as that used in the first peach plant experiment was used to analyse for oxamyl and oxime in leaf and root extracts. Figure 3-6 illustrates the chromatograms of an 8-day leaf sample from the top third of the plant (no oxime or oxamyl present), a standard solution containing 1.6 ppm oxime and 80 ppm oxamyl, and an 8-day leaf sample from the centre third of the same plant. It may be seen from the top-leaf chromatogram that there is no interference from leaf coextractives in the oxime or oxamyl regions of the chromatogram. The chromatogram of the centre-leaf sample indicates that the oxime and oxamyl were readily determined.

The sensitivity of the two-column method at 0.005 AUFS allowed the determination of 0.20 ppm oxime and 0.33 ppm oxamyl. These concentrations gave peak heights of 12 mm each with an average noise level of 6 mm. This translates to minimum detectable quantities of 0.035 µg oxime and 0.058 µg oxamyl.

Comparison of oxime and oxamyl peak heights in duplicate runs of a 1.6 ppm oxime, 80 ppm oxamyl standard and three treated leaf samples gave average coefficients of variation of 2.4% for the standards and 5.9% for the treated leaf samples.

Table 3-3

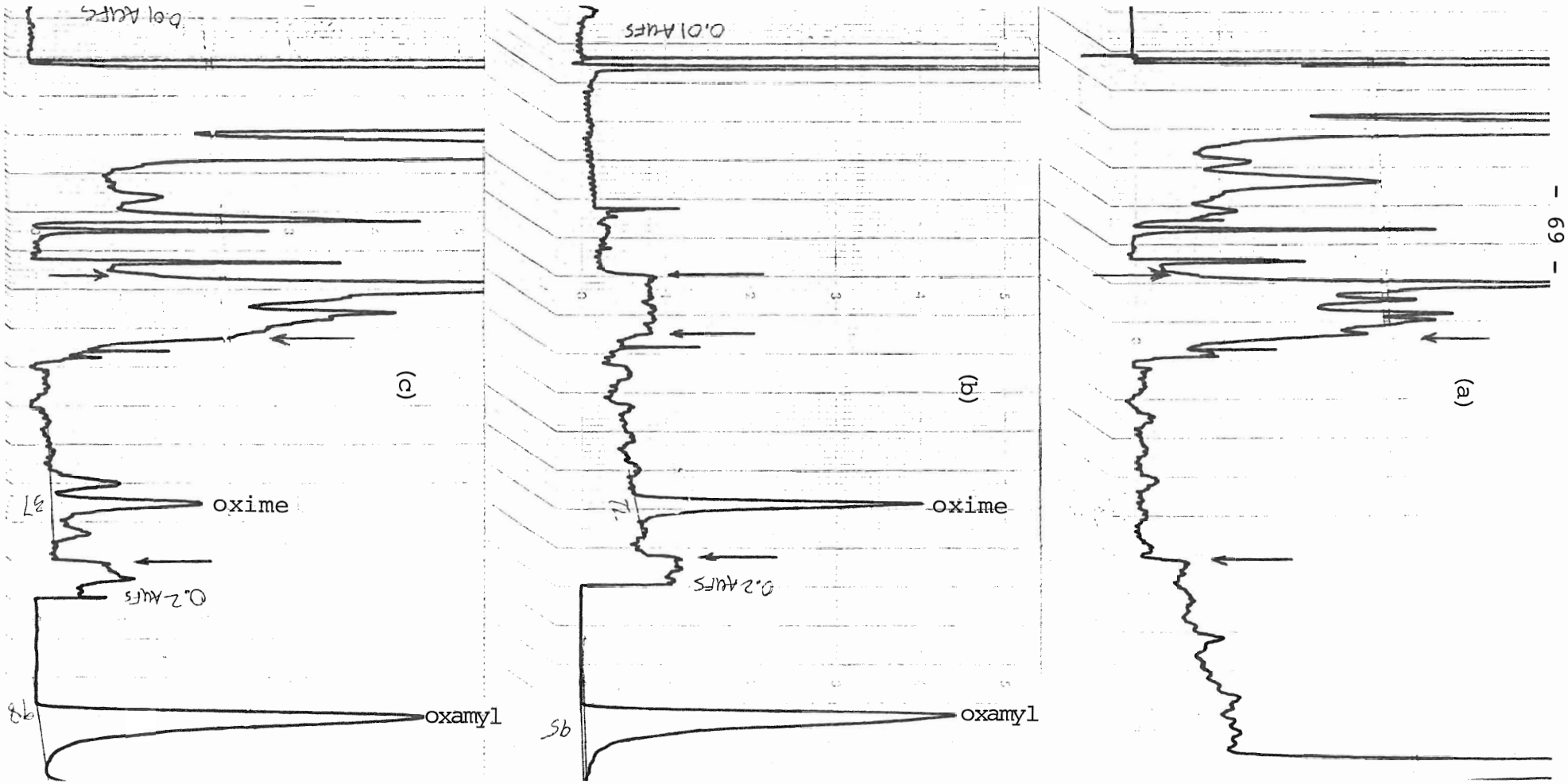
Extraction Efficiency of Oxamyl and Oxime from Peach Leaves
(Second Peach Plant Experiment)

	Replicate 1			Replicate 2		
Extraction	µg oxime	µg oxamyl	oxamyl (as % total)	µg oxime	µg oxamyl	oxamyl (as % total)
1	240	16,400	93.2%	300	9,410	90.1%
2	-	1,100	6.3%	14	930	8.9%
3	ND	93	0.5%	-	100	1.0%
4	ND	ND		ND	ND	
Total Extracted		17,593			10,440	

- indicates value unobtainable due to interference from coextractives
ND - not detected

Figure 3-6 (a) Chromatogram of 8-day top-leaf sample
(b) Chromatogram of 1.6 ppm oxime, 80 ppm oxamyl standard
(c) Chromatogram of 8-day centre-leaf sample

(Second Peach Plant Experiment)
(change in mobile phase indicated with ↓)



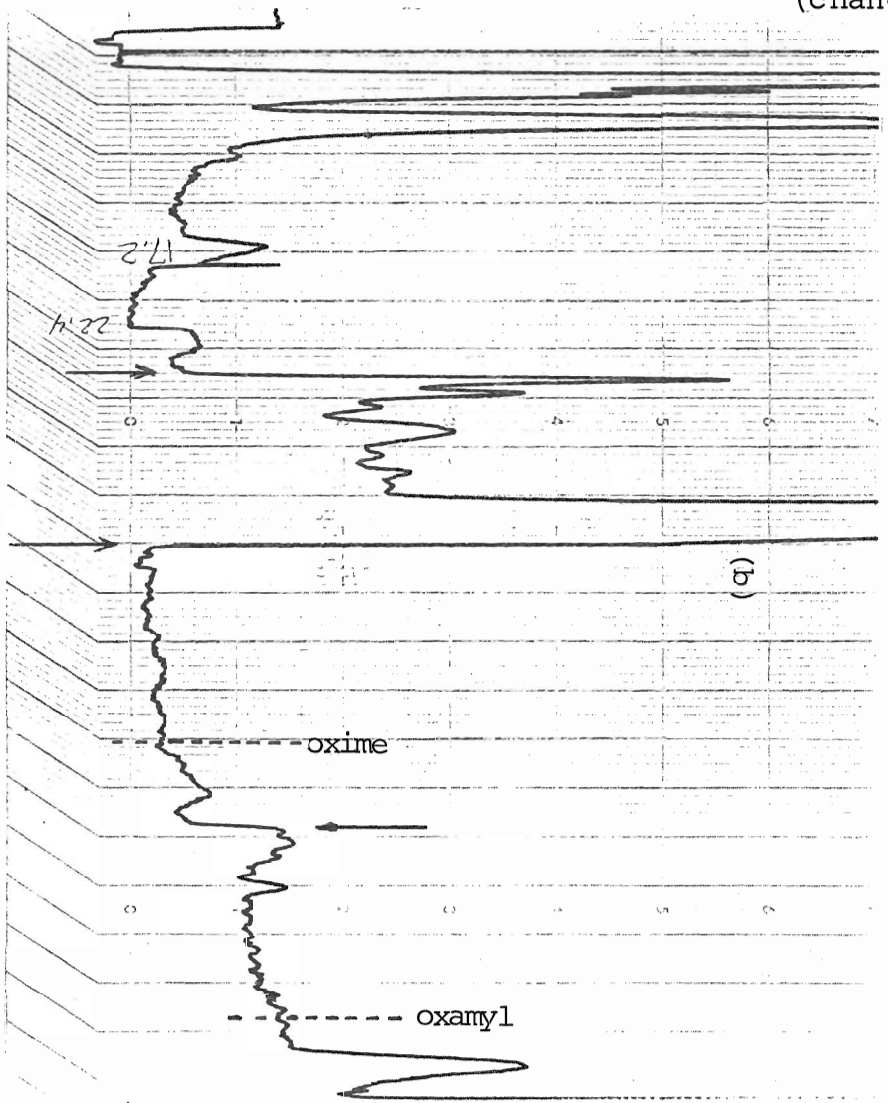
The soil samples were clean enough to allow analysis of oxime on the first column alone. This allowed an increased sensitivity of oxime determination down to 0.11 ppm or a minimum detectable quantity of 0.019 µg oxime. Figure 3-7 illustrates chromatograms of the control soil and control root samples with oxime and oxamyl regions indicated. It is evident that in neither case is there any interference from coextractives.

The results of the analyses of plant parts are tabulated in Table 3-4. The weights of individual plant part samples may be found in the appendix. Two plants were extracted on each sampling day and the results of each replicate are included in the table and on the graph. The top and bottom leaf samples were not analysed until the eighth day due to time constraints in the early stages of the experiment.

It may be said that, in general, the two-column system was effective for the analysis of leaf, root and soil samples for oxamyl and its oxime. A dash in the table indicates that a concentration value was unobtainable due to interference from coextractives in the chromatogram. It may be noticed, especially in the case of the treated leaf samples, that the occurrence of these dashes predominates in the later period of the experiment when there was interference in the oxime region of the chromatograms. It was felt that this may be due to deterioration in the efficiency of the first column due to contamination by strongly adsorbed leaf coextractives. The deterioration would result in a decreased number of theoretical plates

Figure 3-7 (a) Chromatogram of control soil sample
(b) Chromatogram of control root sample

(Second Peach Plant Experiment)
(change in mobile phase indicated with ↓)



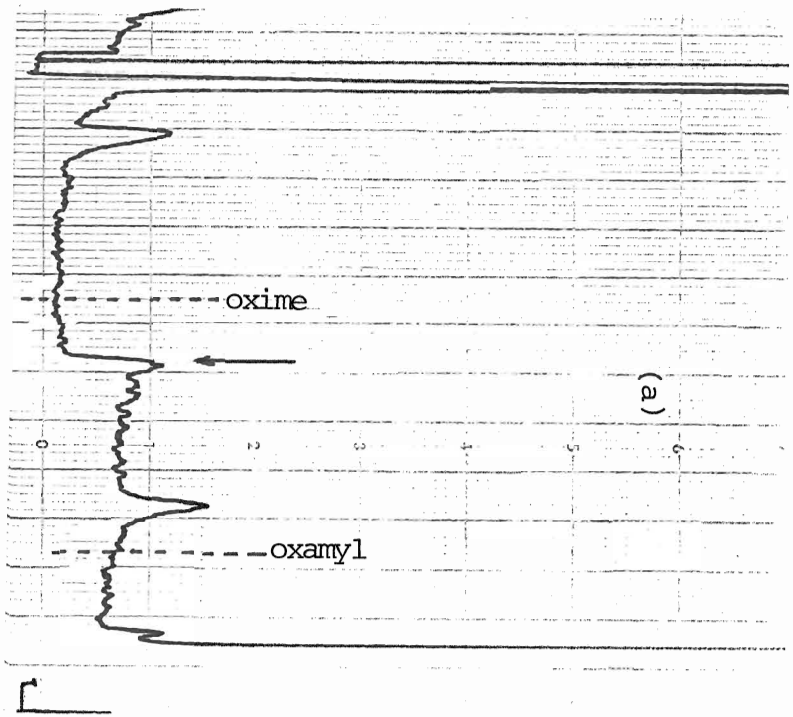


Table 3-4

Oxime and Oxamyl Concentrations in Peach Plant Parts
(Second Peach Plant Experiment)

Sampling Day	Replicate	Oxime, Oxamyl Concentrations (µg/g)					
		Leaf Samples				Root	Soil
		Centre		Top	Bottom		
0	1	78, 2410	2140*				
	2	<21, 1860					
1	1	26, 2180	1600*			ND, ND	ND, ND
	2	27, 1010					ND, ND
4	1	59, 1660	1340*			ND, ND	ND, ≤0.06
	2	<11, 1020				-, ND	ND, ND
8	1	47, 1040	1220*	ND, ND	≤5, ND	ND, -	ND, ND
	2	≤19, 1400		ND, ND	ND, ND	ND, -	ND, ND
14	1	-, 710	690*	-, ND	≤2, 21	ND, ND	ND, ND
	2	-, 680		ND, ND	-, 22	ND, ND	ND, ≤0.06
21	1	-, 501	856*	-, ND	-, ND	ND, -	ND, ND
	2	-, 1210			ND, ND	ND, -	ND, ND
30	1	-, 1310	1270*	ND, ND	ND, ND	ND, ND	ND, ND
	2	-, 1230		-, ND	ND, ND	ND, ND	ND, 0.2

* average oxamyl concentration of two replicates
ND - not detected

and decreased resolution of oxime from coextractives. This in turn would result in an increased volume of coextractives being admitted to the second column, where they would interfere with oxime determination. Toward the end of the experiment it was, in fact, found that the number of theoretical plates provided by the 3 cm guard column and 12 cm C_{18} column had decreased from the original 1560 plates to 1156 plates, a 26% reduction in column efficiency.

It is evident from Table 3-4 that neither oxamyl nor the oxime was conclusively found in either root or soil samples. Of the leaf samples taken from the bottom third of the stem oxamyl was found only in the 14-day samples. Oxamyl and the oxime were not detected in any leaf samples taken from the top third of the plant.

These results pose a problem for interpretation. As indicated in the introduction, translocation in the plant occurs in each of two systems, the apoplast (including the xylem) and symplast (including the phloem). The system involved in exporting materials from leaves is the phloem. Phloem transport is directed from regions of supply ("sources") including photosynthesizing leaves, to regions of demand ("sinks")[63]. The two main sinks in a young herbaceous plant are the shoot apex and the root system. Young leaves (less than 50% full size) also import material from the phloem. Mature leaves, however, are purely sources and do not import from the phloem[64]. It would be possible, therefore, that a leaf treated with a systemic pesticide would export the pesticide in the phloem to immature leaves, to the shoot apex and to the root. In

order for the pesticide to reach mature, untreated leaves it is necessary that it move from the phloem to the xylem. In general this means that the pesticide is translocated in the phloem to the root, where transfer into the xylem may occur, and then translocated in the xylem to the foliage[65].

In this experiment the leaves on the bottom third of the stem were mature leaves, and the leaves of the top third of the plant may have included both mature and young leaves. The problem posed by the experimental results is as follows. No oxamyl or oxime were detected in the top leaf samples to which, conceivably, translocation could have occurred directly from the treated leaves in the phloem. Oxamyl was detected in the bottom leaves of both plants sampled on the fourteenth day. If this oxamyl was present due to internal translocation, it is probable that the source would have been from the root through the xylem. However, no oxamyl was detected on any day, including the fourteenth, in the root extracts. The problem is complicated further by the fact that once transfer to the xylem occurs, translocation in the apoplast should occur to all regions of the plant, yet no oxamyl was detected in the top leaf samples on the fourteenth day. Therefore the reason for the presence of oxamyl in the 14-day bottom leaf samples is not clear. It is possible that contamination may have occurred during extraction or preparation of the 14-day bottom leaf samples for analysis.

It may be observed from Table 3-4 that the concentration of oxamyl on the treated leaves decreased steadily

for the first two weeks. This is in agreement with the results of the previous experiment. After this point however, the concentration appears to increase. Since there was no source of new oxamyl present in the experiment, this effect must be due to sampling problems. It is evident from Table 3-4 that there was a very high degree of variation between individual samples taken on the same day. These differences range from 30 µg/g between the 14-day samples to 1,170 µg/g between the 1-day samples. The large variation between leaf samples extracted on the same day is probably due to sample size. The individual samples in this experiment weighed about 1 g compared with an average of 4.2 g for the previous experiment. Even when the results of the individual samples are averaged together, the combined sample weights are less than half the average sample weight from the previous experiment. It is evident, then, that a larger sample size is necessary in order to obtain reproducible results. This problem had not been encountered in the previous experiment.

A similar problem is encountered on examination of the oxime concentrations of the treated leaf samples. The variations in concentration between individual samples and the lack of oxime data for the final three weeks of the experiment due to interference from leaf coextractives make the interpretation of the fate of the oxime very difficult.

When the results of the two peach plant experiments are combined, the following picture emerges. The results

of the first experiment illustrated in the plot of log oxamyl concentration versus time in Figure 3-5 indicate that the degradation of oxamyl on leaves was rapid and approximately exponential for about the first two-week period. This was followed by a period of very slow degradation. This type of degradation curve has been described by Gunther and Blinn[66]. During the steep initial portion of the curve, dissipation of the surface residue takes place due to evaporation and degradation by UV light. The latter part of the curve depicts the slower degradation of residues which have penetrated into the leaf tissues.

If intact oxamyl and/or its oxime are translocated to the roots or soil, it appears from the results of the second experiment that their concentrations are very low, since they were not detected in these samples except perhaps in very small quantities in the 4-day, 14-day and 30-day soil samples. These results do not support those of Peterson et al.[31] who found significant translocation of intact oxamyl from the leaves to the roots of potato plants. However, these are the only authors who have reported significant quantities of oxamyl in the root, and this result may be due to the fact that the intensity of light used on their treated plants was approximately twice what is normally used. This would result in a rapid translocation of oxamyl from treated leaves to the root, since the rate of translocation is related to the intensity of incident light[67].

Wright et al.[33] reported that very low concentrations

of oxamyl in the root (3 ng/g) were sufficient to protect young cucumber plants from nematode attack. This concentration of oxamyl in the roots resulted from foliar application of oxamyl to the young cucumber plants. The detection limit for oxamyl in peach roots using the two-column HPLC method was about 2.5 µg/g. The concentration found by Wright et al. using [^{14}C] oxamyl detected by liquid scintillation counting is therefore smaller than the minimum detectable concentration of the two-column system by a factor of almost 10^3. It is therefore possible that such low concentrations of oxamyl were maintained in the peach roots by slow, continuous translocation of oxamyl from treated leaves and that protection from nematode attack could be realized in this way. The oxamyl concentration in the root may not accumulate to detectable levels due to decomposition or conjugation in the root or leaching into the soil followed by rapid degradation. Similarly, continuous translocation of oxamyl in low concentration to untreated leaves is not precluded by these results.

The hypotheses that oxamyl decomposes to a toxic metabolite or stimulates production of a nematicidal compound by the plant are also not precluded by these results. It is possible that such compounds were present but undetected in the leaf, root or soil samples.

Corn Seed Experiment

Since problems were encountered in the previous experiment due to small sample size, it was decided that an experiment concerning the degradation of oxamyl should be performed using larger sample sizes.

Attention in this experiment was focused on corn seeds. Damage to corn roots by nematode attack has been shown to result in reduced yields[68-70]. The plants are most susceptible to nematode attack when they are young seedlings trying to get established. If nematodes are not controlled in this early stage, money spent on fertilizer, cultivation and insecticide sprays may be wasted to a large extent since crop yields and quality may be greatly affected by nematode attack[71].

The most common method for controlling nematodes is pre-plant soil treatment with volatile nematicides which have a fumigating action in the soil. This is generally a very expensive procedure. As an alternative to this technique, seed treatment with a nematicide is suggested as a convenient and inexpensive way to control nematode attack in the sensitive early stage of plant growth. Treatment of seeds with fungicides is a common practice, but nematode control by this means is not common since few appropriate chemicals are available.

The seed-treatment technique affords several advantages over other means of nematode control. It makes a much smaller impact on the environment since the nematicide is applied only at the location where protection is required, making general soil application unnecessary. When a systemic nematicide is used, it is expected that the roots and growing stem will be protected through translocation of the chemical from the seed. If the

nematicide persists for 4 - 6 weeks, the plant will be protected during the most vulnerable period of its growth[72]. Seed-treatment is less expensive than soil fumigation because application does not require expensive field equipment and the total quantity of nematicide used is greatly reduced by concentrating application on the site of need.

It has been proposed that treatment of corn seeds with oxamyl prior to planting may be effective in protecting young corn plants from nematode attack[73]. If this is found to be the case, it could prove to be a very important means of protection since corn is the third most important cereal crop in the world[74] and is the leading feed crop in the United States[75]. The purpose of this experiment was to determine how long oxamyl will persist on treated corn seeds in order to learn whether it will be suitable for this purpose. The formation of metabolites of oxamyl in corn seeds was also monitored in order to further investigate the mode of action of oxamyl. Corn seeds were thought to be advantageous from the point of view of experimental design, since a relatively large number of seeds could be handled in each sample, ensuring accurate representative samples.

The design of the experiment was as follows. Corn seeds were soaked in either 16,000 ppm oxamyl (treated seeds) or distilled water (control seeds) for 16 h. Control and treated seeds were planted in separate flats as described in the experimental section. In order to investigate the effect of microbial action on the rate of degradation of oxamyl two types of soil were used for both treated and control seeds: sterilized and natural. At one week intervals seed samples

were removed from the flats, extracted and analysed by HPLC.

Several extraction procedures were investigated using treated, unplanted seeds in order to determine the most efficient procedure for recovery of oxamyl and oxime. Extraction volumes of 30 mL and 70 mL methanol and a variety of extraction times and methods, including use of an ultrasonic probe, were attempted. It was found that allowing the seeds to sit in three consecutive 70 mL volumes of methanol for several hours each time was an efficient extraction procedure for the unplanted seeds. The recovery data for four 70 mL extractions is presented in Table 3-5. A similar extraction procedure was used for the 1-week and 2-week seed samples.

As time went on and the planted seeds became softer, however, it was found that long extraction periods resulted in increased interference from coextractives and were not necessary to efficiently extract oxamyl and its metabolites. The extraction procedure was therefore changed for the 3-week, 5-week and 6-week samples. Each time the extraction procedure was changed, the extraction efficiency was monitored. The extraction efficiency data from these procedures is also summarized in Table 3-5. It may be observed that three extractions of the unplanted, 3-week and 6-week seeds recovered at least 96% of the oxamyl and its metabolites which were extracted in four extractions. Three extractions were therefore used for the other samples as summarized previously in Table 2-4. The first three extractions of 5-week seeds from sterilized soil were short (1 h each) and a total of six extractions were needed to efficiently extract oxamyl and its metabolites.

Table 3-5

Extraction Efficiency of Oxamyl, Oxime and DMCF From Corn Seeds

Extraction	Time	Oxamyl		Oxime		DMCF	
		µg	% total	µg	% total	µg	% total
(Unplanted Seeds)							
1	17 h	56,000	74.5	2430	75.9	1300	84.4
2	9	10,000	13.3	450	14.1	180	11.7
3	15	6,630	8.8	250	7.8	49	3.2
4	10	2,510	3.3	71	2.2	11	0.7
(3-week seeds from sterilized soil)							
1	21	310	80.9	-		250	
2	2	50	13.1	14		11	
3	1	19	5.0	-		-	
4	24	~4	1.0	ND		-	
(5-week seeds from sterilized soil)							
1	1	56	81.1	55	75.3	19	44.7
2	1	7	10.1	8	11.0	5.4	12.7
3	1	1	1.4	3	4.1	2.9	6.8
4	3	3	4.3	3	4.1	3.1	7.2
5	22.5	2	2.9	2	2.7	7.4	17.4
6	19.3	ND		2	2.7	4.7	11.0
(6-week seeds from sterilized soil)							
1	4	ND		5	100	3.9	51.3
2	3.5	ND		ND		1.7	22.4
3	15.7	ND		ND		1.7	22.4
4	27	ND		ND		0.3	3.9

- indicates value unobtainable due to interference
ND - not detected

It was possible to analyse for oxamyl and the oxime in the unplanted, 1-week and 2-week corn seed samples using only the first column of the HPLC system used in the previous experiment. In the 3-week samples, however, interference from seed coextractives prevented the analysis of the oxime. As mentioned previously the performance of this column had deteriorated during the previous experiment. However, when the first column was replaced with a new 12 cm C_{18} column interference still occurred in the oxime region. Even when the two-column HPLC system was used for analysis of the oxime resolution of the oxime peak from interfering coextractive peaks was not achieved.

It was decided to attempt the analysis of the oxime and oxamyl using a size-exclusion column as the first column of the two column system. In a size-exclusion column the components of a sample are separated according to the size of their molecules. The column is packed with porous silica microspheres. Small molecules are able to enter the pores of the packing and will be retained longer on the column than large molecules which will not fit into the pores and pass quickly through the column[76]. The second column was a reverse phase column. It was felt that by the combination of two mechanisms of separation, size-exclusion and partition, better resolution of the oxime might be achieved.

The method which was developed has been outlined in the experimental section. It was found that with small molecules the size-exclusion column behaved in an adsorption mode with the characteristics of a reverse-phase column. That is the oxime was found to elute first, followed by oxamyl with good resolu-

tion of the peaks. This would not have been the case if the column were operating in a purely size-exclusion mode, since the oxime is smaller than oxamyl.

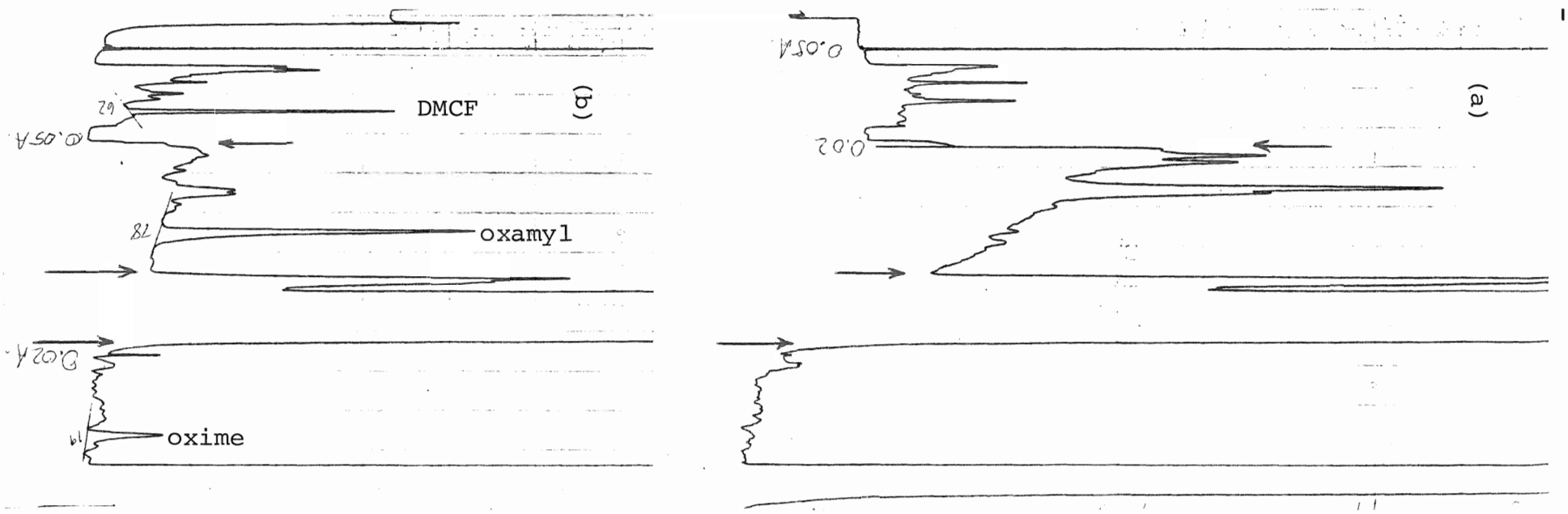
Because of the observation that column performance declined when extracts were run without cleanup, it was felt that in order to prolong the life of the column a cleanup which removed strongly adsorbing materials would be appropriate. For this purpose short disposable C_{18} columns known as "SEP-PAK cartridges" were used as described in the experimental section. It was found that following loading of an oxamyl and oxime standard onto the SEP-PAK cartridge, the oxamyl and oxime could be quantitatively eluted with 2 mL of a 33% methanol, 66% water mobile phase. Most of the colour of a corn seed extract remained in the SEP-PAK cartridge, giving an almost transparent, clear sample which did not require filtering or centrifuging. The strongly adsorbed materials were therefore left behind on the SEP-PAK cartridge and could not contaminate the HPLC column. Comparison of the number of theoretical plates provided by the guard column and the size-exclusion column before and after the analyses were performed indicated a decrease in performance of only 6%.

The HPLC method was very effective in analysing for oxamyl and the oxime. It also proved to be effective for the analysis of DMCF. The DMCF peak was observed when samples cleaned up by the SEP-PAK method began to be run on the two-column HPLC system. Figure 3-8 illustrates the chromatograms of the 4-week untreated corn seed sample from sterilized soil with the DMCF, oxime and oxamyl regions indicated, and the 4-week treated corn seed sample from sterilized soil. It

Figure 3-8 (a) Chromatogram of 4-week untreated seed sample from sterilized soil
(b) Chromatogram of 4-week treated seed sample from sterilized soil

(Corn Seed Experiment)

(change in mobile phase indicated with ↓)



is evident from the former chromatogram that there is no interference in the oxime and DMCF regions, and only a very small interfering peak in the oxamyl region which does not hinder the analysis of oxamyl in the treated corn seed sample.

An indication of the reproducibility of the HPLC method is provided by duplicate runs of the 3-week sample from sterilized soil, the 4-week sample from natural soil and a standard solution of DMCF oxime and oxamyl which gave average coefficients of variation of 2.4%, 3.2% and 4.3% respectively. The method allowed detection of 0.03 ppm DMCF, 0.1 ppm oxime and 0.1 ppm oxamyl giving minimum detectable quantities of 0.005 µg, 0.02 µg and 0.02 µg respectively.

The results of the corn seed analyses are summarized in Table 3-6 and illustrated graphically in Figure 3-9. The concentration of DMCF in the unplanted seeds was estimated from the height of the DMCF peak in the chromatogram of each extraction compared with a DMCF standard run several days later. All the values of DMCF concentration may be lower than the true values since it was discovered after the analyses were completed that some DMCF may be lost in the concentration step of sample preparation. The concentration step needs to be perfected for future analyses of DMCF.

DMCF was evidently produced through the degradation of oxamyl, since the Vydate used to treat the seeds contained only a trace of DMCF (0.06%) in terms of DMCF + oxamyl, while the oxime concentration in the Vydate was 2.7% in terms of oxime + oxamyl. In the corn seed extracts of the 2-week, 3-week and 4-week samples the DMCF concentration is higher than the oxime concentration, indicating that it is a signifi-

Table 3-6

Concentrations of Oxamyl, Oxime and DMCF in Treated Corn Seeds

Sampling Time	Sample	Concentration (µg/g seed) Oxamyl	Oxime	DMCF
unplanted		4750	200	100
1 week	sterilized soil	1190	34	
	natural soil	410	14	
2 weeks	sterilized soil	320	20	83
	natural soil	110	18	51
3 weeks	sterilized soil	80	12	49
	natural soil	36	2	5.6
4 weeks	sterilized soil	57	5.7	25
	natural soil	97	9.8	7.2
5 weeks	sterilized soil	20	21	12
	natural soil	16	12	-
6 weeks	sterilized soil	ND	3	3.4
	natural soil	6.1	5	ND

- value unobtainable due to interference
ND - not detected

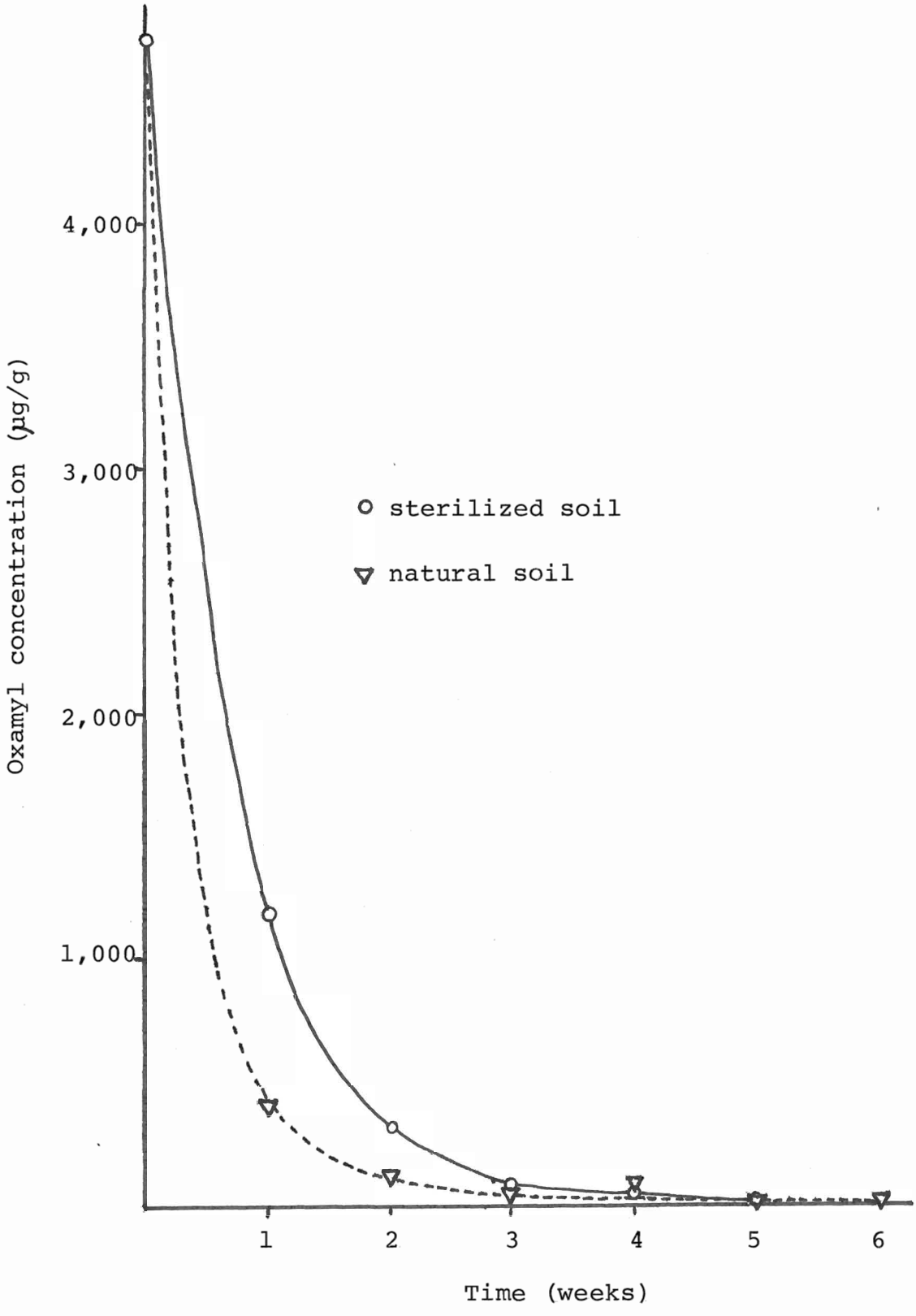


Figure 3-9 Concentration of oxamyl in treated corn seeds from sterilized and natural soil vs. time

cant metabolite of oxamyl in corn seeds. Harvey and Han[77] have shown that oxamyl in the presence of rat liver enzymes decomposes by two routes, hydrolytically to the oxime and enzymatically to DMCF. It may be that degradation of oxamyl to DMCF in corn seeds also occurs via an enzymatic pathway, since the degradation of oxamyl in distilled water and tap water resulted in only traces of DMCF being produced, the major metabolite being the oxime.

It may be observed from Figure 3-9 that decomposition of oxamyl was most rapid in the first 3-week period, during which time about 99% of the oxamyl originally applied had dissipated. Degradation of oxamyl after this point was slow, with a small amount of oxamyl remaining in the 6-week corn seed sample from natural soil. No oxamyl was detected in the 6-week corn seed sample from sterilized soil. Oxamyl may be lost through degradation to the oxime or DMCF or through translocation to soil or to the growing root, stem and leaf portions of the plant.

It is evident that oxamyl concentrations well in excess of those found by Wright el al. in cucumber roots persist in treated corn seeds for at least five weeks. It is possible that low concentrations of oxamyl may be maintained in the root for the same period of time by translocation from the seed. This needs to be confirmed by analysis of the root samples. Based on the results of this experiment, it may be said that oxamyl persists long enough on treated corn seeds to be considered for possible application as a seed treatment. The next step is to test the oxamyl seed-treatment technique for its effectiveness in protecting young corn roots from

nematode attack.

During the period of most rapid loss of oxamyl in the first three weeks, the oxamyl concentration in the corn seeds planted in natural soil was less than half the oxamyl concentration in those planted in sterilized soil. This result indicates that soil microorganisms may play an important role in degradation of oxamyl. The plastic bags which protected the sterilized soil from airborn contamination had been removed five days after planting because of the growth of the corn plants. It would be expected that air-born spores and bacteria would begin to contaminate the soil to the depth of the seeds within a couple of days of the removal of the bags. It would, however, take two or three weeks before the sterilized and natural soils could be regarded as identical[72]. After this point it would not be possible to claim any distinction between them and in fact in the later period of the experiment there seems to be no identifiable difference in the rate of loss of oxamyl between the two soil types.

The concentration of oxamyl used to treat the corn seeds was observed to have a phytotoxic effect on seed germination and plant growth. The weights of corn plant parts may be found in the appendix and the weight of the leaf and stem portion is plotted against time in Figure 3-10 for the four types of samples. The fastest growth rate for the 6-week period was exhibited by untreated seed grown in natural soil. The treated seed grown in sterilized soil consistently showed the slowest growth rate. There appears to be little significant difference in rate of growth between the treated seed planted in natural soil and the untreated seed planted in sterilized soil. It

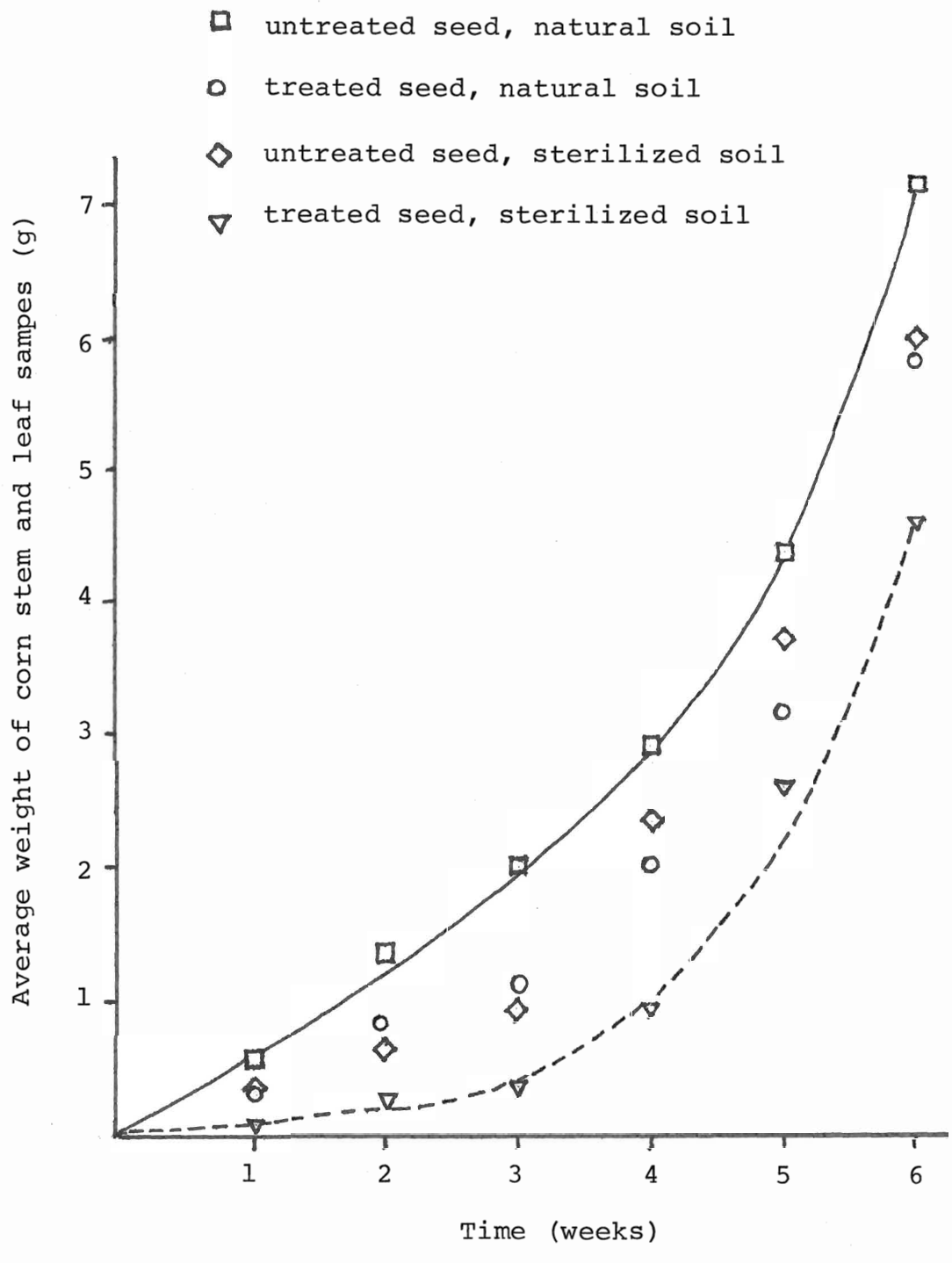


Figure 3-10 Average weight of corn stem and leaf samples vs. time

appears therefore that both the treatment of the seed with oxamyl and planting in sterilized soil had an inhibitory effect on plant growth.

Planting in sterilized soil appeared to have little effect in itself on germination of the corn seeds as the untreated seeds in natural and sterilized soil exhibited 98% and 97% germination respectively. The treated seeds planted in natural soil exhibited 91% germination, while the combination of planting treated seeds in sterilized soil produced only 78% germination. It is evident that a lower concentration of oxamyl should be used for corn seed treatment in order to avoid these phytotoxic effects.

Toxicological Studies

Since DMCF was found in the treated corn seed in significant concentrations it was thought that perhaps DMCF may be a nematicidal agent which provides protection against nematode attack. Since no information on the toxicological properties of DMCF was found in the literature, it was decided to compare the effects of various concentrations of oxamyl, the oxime and DMCF on the mobility of nematodes.

DMCF was synthesized according to the reaction described in the experimental section. The reaction was first attempted using dimethylformamide as a solvent based on the method of Harvey and Han[77], but it was not possible to achieve a good separation of the product from the solvent by fractional distillation or liquid-liquid extraction. Harvey and Han had performed the reaction on a micro scale to synthesize ^{14}C-labelled DMCF and used HPLC to purify the product. Dichloromethane was also tried as a solvent but the yield was very poor,

probably due to the low solubility of NaCN in the solvent. The use of acetone as a solvent gave a good yield (74%) and the product was purified by washing with water to hydrolyse the unreacted starting material and extracting with diethylether. The mass spectrum of the product may be found in the appendix and compares favourably with the mass spectrum of a small sample of the pure compound obtained from DuPont.

The in-vitro effects of oxamyl, the oxime and DMCF on the ability of nematodes to continue normal motion were compared by placing 10 nematodes in aqueous solutions of various concentrations of the chemicals. In 8,000 ppm solutions M.incognita larvae were immobilized within 5 - 10 min in oxamyl and DMCF. In the case of nematodes in 8,000 ppm oxime solution, however, slow movement was observed for a period of 23.5 h, except at 17.5 h when they were observed to be motionless. The difference in biological activity between the oxime and the other two chemicals was very dramatic. According to Harvey[78], the lack of biological activity of the oxime is due to the absence of the methyl carbamate moiety. He gives the acute oral LD_{50} of the oxime as 11,000 mg/kg body weight for rats, compared to 5.4 mg oxamyl/kg body weight.

The effect of lower concentrations of oxamyl and DMCF on nematodes was monitored down to 4 ppm for oxamyl and to 500 ppm for DMCF. The behaviour of nematodes in the 4,000, 2,000, 1,000 and 500 ppm oxamyl solutions was virtually identical to that in the 8,000 ppm oxamyl solution. Figure 3-11 illustrates a plot of the time required to immobilize 50% of the nematodes versus the concentration of oxamyl up to 500 ppm. As the concentration decreased the amount of time required to

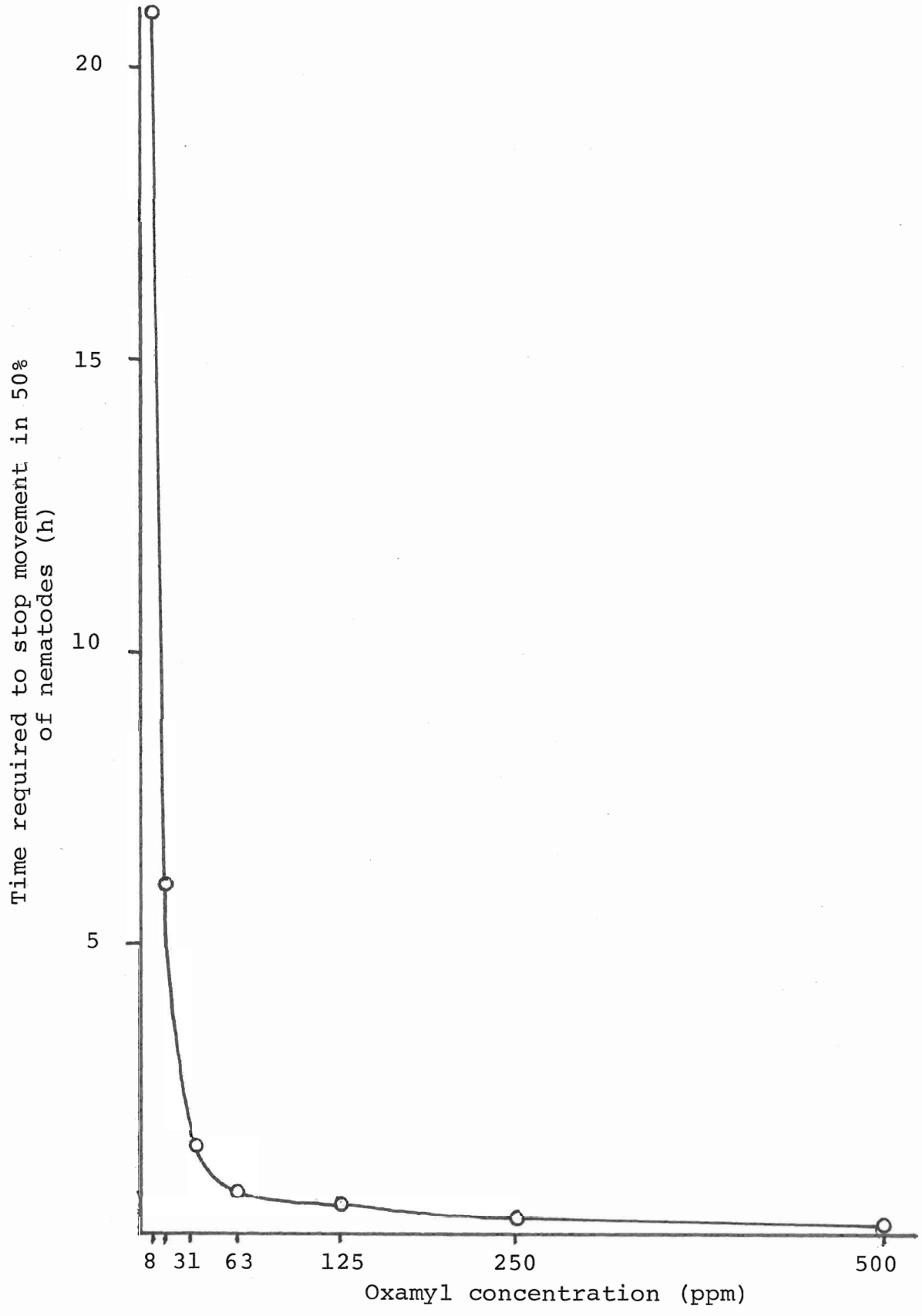


Figure 3-11 Time required to stop movement in 50% of nematodes immersed in oxamyl solution vs. oxamyl concentration

immobilize 50% of the nematodes increased slowly until 31 ppm oxamyl was reached, after which point an abrupt change occurred. Six hours were required in 16 ppm oxamyl and 21 h in 8 ppm oxamyl while in 4 ppm solution all the nematodes still showed slow movement after 23 h.

In the solutions of high concentration ($\geq$500 ppm oxamyl) the nematodes were quickly immobilized and would exhibit only some twitching of the tail or spasmodic convulsion about mid-point in the body. After several minutes, motion was completely stopped and the nematodes were in random configurations. When observed between 10 and 20 h later, the nematodes were all straight and motionless. In this position the longitudinal muscles are completely relaxed, and this is the position assumed upon the death of the nematode.

In the lower concentrations of oxamyl ($\leq$250 ppm) straightening of the nematodes was not observed for up to 20 h in solution. As the concentration decreased below 250 ppm movement remained vigorous for longer periods of time, followed by a general slowing of movement, until only slow flexing and then intermittent twitching were observable. In concentrations down to 31 ppm oxamyl, all nematodes stopped moving within 2.5 h. In lower concentrations some nematodes still exhibited slow movement after 21 h. These observations are similar to those made by Wright et al.[33] who performed a similar study in oxamyl solutions ranging from 0.5 ppm to 32 ppm. They observed some slowing of activity after 8 h in 2 ppm oxamyl and 1 h in 8 ppm oxamyl. They did not observe any effect on M.incognita larvae immersed in 32 ppm oxime.

It is evident therefore that at low concentrations down

to 2 ppm, oxamyl will cause some reduction in activity of M. incognita larvae.

In a 4,000 ppm DMCF solution, the larvae behaved essentially the same as they did in 8,000 ppm DMCF, being immobilized within 10 min. In 2,000 ppm solution all 10 larvae were still moving slightly after 12 h and at 18.5 h the larvae were moving vigorously. No effect was observed in 1,000 ppm and 500 ppm solutions up to 18.5 h. Therefore the ability of DMCF to reduce activity of nematodes in this mobility test is much weaker than that of oxamyl, and it is apparent that DMCF would be effective in slowing nematode movement only in high concentration ($\geq$2,000 ppm).

Solutions of 4,000 ppm oxamyl and DMCF appeared to have similar effects on the mobility of M. incognita larvae, but the ability of the larvae to recover after transfer to water differed considerably. The results of these studies are summarized in Tables 3-7 and 3-8. The percentages in the final column indicate the percentage of larvae which recovered some degree of motion. Larvae immersed in 4,000 ppm oxamyl for longer than 30 min did not recover apart from some slight movement noticed after 28 h in the 40-min group. On the other hand some recovery was possible after immersion in 4,000 ppm DMCF for as long as 24 h. The nature of the biological activity of DMCF appears to be quite different from that of oxamyl. DMCF does not have a carbamate group and thus probably does not act to inhibit acetylcholinesterase. It appears instead to have a nematostatic effect, and allows a high degree of recovery after transfer of the nematodes to water.

The only criterion used in this study to assess the

Table 3-7

Effect of Time of Exposure to 4,000 ppm Oxamyl
on Recovery of M. Incognita
after transfer to water

Time of Exposure	Initial Recovery Observed	Final Recovery (up to 30 h)
10 min	4 h	24 h (100%)
20	6	24 h (70%)
30	24	30 h (90%)
40	28	-
50	no recovery within 30 h (all straight)	
60	"	
70	"	
80	"	
90	"	

Table 3-8

Effect of Time of Exposure to 4,000 ppm DMCF
on Recovery of M. Incognita
after transfer to water

Time of Exposure	Initial Recovery Observed	Final Recovery (up to 24 h)
4 h	18 min	30 min (100%)
8	5 h	24 h (60%)
12	12 h	12 h (30%)
24	2.5 h	3 h (30%)*

*final recovery up to 12 h.

biological activity of the chemicals was reduction in the mobility of nematodes. It is therefore not impossible that the compounds may be biologically active in other ways, such as by repelling or preventing orientation of nematodes to the root, or interfering with feeding or invasion of the root.

Since the concentrations of DMCF which are produced in a plant due to decomposition of oxamyl are much lower than 2,000 ppm, it appears that DMCF does not act to reduce mobility of nematodes in the vicinity of the root. This does not rule out the possibility that DMCF acts by one of the mechanisms mentioned above. In the study by Wright et al. it was found that the 3 ng/g concentration of oxamyl found in the root was enough to substantially inhibit invasion of the root by M. incognita larvae. However the same concentration did not inhibit the development of larvae which were already present in the root, and 0.5 ppm oxamyl did not reduce the mobility of the larvae in vitro even after 24 h exposure. Therefore it appears that the root protection provided by foliar treatment with oxamyl was due to some mechanism other than inhibition of nematode mobility. On the other hand, Wright et al. also found that 0.5 µg/mL oxamyl on an agar plate interfered to some degree with the ability of the larvae to orient themselves to a cucumber root, and interfered to an even greater extent with their ability to invade the root.

It is therefore possible that oxamyl itself is the active agent which prevents nematode attack, not by killing or reducing the rate of movement of the nematodes, but perhaps by inhibiting their ability to orient toward the root and coordinate the complex behavioural sequences necessary for invasion.

CONCLUSION

A convenient, efficient two-column HPLC method was developed for the simultaneous determination of oxamyl and its oxime in peach leaves, roots and soil, and treated corn seeds. The method used for peach samples involved two reverse phase columns in series, separated by a switching valve. The method used for corn seeds involved a size-exclusion column and a reverse phase column, and was effective not only for the analysis of oxamyl and its oxime but also for detection of DMCF. In both methods the first column served to provide a preliminary cleanup of the sample by isolating the oxime and (in the method for peach samples) oxamyl fractions for analysis on the second column.

On treated peach leaves, oxamyl was found to dissipate rapidly during the first two-week period, followed by a period of slow decomposition lasting at least another two weeks. Movement of oxamyl or its oxime did not occur in the plant in detectable quantities, but may have occurred in very low concentrations.

Oxamyl was found to persist in treated planted corn seeds for a period of 5 - 6 weeks. This degree of persistence is sufficient to permit oxamyl to be considered for possible application as a seed treatment to protect young corn plants from nematode attack. During the period of rapid decomposition in the first three weeks it was found that degradation of oxamyl was consistently more extensive in natural soil than in sterilized soil, indicating that microbial action may be involved in the degradation of oxamyl. Both the oxime and DMCF were

detected as metabolites of oxamyl. Treatment of corn seeds in 16,000 ppm oxamyl for 16 h was found to have a phytotoxic effect on the germination of the seeds and the rate of growth of the young plants.

At high concentrations ($\geq$ 2,000 ppm) DMCF was found to have a nematostatic effect on nematodes, from which some degree of recovery was possible even after 24 h exposure to a 4,000 ppm solution. At lower concentrations of DMCF ($\leq$ 1,000 ppm), including the range of DMCF concentrations found in oxamyl-treated corn seeds, no effect on the mobility of nematodes was observed. Oxamyl was found to reduce the mobility of nematodes in concentrations down to 4 ppm. Recovery of M. incognita larvae from exposure to 4,000 ppm oxamyl was not nearly as great as that from exposure to 4,000 ppm DMCF.

As a result of this study, the HPLC method which was developed may be applied to practical problem-solving and routine residue determination. It may also be used in further studies on the persistence, metabolism and translocation of oxamyl in plants and its mode of action against soil nematodes.

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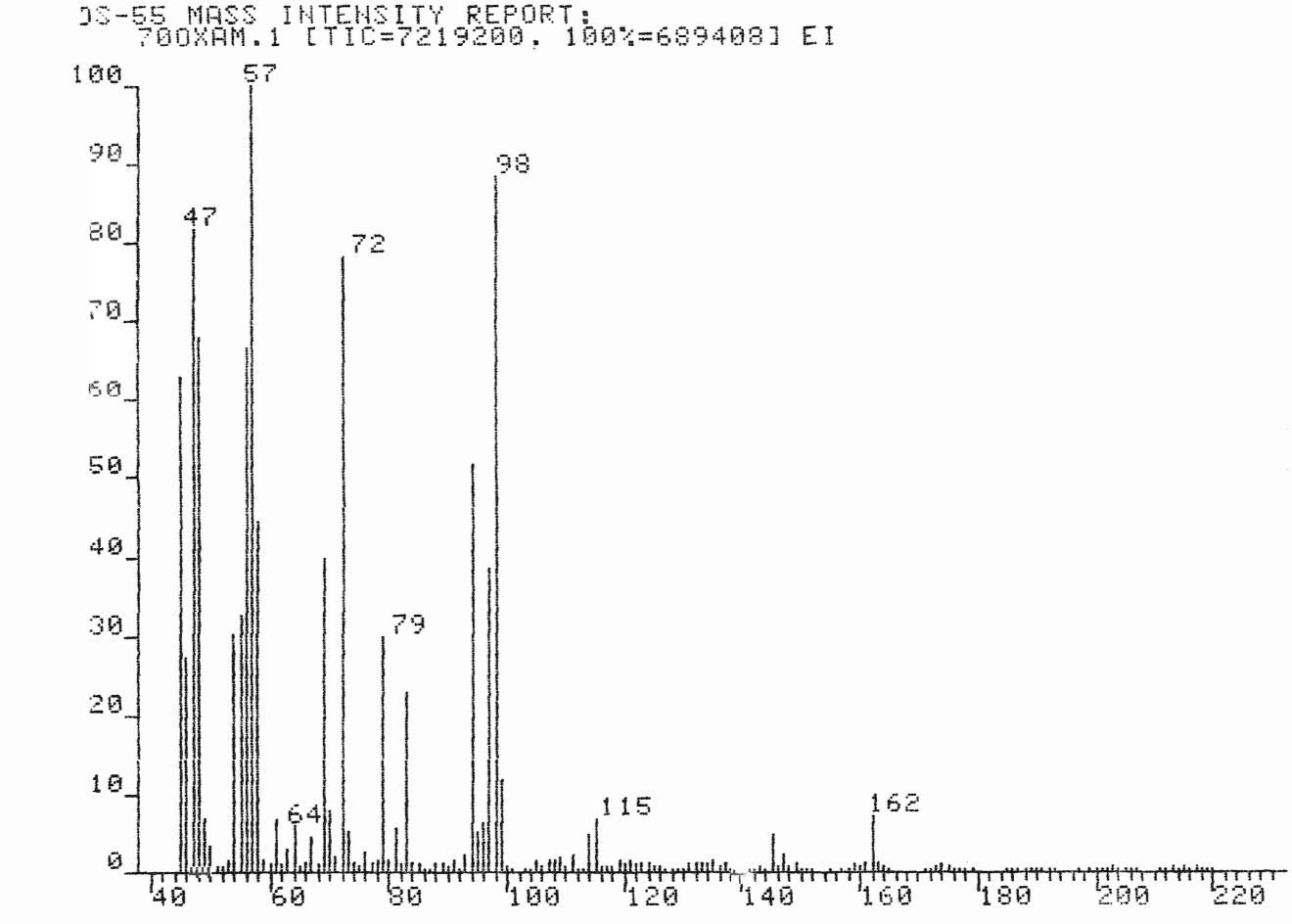
APPENDIX



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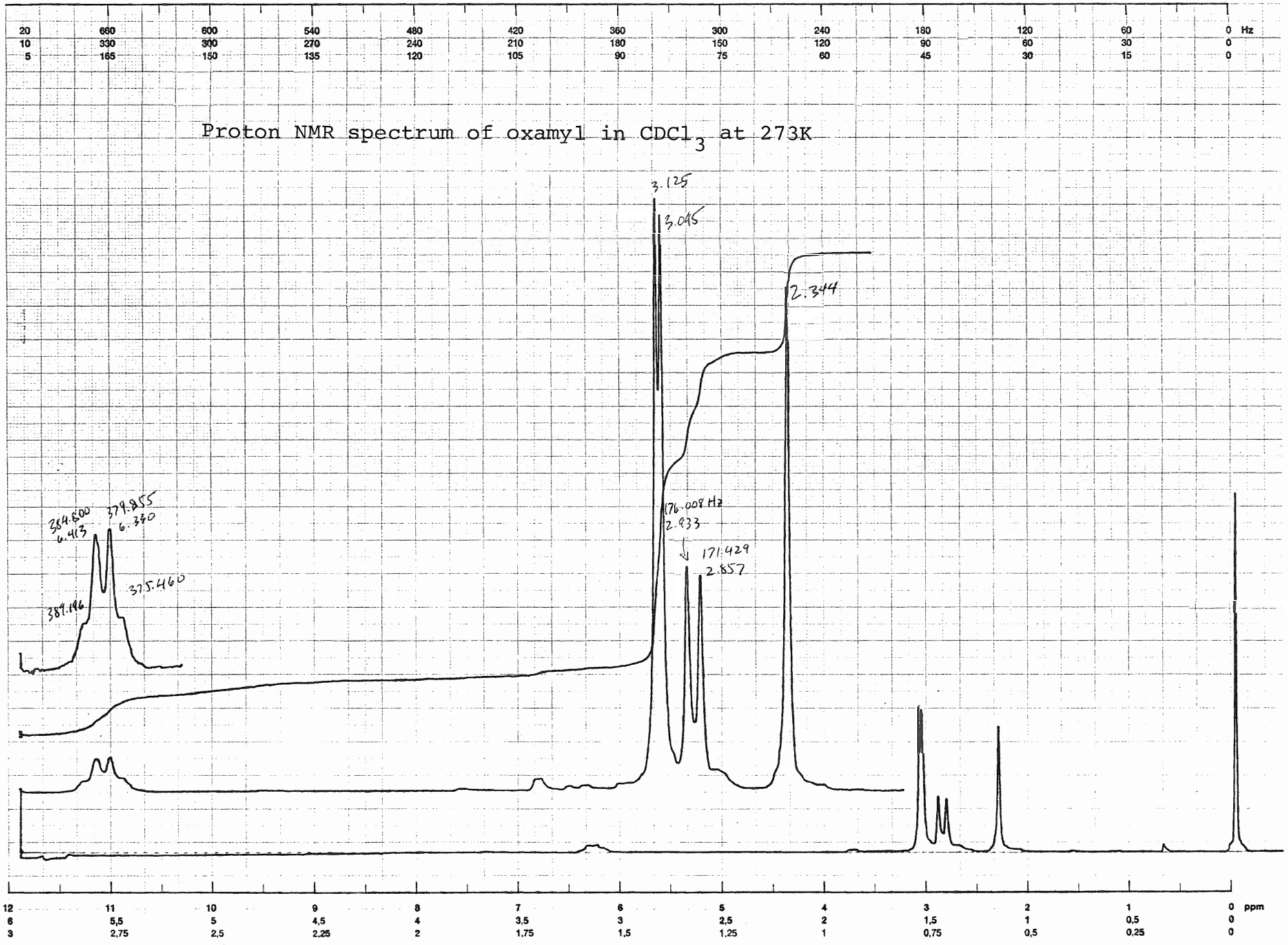
PROBE TEMPERATURE:50-80C
ELECTRON VOLTAGE:70EV
ACCELERATING VOLTAGE:4KV

Mass spectrum of oxamyl



No

BRUKER
SPECTROSPIN

60 MHz - ^{1}H - Spectrum

Sample OXAMYL

Conc. % in $CDCl_3$

Int. Standard TMS

Temp. amb. ☐

273 °K ☐

- 106 -

Signal f_1

Puls width (PW) 1.3 µ

Points (SI) 8 k/ 4

Spectrum

width (SW) f_1 750 Hz

Offset (A) f_1 2625 Hz

Time 5.46

times (NS) 16 sc

Time constant (TC) 0

Decoupling f_2

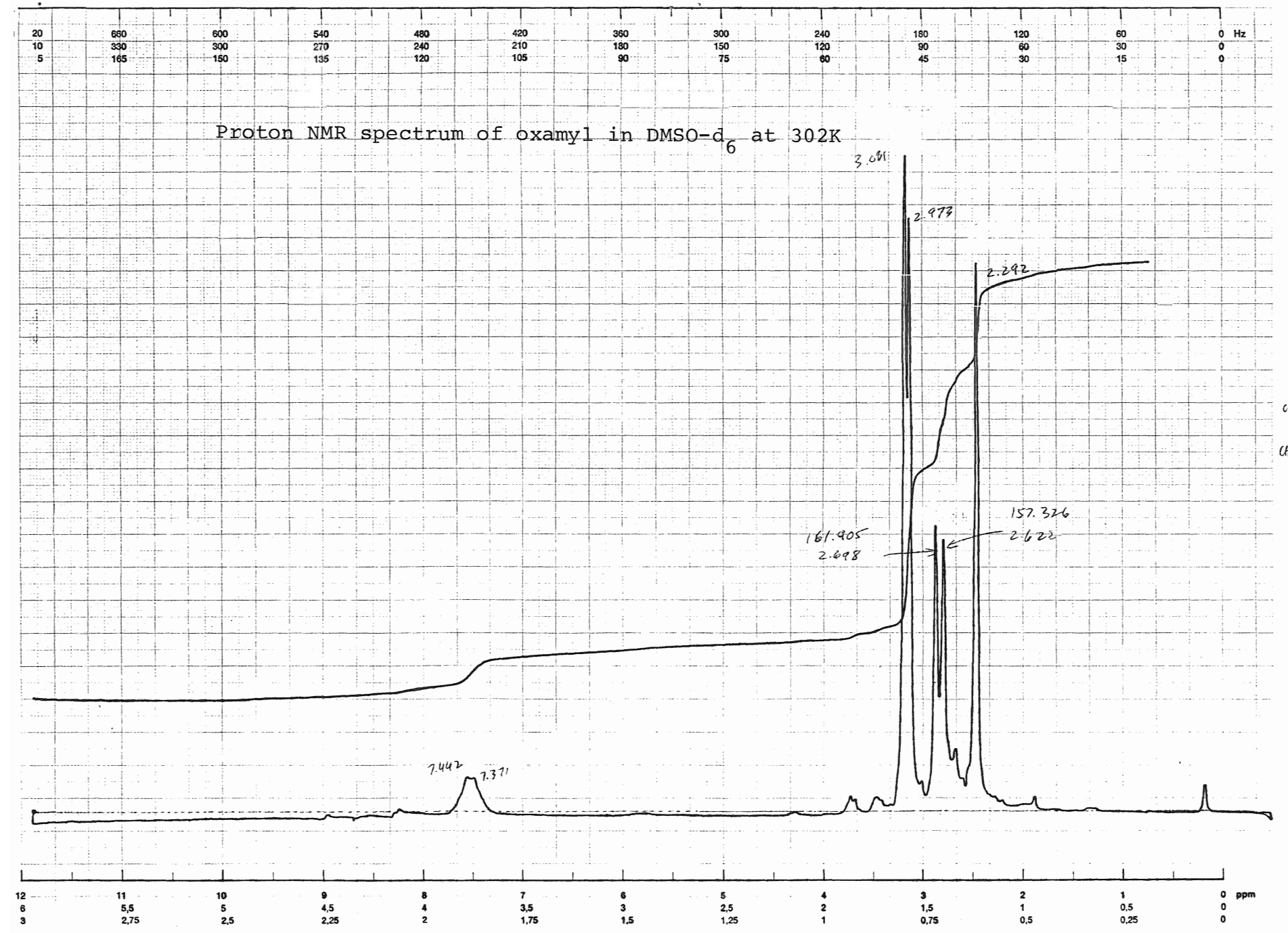
Frequency

Power

Date 6m. 23/80

Operator BM

Proton NMR spectrum of oxamyl in DMSO-d_6 at 302K



No

BRUKER
SPECTROSPIN

60 MHz - ^{1}H - Spectrum

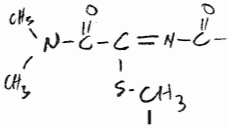
Sample OXAMYL

Conc. % in DMSO

Int. Standard TMS

Temp. amb. ☐

302 °K ☐



107

Signal f_1

Puls width (PW) 1.3 µ

Points (SI) 8 k / 4

Spectrum

width (SW) f_1 250 Hz

Offset (A) f_1 2400 Hz

Time 6.46

times (NS) 16 sc

Time constant (TC) 0

Decoupling f_2

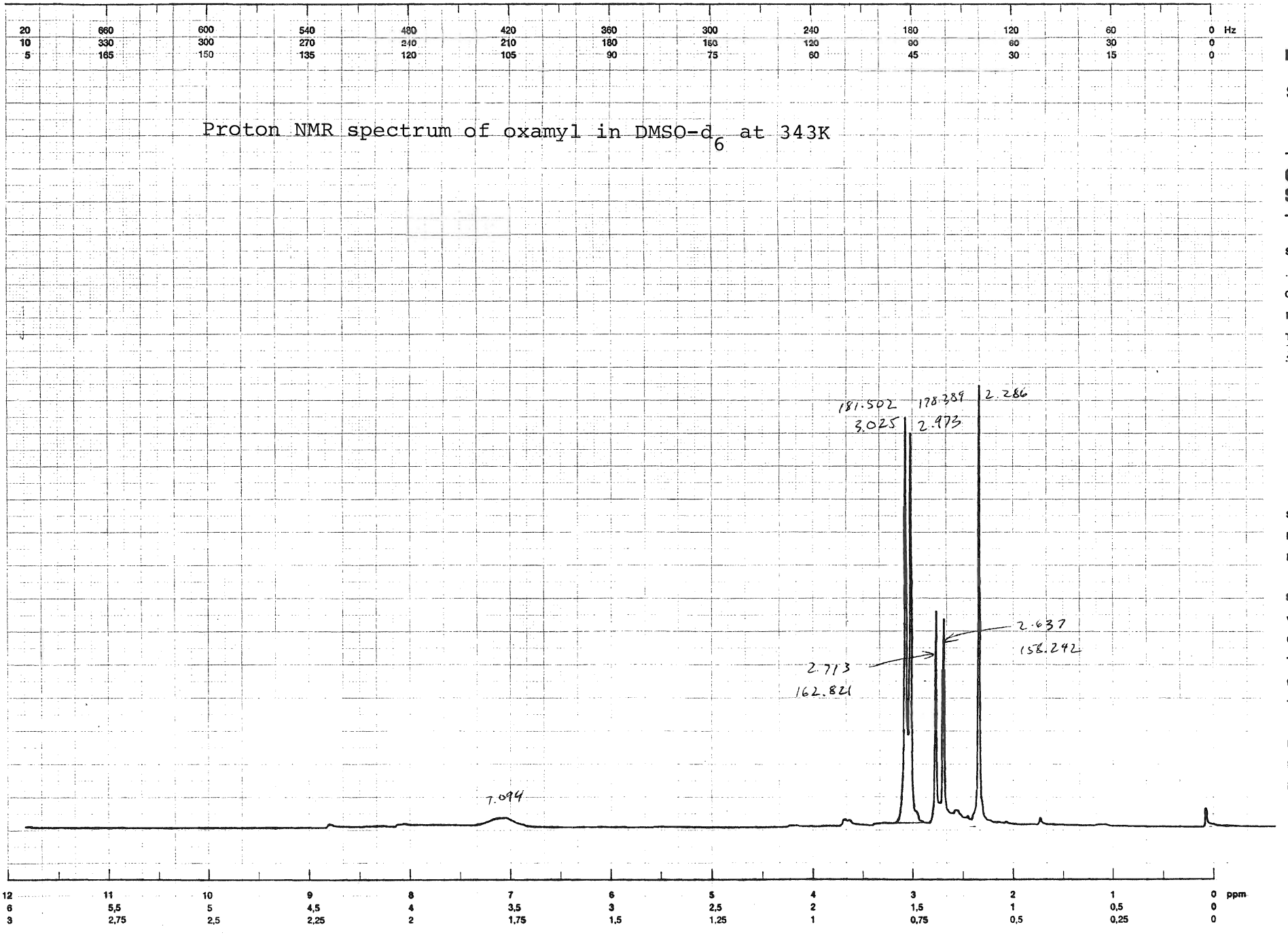
Frequency

Power

Date Gen. 23/8

Operator BDM

Proton NMR spectrum of oxamyl in DMSO-d_6 at 343K



No

BRUKER SPECTROSPIN

60 MHz - ^{1}H - Spectrum

Sample OXAMYL

Conc. % in DMSO

Int. Standard TMS

Temp. amb. ☐

343 °K ☐

108

Signal f_1

Puls width (PW) 1.3 µs

Points (SI) 8 k/ 4

Spectrum

width (SW) f_1 750 Hz

Offset (A) f_1 2400 Hz

Time 5.46

times (NS) 16 sc

Time constant (TC) 0

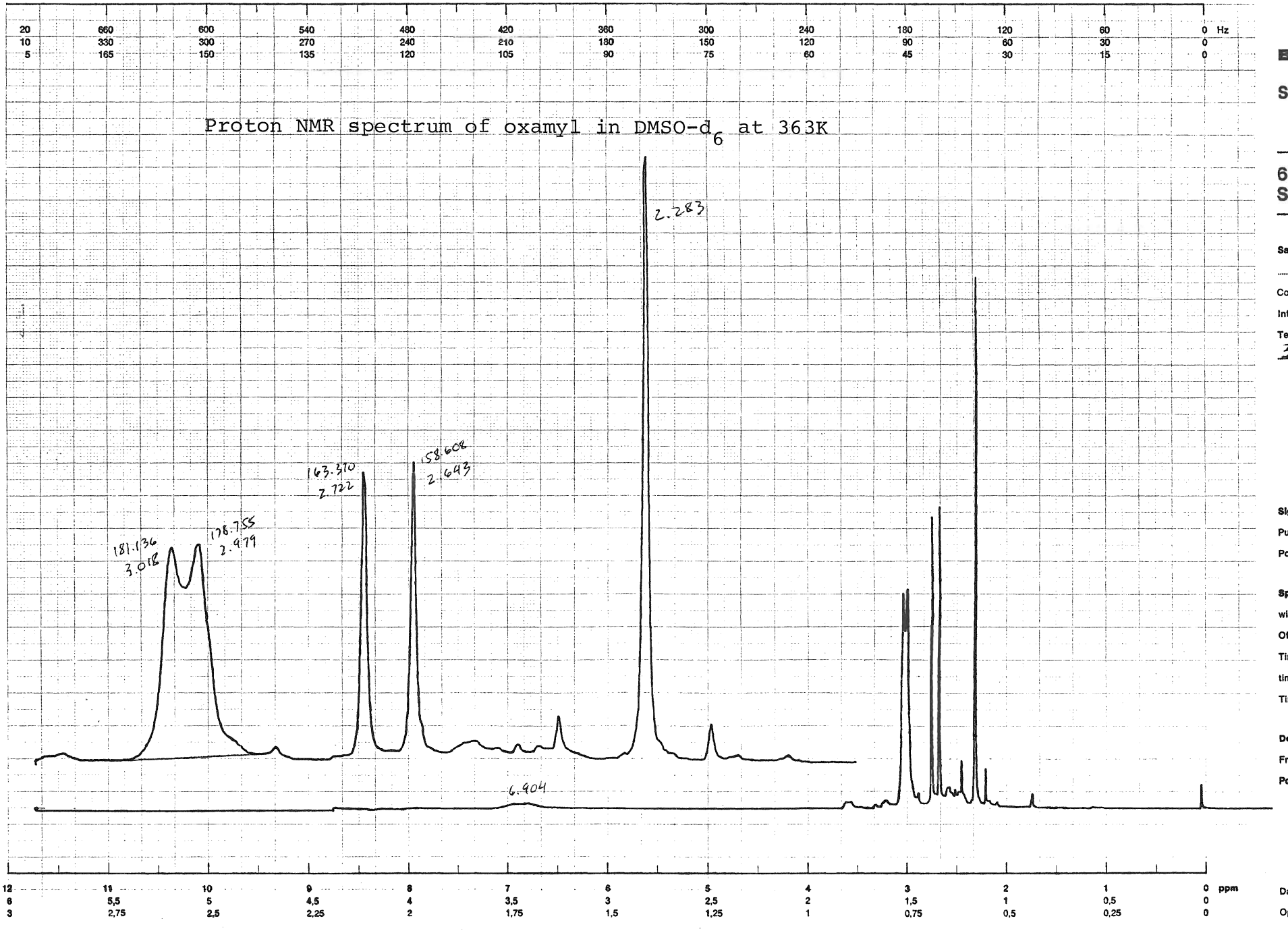
Decoupling f_2

Frequency

Power

Date Apr. 23/80

Operator BRM



No .....

BRUKER
SPECTROSPIN

60 MHz - ^{1}H - Spectrum

Sample ..... OXAMYL

Conc. ..... % in ..... DMSO

Int. Standard ..... TMS

Temp. amb. ☐

363 °K ☐

- 109 -

Signal f$_1$

Puls width (PW) ..... 6.3 µs

Points (SI) ..... 8 k/ 4

Spectrum

width (SW) f$_1$ ..... 750 Hz/

Offset (A) f$_1$ ..... 2400 Hz

Time ..... 5.46 s

times (NS) ..... 16 sc

Time constant (TC) 0

Decoupling f$_2$

Frequency .....

Power .....

Date ..... Apr 23/80

Operator ..... B.D.M

No .....

BRUKER
SPECTROSPIN

60 MHz - ^{1}H - Spectrum

Sample OXAMYL

Conc. ..... % in DMSO

Int. Standard TMS

Temp. amb. ☐

365 °K ☐

- 110 -

Signal f_1

Puls width (PW) 1.3 µs

Points (SI) 8 k/ 4

Spectrum

width (SW) f_1 750 Hz/

Offset (A) f_1 2900 Hz

Time 5.46

times (NS) 16 sc

Time constant (TC) 0

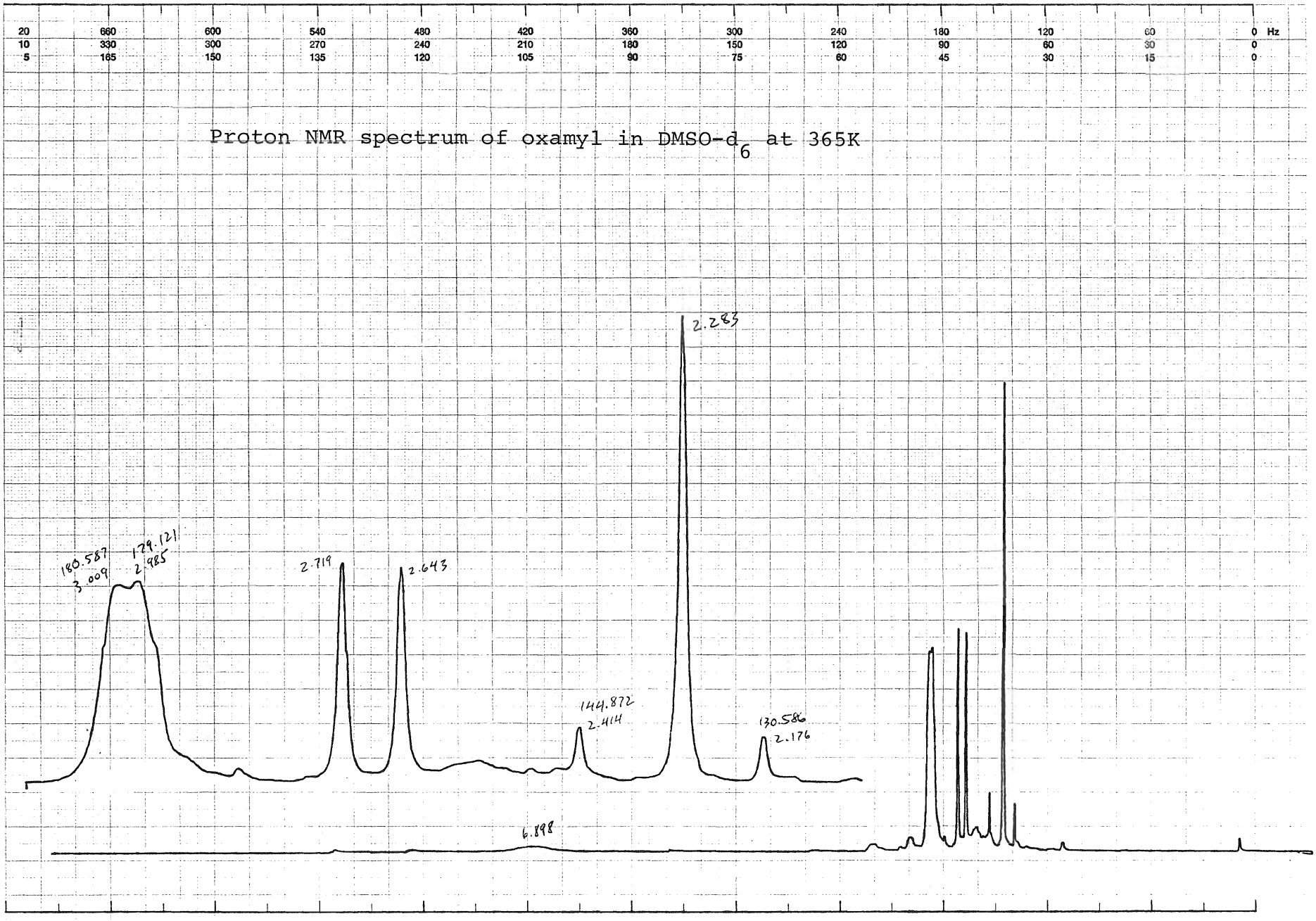
Decoupling f_2

Frequency .....

Power .....

Date Apr. 23/8(

Operator BDM

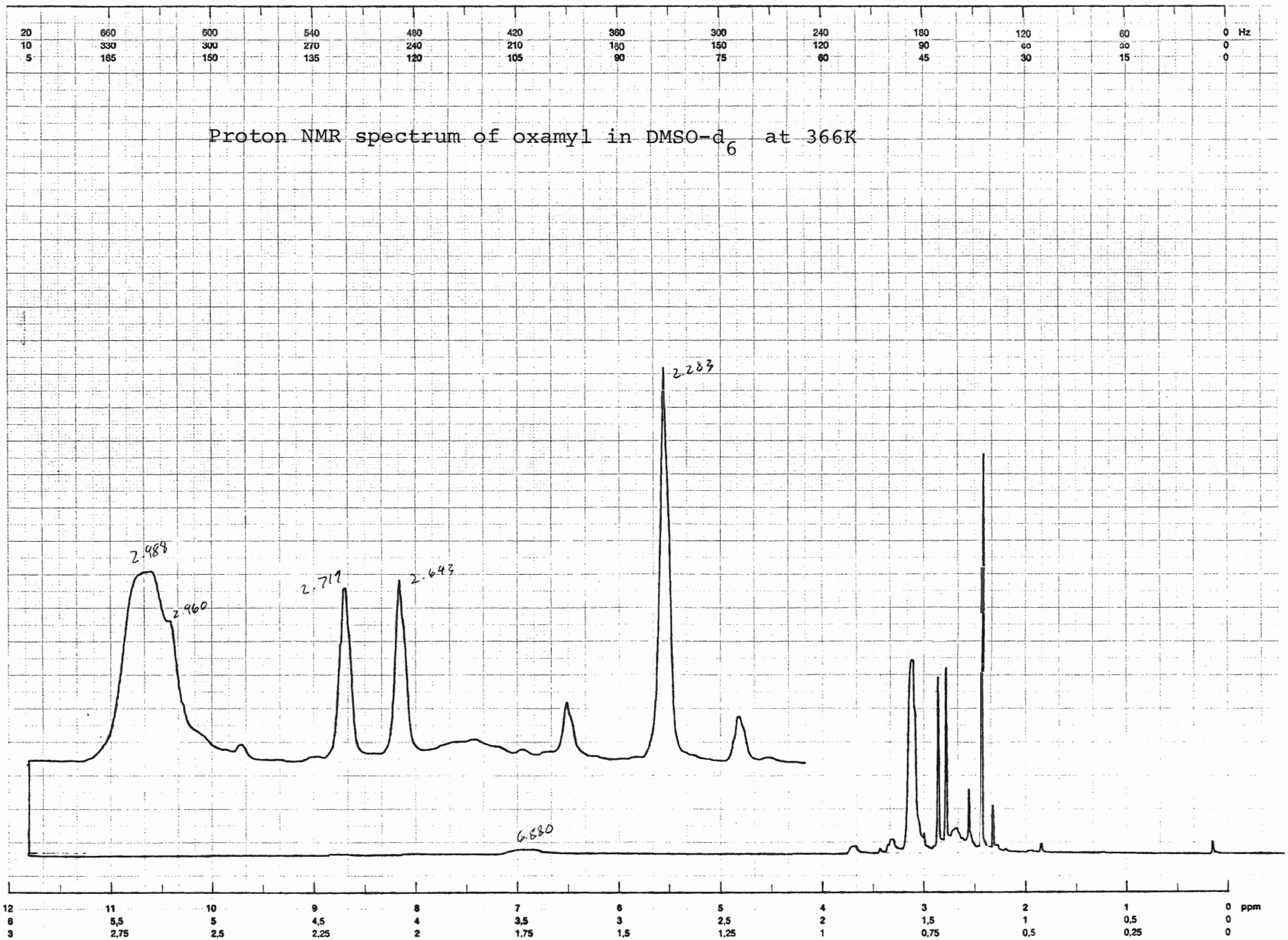


No .....

BRUKER

SPECTROSPIN

Proton NMR spectrum of oxamyl in DMSO-d_6 at 366K



60 MHz - ^{1}H - Spectrum

Sample OXAMYL

Conc. ..... % in DMSO

Int. Standard TMS

Temp. amb. ☐

366 °K ☐

- 111 -

Signal f_1

Puls width (PW) 1.3 µs

Points (SI) 8 k/ 4

Spectrum

width (SW) f_1 750 Hz/

Offset (A) f_1 2900 Hz

Time 5.46 s

times (NS) 16 sc

Time constant (TC) 0

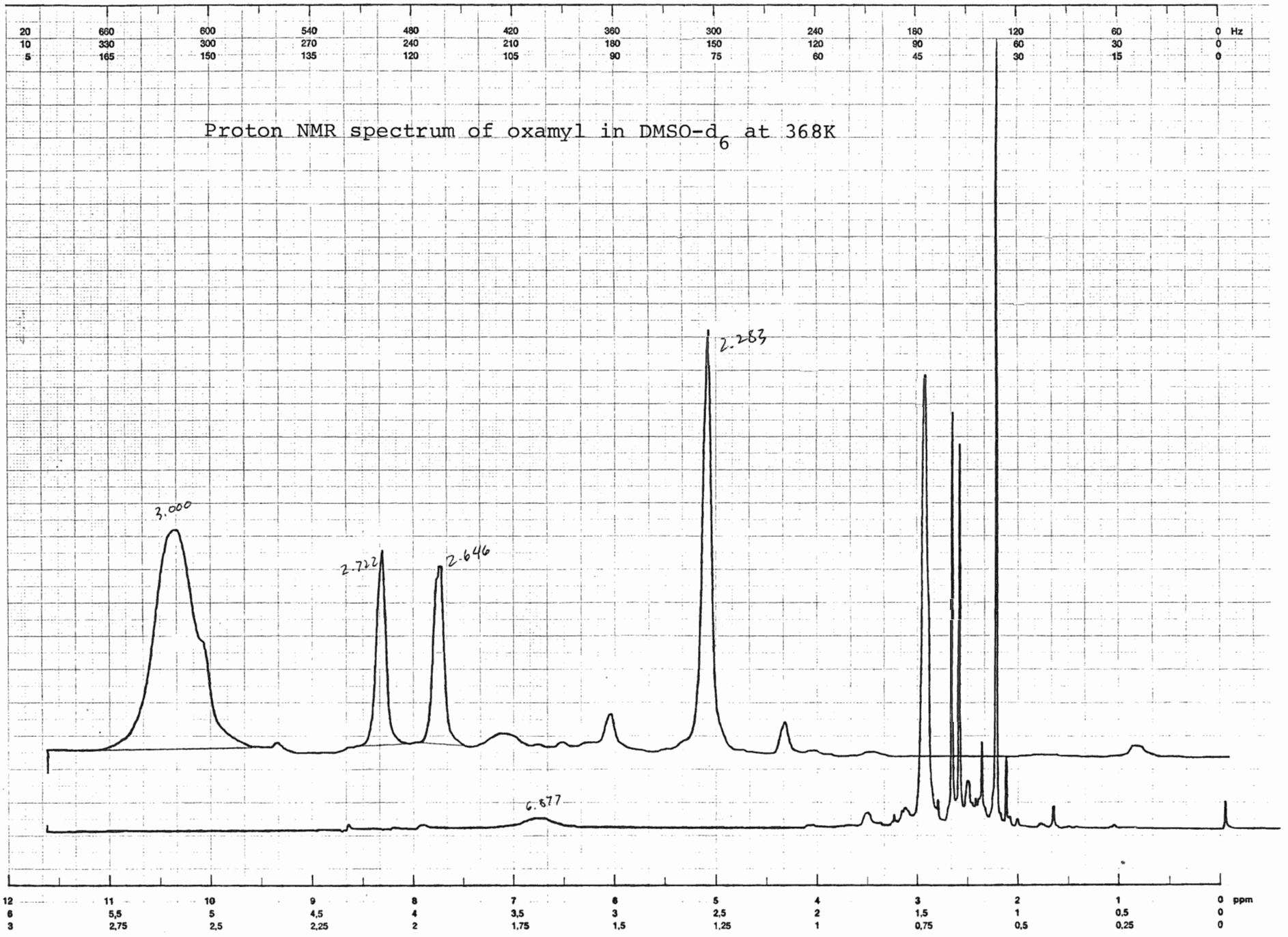
Decoupling f_2

Frequency .....

Power .....

Date Apr 23/80

Operator BDM



No .....

BRUKER
SPECTROSPIN

60 MHz - ^{1}H - Spectrum

Sample OXAMYL

Conc. ..... % in DMSO

Int. Standard TMS

Temp. amb. ☐

368 °K ☐

- 112 -

Signal f_1

Puls width (PW) 1-3 µs

Points (SI) 8 k/ 4

Spectrum

width (SW) f_1 750 Hz/

Offset (A) f_1 2900 Hz

Time 5.46

times (NS) 16 sc

Time constant (TC) 0

Decoupling f_2

Frequency .....

Power .....

Date Apr. 23/80

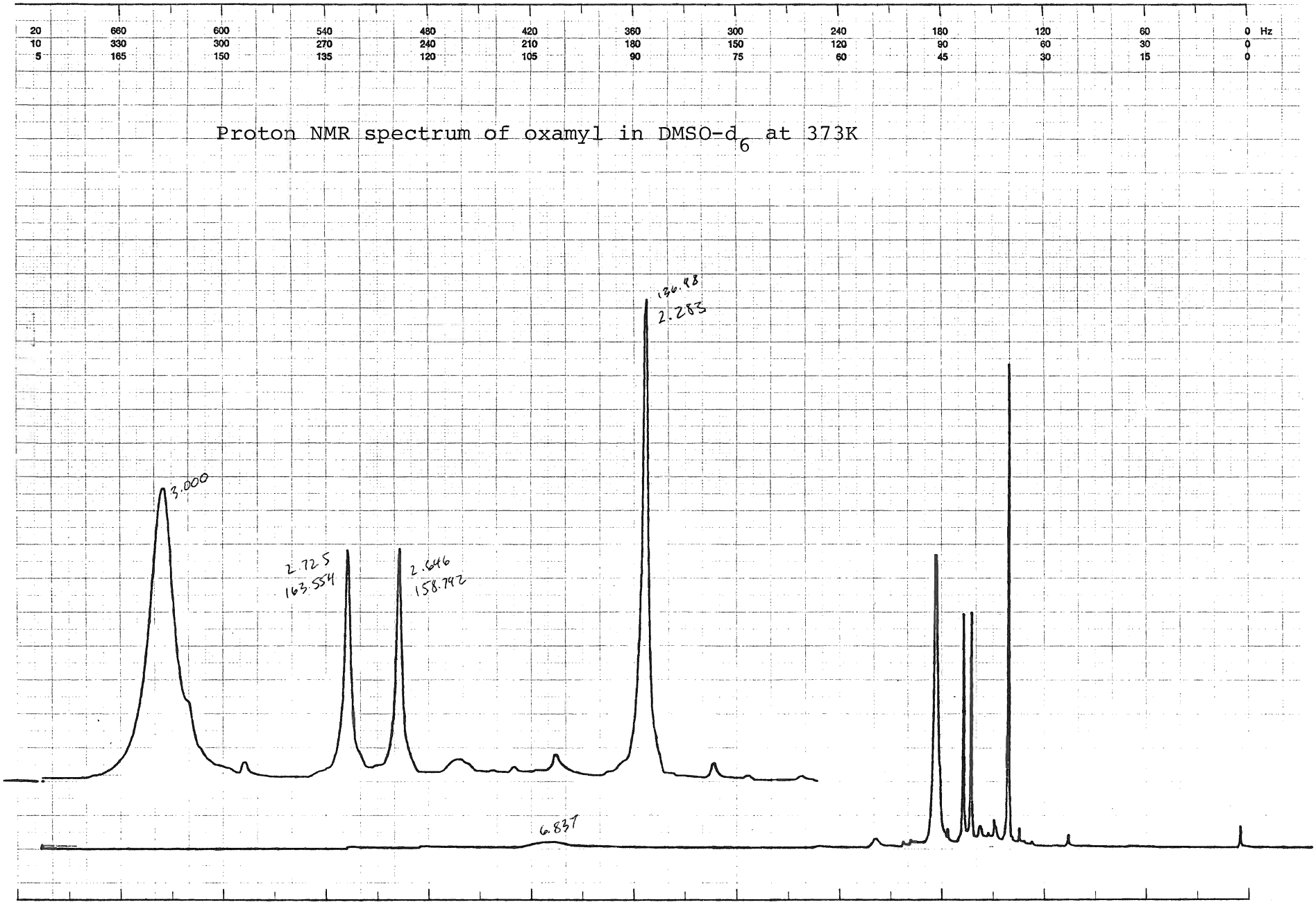
Operator JDM

No .....

BRUKER
SPECTROSPIN

60 MHz - ^{1}H - Spectrum

Proton NMR spectrum of oxamyl in DMSO-d_6 at 373K



Sample OXAMYL

Conc. ..... % in DMSO

Int. Standard TMS

Temp. amb. ☐

373 °K ☐

- 113 -

Signal f_1

Puls width (PW) 1.3 µs

Points (SI) 8 k/ 4

Spectrum

width (SW) f_1 750 Hz

Offset (A) f_1 2400 Hz

Time 5.46

times (NS) 16 sc

Time constant (TC) 0

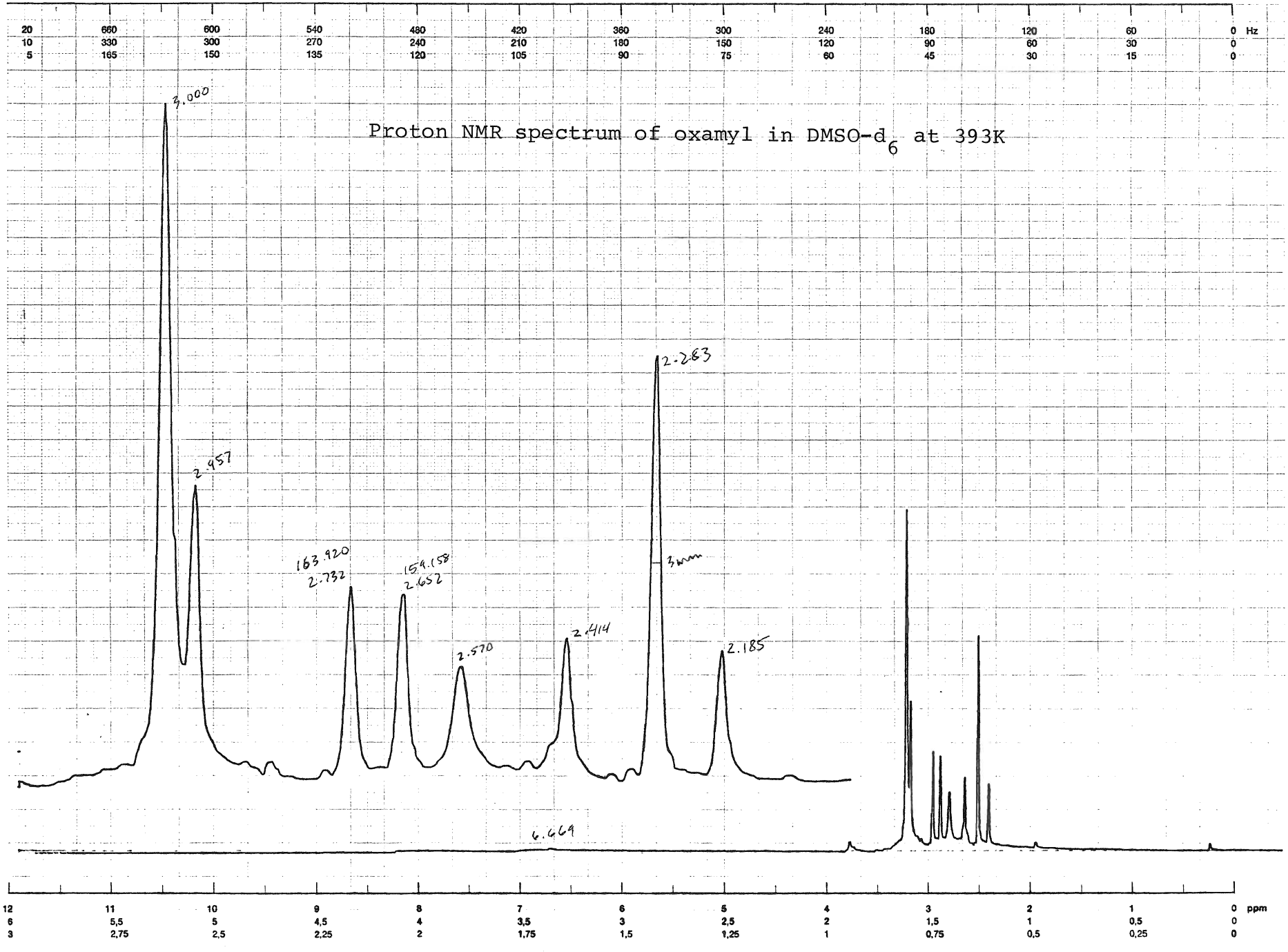
Decoupling f_2

Frequency .....

Power .....

Date Apr 23/80

Operator BZ.4



No .....

BRUKER
SPECTROSPIN

60 MHz - ^{1}H - Spectrum

Sample OXAMYL

Conc. ..... % in DMSO

Int. Standard TMS

Temp. amb. ☐

393 °K ☐

- 114 -

Signal f_0

Puls width (PW) 1.3 µs

Points (SI) 8 kW 4

Spectrum

width (SW) f_1 250 Hz

Offset (A) f_1 2400 Hz

Time 5.46

times (NS) 16 sc

Time constant (TC) 0

Decoupling f_2

Frequency .....

Power .....

Date Apr 23/80

Operator BCM

Weights of Peach Plant Parts

(Second Peach Plant Experiment)

Sampling Day	Replicate	Centre Leaf	Top Leaf	Bottom Leaf	Root	Soil	% H_2O in Soil
0	1	1.1536 g					
	2	0.9149					
1	1	0.9367	0.6352 g	1.1263 g	2.5564 g	92.8 g	3.00%
	2	1.0295	0.7452	0.6509	2.7198	94.0	3.26
4	1	1.1596	1.0827	1.0268	3.0967	93.2	3.21
	2	1.0673	0.9344	1.3961	3.0069	104.5	4.19
8	1	1.1205	1.0455	1.0441	1.9440	106.3	3.18
	2	1.1437	1.0654	1.0918	2.4600	90.0	2.59
14	1	1.1259	1.2352	1.1074	1.9371	97.3	8.80
	2	1.1277	1.1227	1.1594	2.2141	88.1	9.62
21	1	1.1362	1.1058	0.8510	3.2072	101.6	4.47
	2	1.1219	1.0907	1.0158	3.1476	87.5	4.46
30	1	1.1869	1.1223	0.8485	2.7604	93.1	5.55
	2	1.1335	1.1293	1.0703	3.1145	87.6	5.87
Control		1.1870			5.1971	95.2	6.55

Weights of Plant Parts

(Corn Seed Experiment)

Seed

Week	Untr. Nat.	Tr. Nat.	Untr. Ster.	Tr. Ster.
1	15.0 g	16.1 g	17.3 g	13.9 g
2	6.2	6.9	7.7	4.8
3	3.0	5.1	3.9	4.6
4	2.3	3.7	3.5	4.2
5	2.0	2.6	3.3	3.5
6	1.9	2.4	2.5	3.1

Root

Week	Untr. Nat.	Tr. Nat.	Untr. Ster.	Tr. Ster.
1	2.4 g	3.5	6.4 g	0.7 g
2	20.1	18.5	14.4	12.4
3	17.1	14.9	17.3	9.6
4	21.2	15.9	32.3	12.9
5	46.1	41.6	41.9	18.8
6	39.6	72.4	122.2	42.1

Leaf

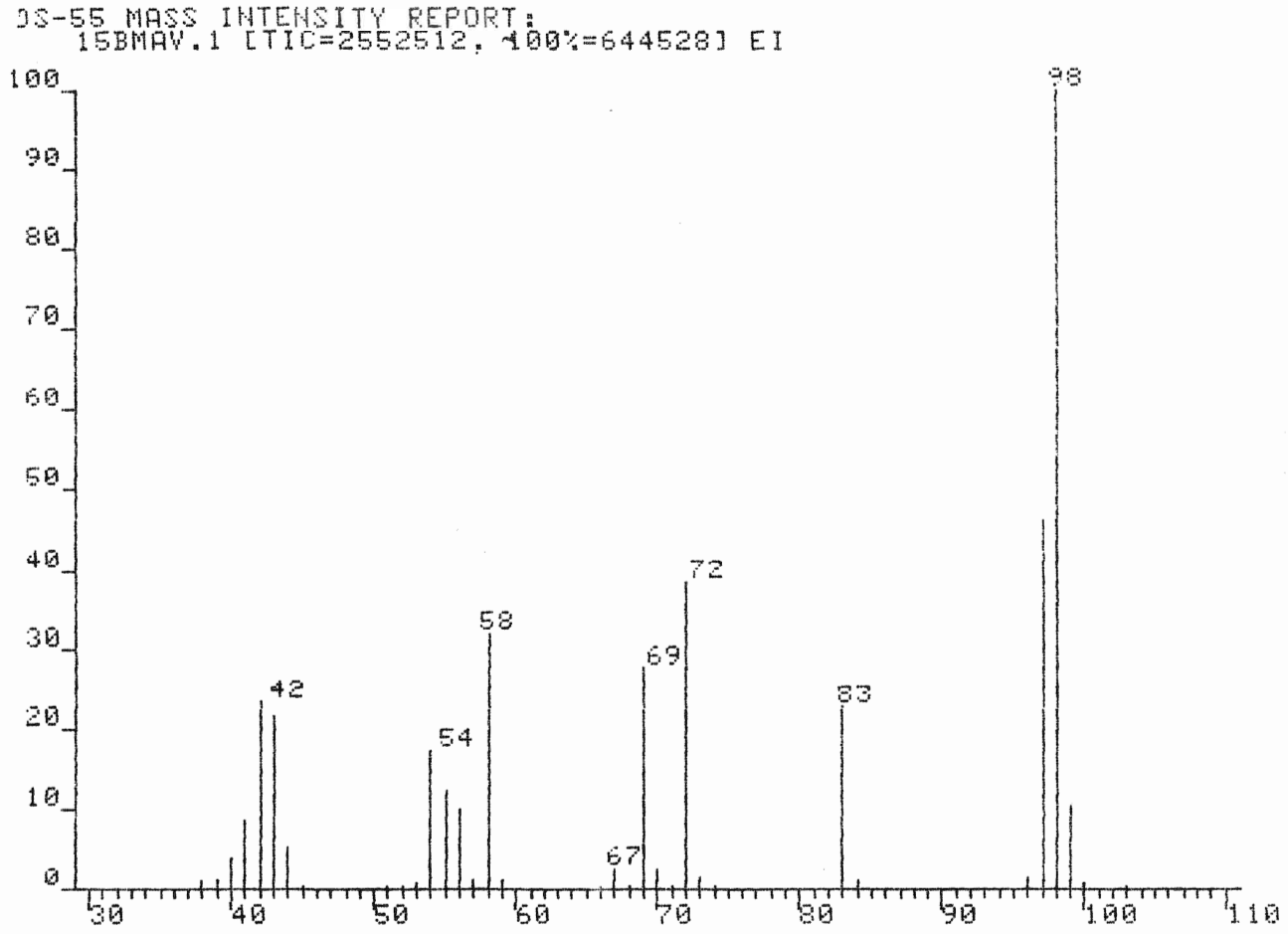
Week	Untr. Nat.	Tr. Nat.	Untr. Ster.	Tr. Ster.
1	23.4 g	11.2 g	13.9 g	2.6 g
2	58.3	35.2	26.4	8.3
3	89.6	44.4	38.9	13.9
4	126.7	83.2	100.0	29.9
5	196.3	123.0	168.2	98.4
6	306.8	242.7	263.9	138.1

Untr. - Untreated seed

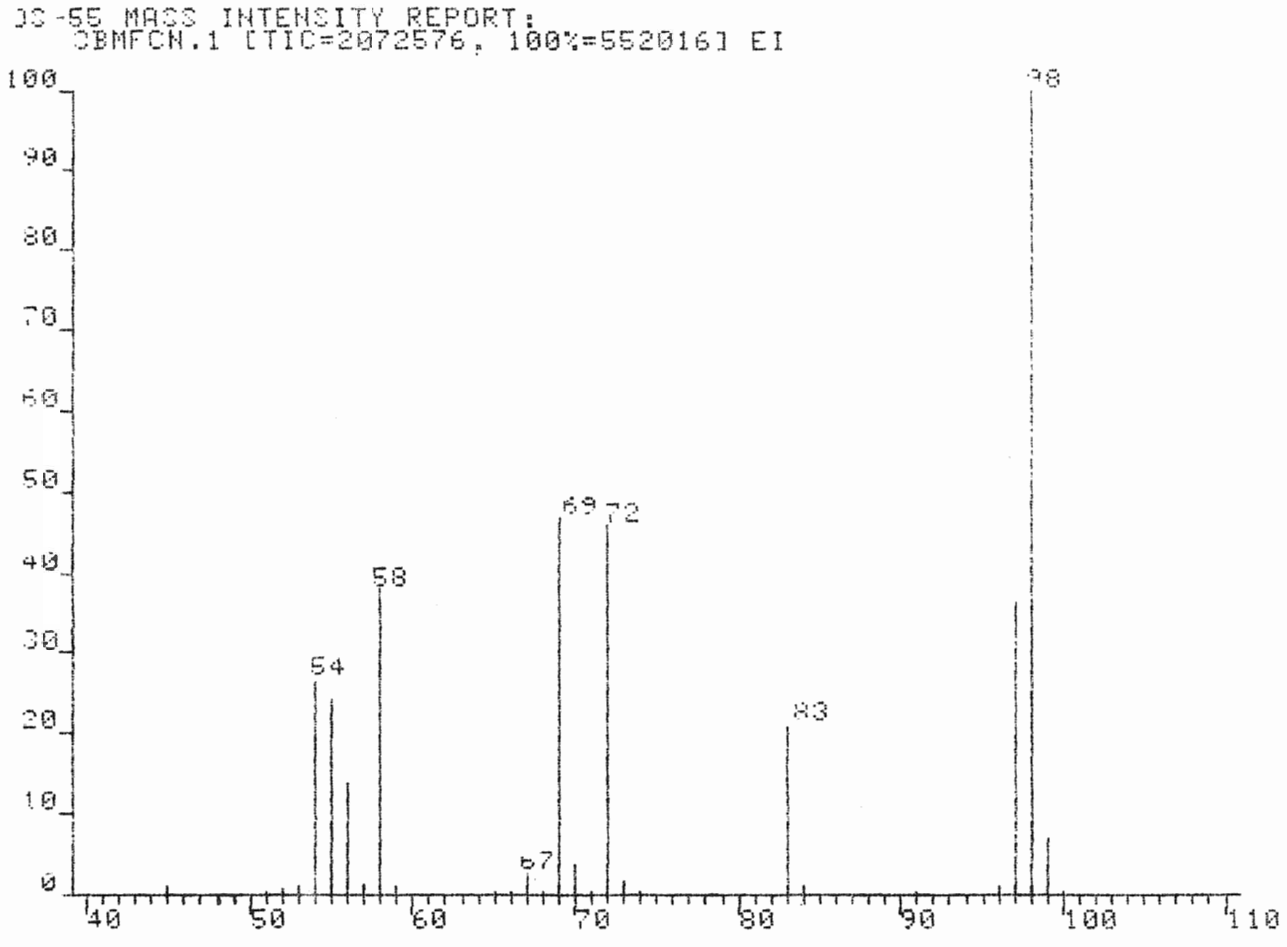
Tr. - Treated seed

Nat. - Natural soil

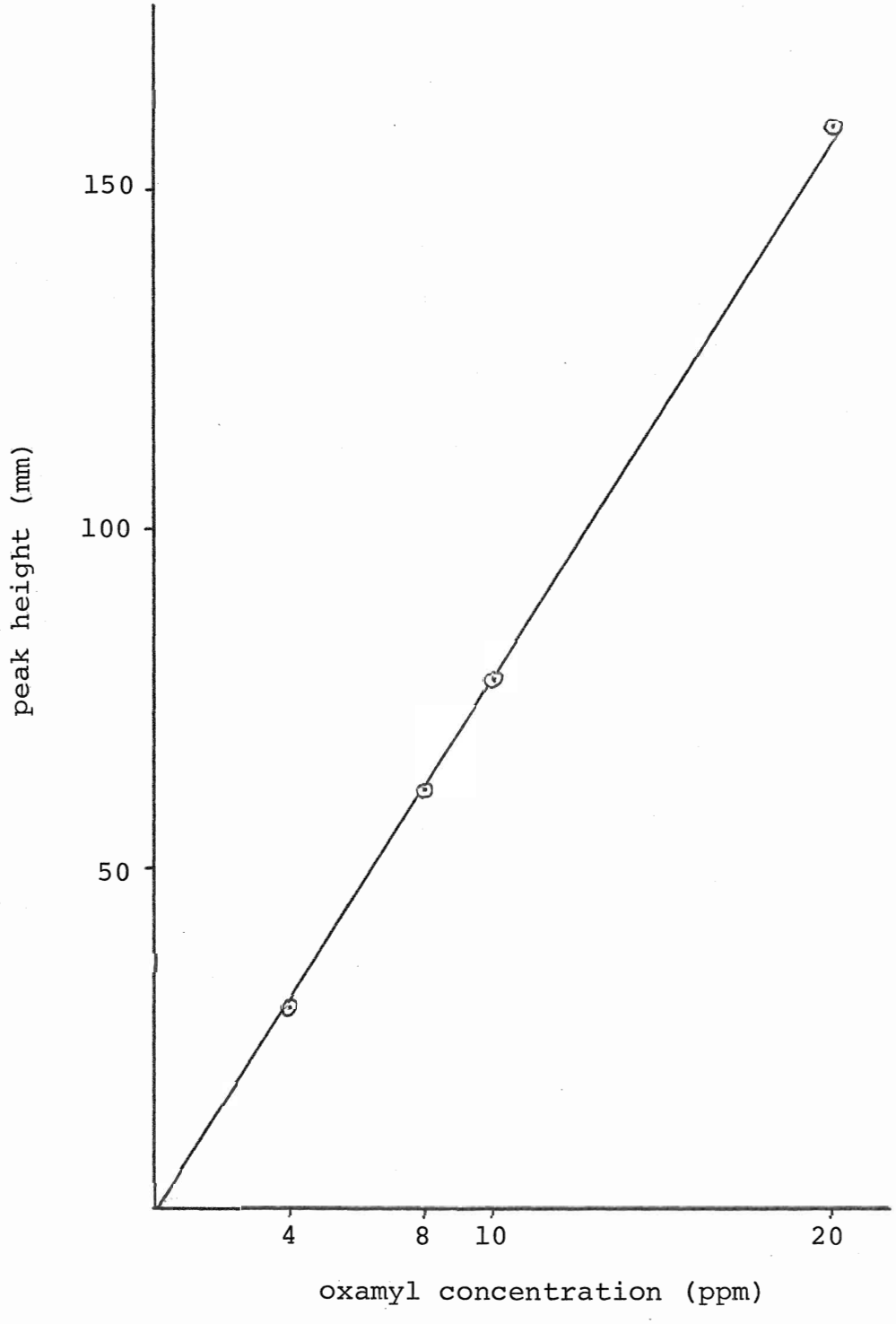
Ster. - Sterilized soil



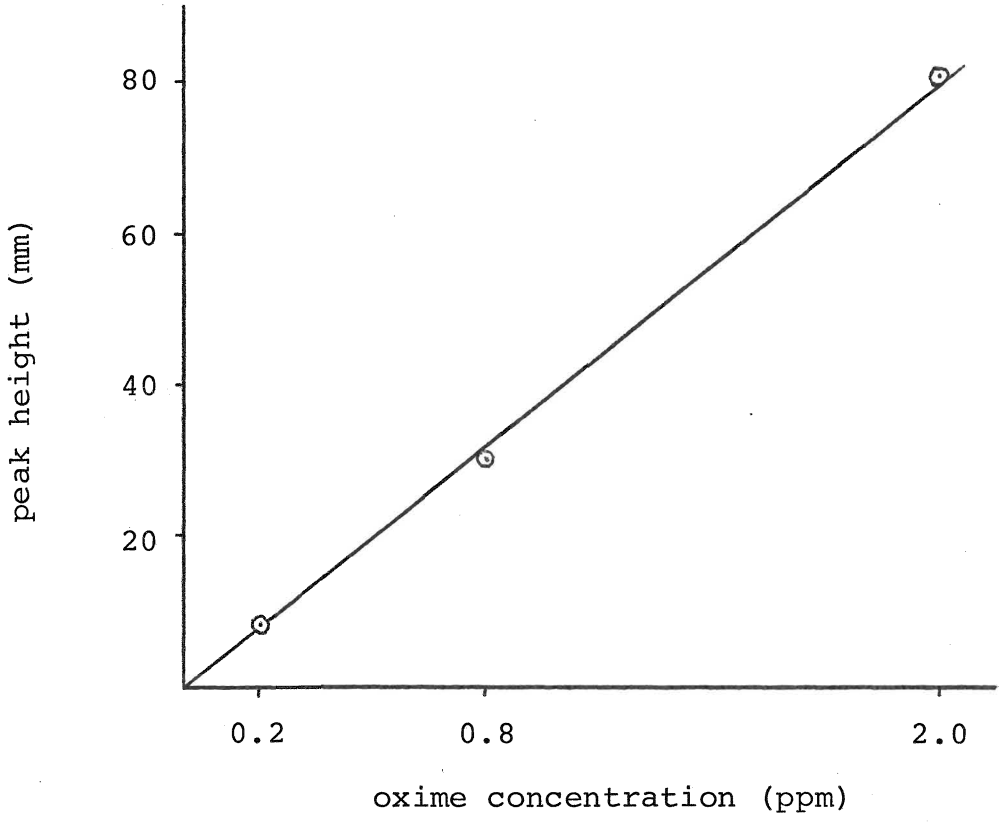
Mass spectrum of DMCF synthesized at Brock



Mass spectrum of a pure sample of DMCF obtained from DuPont



Calibration curve of oxamyl standards



Calibration curve of oxime standards