The Characterization of New Benzimidazole Fungicides

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ABSTRACT

Extensive studies have been initiated to generate enough data to register the methyl homologue (MBC-MIC, see List of Abbreviations, page 14) of benomyl (MBC-BIC) as a commercial product through a joint effort between the federal government and Canadian industry. The objective of this study, as part of the whole project, was to generate fundamental data on the physical properties of the series of benomyl homologues (MBC-MIC, MBC-EIC, MBC-PIC and MBC-BIC). These data include the half lives of these compounds in water at the pH range from 2 to 12; they ranged from 0.7 to 10.1 hours. Standard solutions of these compounds in concentrated acid were found to be stable for at least two weeks, and in the case of MBC-MIC it was stable at least 1 month.

Another major goal of this study was to determine the solubility of each compound in water at different pHs in the range of 1 to 12. The solubility of the compounds ranged from 0.6 µg/mL to 396 µg/mL. In addition, it was possible to prepare stable stock solutions at concentrations > 1000 µg/mL in concentrated nitric acid.

Several aspects of analytical methods have been improved to accurately assess the solubility and rate of degradation of benomyl and its homologues in alkaline conditions.

The determination of melting points was attempted but all compounds decomposed before melting.

To complement the studies of the benomyl homologue series attempts were made to explore the presence of any relationships between the structures of the compounds and their properties. Although there were some exceptions, the compound's solubility decreased and half life increased as the molecular size increased from the methyl to the butyl analogue.

ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Dr. Mikio Chiba for his guidance and patience, and for giving me the opportunity to work in his laboratory. The experience gained from performing my research at the Agriculture and Agri-Food Canada Pest Management Research Centre was invaluable.

From Brock University I would like to thank Dr. Richardson, Prof. Ian Brindle, Dr. Brennan, and Dr. Hartman for their helpful suggestions and encouragement. I would also like to extend my thanks to Tim Jones for obtaining the mass spectra used in this thesis.

In addition, I would like to acknowledge the staff, past and present, and people I became acquainted with during my work at the Research Centre for their advice and assistance.

DEDICATION

I would like to take this opportunity to thank my parents for their love, support and understanding throughout my academic career, which until this time, has been my life. Without their efforts this work would have been much more difficult to complete and so it is to them I make this dedication.

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LIST OF ABBREVIATIONS

2-AB	2-aminobenzimidazole
BBU	1-(2-benzimidazolyl)-3-*n*-butylurea
EBU	1-(2-benzimidazolyl)-3-*n*-ethylurea
XBU	The acronym used to classify the MBU, EBU, PBU and BBU compounds
HPLC	High Performance Liquid Chromatography
MBC	Methyl 1H-benzimidazol-2-ylcarbamate or carbendazim
MBC-BIC	Methyl [1-(butylcarbamoyl)-1H-benzimidazol-2-yl]carbamate
MBC-EIC	Methyl [1-(ethylcarbamoyl)-1H-benzimidazol-2-yl]carbamate
MBC-MIC	Methyl [1-(methylcarbamoyl)-1H-benzimidazol-2-yl]carbamate
MBC-PIC	Methyl [1-(propylcarbamoyl)-1H-benzimidazol-2-yl]carbamate
MBU	1-(2-benzimidazolyl)-3-*n*-methylurea
PBU	1-(2-benzimidazolyl)-3-*n*-propylurea
RP-HPLC	Reversed Phase - High Performance Liquid Chromatography
STB	3-butyl-2,4-dioxo-*s*-triazino[1,2-a]benzimidazole
STE	3-ethyl-2,4-dioxo-*s*-triazino[1,2-a]benzimidazole
STM	3-methyl-2,4-dioxo-*s*-triazino[1,2-a]benzimidazole
STX	The acronym used to classify the STM, STE, STP and STB compounds

INTRODUCTION

1. Pesticides in Agriculture

1.1 Definitions, Terms, and Classifications

Dramatic changes in farming practices during the second half of this century have produced larger farms and areas where only one crop is grown. In this monoculture each farm tends to specialize in one or a few crops and there is less use for crop rotation. Such regions are ideal targets for pests and disease (Hassell, 1990).

Generally, a pest may be defined as that which disturbs animals or plants. Common pests can be classified into four groups (Table 1). The first group is the invertebrates which includes insects, nematodes, molluscs, mites and ticks. The second group is vertebrates which includes rodents, birds, and fish. The third group is microorganisms which includes fungi, bacteria, algae and viruses. Finally, the plant group includes all weed plant life.

Pests can be controlled by a variety of methods (e.g., physical removal of weeds, biopesticides such as ladybugs for aphids), however, at the present time pesticides are the most often used method on most farms in most developed countries (Hassell, 1990).

The type of action a chemical compound takes upon a pest is generally described by its naming as a "-cidal" or "-static" (e.g., fungicidal or fungistatic) agent. The former description is used to classify those compounds that kill its target outright while the latter is used to classify those agents that eliminate the pest through an indirect manner (e.g., prevent growth or reproduction).

Some of the most common chemicals used to control pests are carbamates, organochlorines, and organophosphorus compounds. Each chemical class is not limited to the control of a single group of pest and examples are shown in Table 1.

Table 1: Groups of Pests, their members, and the chemical classes used to control them.

Group	Member	Chemical Classes	Example
Invertebrates	Insects	Carbamates	Carbaryl
		Organosulfurs	Tetradifon
		Organochlorines	DDT
		Organophosphorus	Malathion
	Nematodes	Carbamates	Aldicarb
		Halogenated hydrocarbons	Methyl bromide
		Isothiocyanates	Vorlex
		Organophosphorus	Phorate
	Molluscs	Carbamates	Carbaryl
		Organochlorides	Niclosamide
	Mites & Ticks	Organosulfurs	Tetradifon
Vertebrates	Rodents	Coumarins	Warfarin
		Organochlorines	DDT
	Birds	Organochlorines	Starlicide®
	Fish	Organic	Rotenone
Microorganisms	Fungi	Inorganics	Sulfur, Copper
		Dithiocarbamates	Ferbam
		Carbamates	Benomyl
		Organosulfurs	Captan
		Organophosphorus	Fosetyl
	Bacteria	Antibiotics	Streptomycin
	Algae	Inorganics	Chlorine salts
			Copper trieth-anolamine

Group	Member	Chemical Classes	Example
		Organics	Dichlone
	Viruses	Phenols	Lysol®
		Halogens	Chlorine gas
		Alcohols	Ethanol
Plants		Inorganics	Sodium Chlorate
		Organochlorines	Metolachlor
		Amides	Propanil
		Carbamates	Asulam
		Organophosphorus	Glufosinate
		Organosulfurs	Chlorosulfuron

Note: Not all types of pests and their compounds of control are listed. The examples listed may not be the best chemicals for the control of the indicated pests.

1.2 Surfactants

The most frequently used method for applying pesticides is to spray them as aqueous solutions or suspensions (Hassell, 1990). Probably due to its cheapness, availability, and lack of phytotoxicity, water is considered to be an excellent spray component. Unfortunately, the high surface tension of water is the source of a problem associated with the efficiency of spraying. Water has a low affinity for waxy leaf surfaces. Molecules of water arrange themselves so that they are relatively close together. For any molecule in general, forces of cohesion increase as the distance between molecules becomes shorter. These forces of cohesion give water its high surface tension. Small droplets from spraying tend to be spherical in nature and roll or bounce off the lipophilic leaf surfaces. This action is responsible for the loss of active ingredient.

Another problem can occur when water insoluble pesticide particles in a suspension settle under

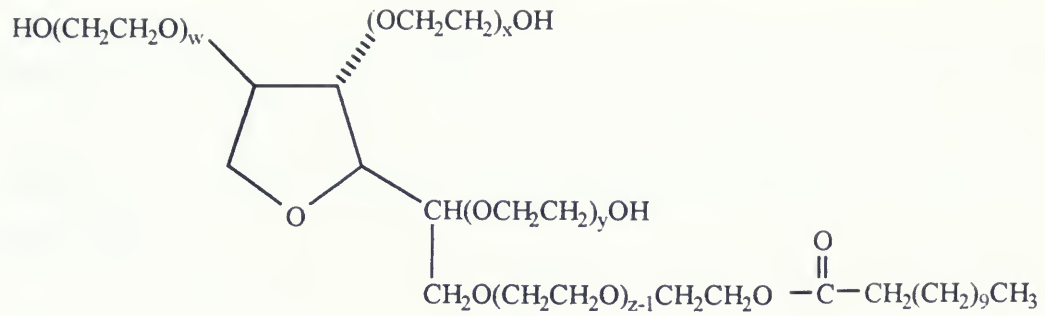
the force of gravity during spraying. Concentration gradients of pesticide occur throughout the mixture and can result in non-uniform pesticide applications.

Spray supplements are used to minimize the problems associated with pesticide applications. Emulsifiers and dispersing agents enable heterogeneous mixtures to be kept uniform, while spreading or wetting agents reduce the surface tension of the solvent and increase the contact between the spray droplets and the sprayed surface. These agents are known as surfactants.

Surfactants can be classified into 3 main groups. Anionic surfactants consist of molecules with long non-polar sections and a compact hydrophilic, negatively ionized end group (e.g., sulfated alcohols and sulfonated hydrocarbons). Cationic surfactants are similar in nature to anionic species except that they carry a compact positively charged group (e.g., quaternary ammonium compounds and quaternary heterocyclic compounds such as pyridinium derivatives). The third class of surfactants belongs to non-ionic species. Many are polyethylene oxide derivatives and include the Tween® series of compounds (Figure 1).

Anionic and cationic surfactants act as emulsifier and dispersing agents when added separately to a suspension of pesticide solid particles or oil droplets in water. The surfactant ions surround the suspended particles, creating spheres of charge which act to repel one another. These repulsions oppose the forces of gravity and deter the settling of suspended particles.

Wetting agents act by reducing the forces of cohesion between water molecules. The surfactants separate preferentially at the surface and separate the close-packed water molecules. The increase in distance between water molecules reduces cohesion forces while lower adhesion forces between surfactant and water molecules partially take their place. The decrease in surface tension reduces the spherical nature of the water droplets and increases the contact area of the pesticide and the



$$\text{Sum of } w + x + y + z = 20$$

Figure 1: The molecular structure of Tween 20®, polyoxyethylene (20) sorbitan monolaureate (Merck Index, 1990).

sprayed surface.

Since the rate of degradation of a particular pesticide in water may be influenced by its solubility (Northover and Chiba, 1989), surfactants may play an important role in the stability of some control agents.

1.3 Pesticide Market and the Need for New Pesticides

In spite of the potential impacts of pesticides and surfactants on the environment from improper use, their role as crop protecting agents is crucial in current agricultural practices. Crops are susceptible to over 120 000 pests worldwide (Ware, 1989). Between 80 000 - 100 000 diseases are caused by viruses, bacteria, mycoplasma like organisms, rickettsias, fungi, algae, and parasitic higher plants(Ware, 1989). About 30 000 species of weeds compete with crops for soil nutrients and growth space and approximately 1800 of these cause substantial economic losses (Ware, 1989). The number of nematode species that attack crop plants is near 3000 and over 1000 of these cause serious damages (Ware 1989). Furthermore, among the insect species there are about 10 000 that are plant eating (Ware 1989).

At present, the shortage of available effective chemical agents has incited a demand for new commercial compounds. Companies across the world are synthesizing thousands of compounds yearly in attempts to create new agents. Few are found to be effective and even fewer are commercially viable.

Recently it has been estimated that the worldwide demand for pesticides will grow 4.4% annually to $34 billion in 1998 (Freedonia Group, 1996). These gains are primarily expected from above average growth in developing markets (e.g., China, Brazil). Slower or declining demand in highly developed markets are expected since changes in consumer attitudes favour higher priced agents that can be applied in lower doses (Freedonia Group, 1994). In effect, the growth in volume of consumption is estimated to be only 1.1% per year to 2.6 million metric tons in 1998. Additionally, the use of alternative pest controls may reduce the quantity of pesticides sold but recent cuts in research have eliminated many such programs and may in fact increase the growth of the pesticide market more than originally estimated by Freedonia.

Today, the higher price of new pesticides reflects the rising costs of research and registration costs. New products may be subject to as many as 120 tests with investments between $35-60 million and 5-7 years of research for a single chemical (Zarcone, 1994). Some estimate that for every 8000 substances synthesized only one is likely to be successfully marketed (Hassell, 1990) while others estimate only one molecule in 15-20 000 become a developmental compound (Marchington, 1987).

The high risk in new pesticide research and development investments limits the pesticide market to those who have the resources to invest. To help ensure profitability joint marketing ventures between independent groups are common and have proven to be successful in the past (Zarcone, 1994). Such a joint venture was conducted by the Canadian Federal Department of Agriculture and Agri-Food Canada and Wilson Laboratories Inc. (a Canadian pesticide company) in an effort to develop a new compound to replace the widely used but resistant prone benzimidazole fungicide benomyl.

The need for the development of new pesticides is largely driven by the selection of resistant species. Over time pests may evolve a variety of mechanisms to resist the toxins that act against them. As resistant species populations spread pesticide applications are usually increased in quantity and frequency creating a potentially harmful effect for the environment. Eventually the control agent may become ineffective and new pesticides are required.

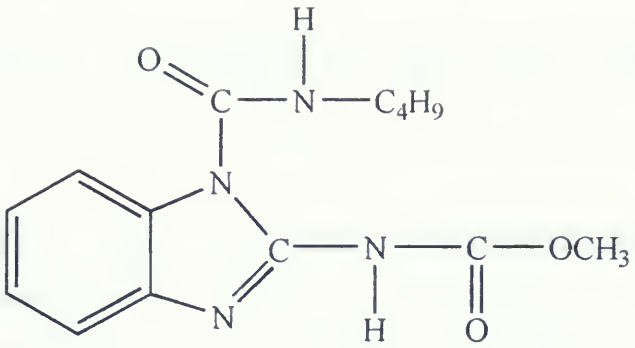
2. Benomyl

2.1 History, Uses, Mode of Action, and Resistance

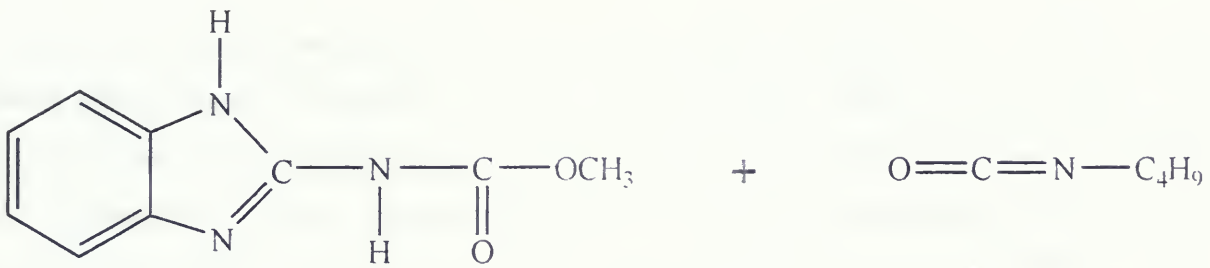
When first introduced as a commercial fungicide in 1969 benomyl (methyl [1-(butylcarbamoyl)-1H-benzimidazol-2-yl]carbamate, Figure 2) was an effective crop protection agent against many diseases including *Botrytis cinerea* Pers: Nocca & Balbis. Although its predominant use is for rice and soybean crops, benomyl has registered uses for croplands, greenhouses, ornamental plants, turf sites, preplant dips, and transplants which provided sales approximating $100 million annually worldwide in 1992 (Hanson, 1992).

The repeated use of commercial benzimidazole compounds has resulted in the evolution of resistant fungal strains (Dekker, 1976; Ishii *et al.*, 1985; Ishii *et al.*, 1984). Locally, on the Niagara peninsula of Ontario, Canada, *Botrytis cinerea* is an important pathogen of grapes and benzimidazole resistant fungal strains have evolved (Northover, 1986; Northover and Matteoni, 1986) making benomyl inefficient (Northover and Chiba, 1989).

The fungicidal action of benomyl is thought to be derived from MBC (methyl 1H-benzimidazol-2-ylcarbamate or carbendazim) (Figure 2), its natural degradation product. Although it was shown in 1973 that benomyl was more active than MBC in the inhibition of growth in some fungi



Benomyl



Carbendazim Butyl isocyanate

Figure 2: The molecular structure of MBC-BIC (benomyl) and its degradation compounds MBC (carbendazim) and BIC (butyl isocyanate).

(Hammerschlag and Sisler, 1973), past research has used MBC to elucidate benomyl's mode of action. The biochemical mode of action of benzimidazoles involves binding of the agent molecules to fungi tubulin subunits and leads to mitotic arrest (Davidse, 1986). Other effects include a reduction in the rate of linear growth and change in cell shape (Howard and Aist, 1977).

The primary mechanism of resistance of fungal pathogens appears to be from mutations in the

β-tubulin gene which create tubulin subunits with less affinity for benzimidazole molecules (Davidse, 1987).

When a mutation results in resistance to one benzimidazole fungicide, usually a cross resistance occurs and other benzimidazoles become prone to resistance as well. In contrast, there is a special kind of mutation that provides an increase in sensitivity to benzimidazole resistant fungi; this phenomenon is known as negative cross resistance. The increased sensitivity of benzimidazole resistant fungi to other compounds suggests that the resistant strains might be controlled by compounds designed to fit their altered target site (Sisler, 1988). For *Botrytis cinerea*, MDCP (N-(3,5-dichlorophenyl) carbamate) has been found to control benzimidazole resistant strains but not benzimidazole sensitive isolates (Kato *et al.*, 1984).

2.2 New Benzimidazole Fungicides

At the VIth International Congress of Pesticide Chemistry held in Ottawa, Canada in August of 1986, Chiba and Northover introduced a group of new fungicides. They synthesized methyl, ethyl, propyl, and hexyl isocyanate homologues of benomyl (MBC-MIC, MBC-EIC, MBC-PIC and MBC-HIC, respectively) (Figure 3) and compared their toxicities toward benomyl-sensitive (S) and benomyl-resistant (R) isolates of *Botrytis cinerea* using a spore germination test. MBC-MIC, MBC-EIC and MBC-PIC were equally as effective as MBC-BIC in the inhibition of germ tube length for (S) isolates but were more effective than MBC-BIC against (R) isolates. MBC-HIC was less effective than MBC-MIC, MBC-EIC and MBC-PIC against both isolates. MBC-MIC was as effective in inhibiting spore germination as the other homologues against the (R) isolate and was more active than the other homologues against the (S) isolate. The activity of MBC-EIC and MBC-PIC for the inhibition of spore germination of (S) and (R) isolates was the reverse to the relative activity of benomyl on (S) and (R) isolates. In these studies the (R) isolate was more sensitive to MBC-EIC and MBC-PIC than the (S) isolate, an exhibition of negative cross resistance. Further tests were conducted to compare the efficacy of MBC-EIC, MBC and benomyl for the protection

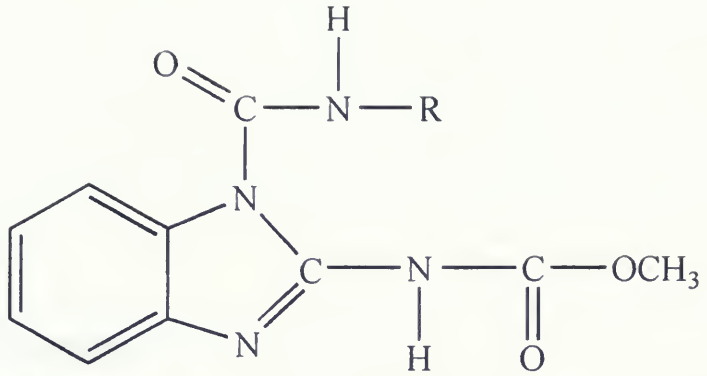


Figure 3: The basic molecular structure for benomyl and its homologues where R is the methyl, ethyl, propyl or butyl group corresponding to MBC-MIC, MBC-EIC, MBC-PIC and MBC-BIC respectively.

of wounded apples. While MBC-EIC was slightly less effective against the (S) isolate than benomyl and MBC, it was more effective against the (R) isolate than benomyl and MBC.

Additional work by Northover and Chiba (1989) compared the stability of MBC-MIC, MBC-EIC, and MBC-HIC and their efficacy against S and R isolates of *Botrytis cinerea* in comparison to that for benomyl. Tests for protection of apples wound-inoculated with S and R isolates were conducted at storage temperatures of 1°C and/or 20°C. In separate trials of 20°C and combined 1°C and 20°C programs MBC-EIC was comparable to benomyl and considered "superior" to MBC-MIC against the S isolate. Although MBC-EIC and MBC-MIC were cross resistant to benomyl against the R isolate both were much more active than benomyl. Meanwhile, at 1°C, MBC-MIC was negative cross resistant to the R isolate. This was an example of temperature dependant cross resistance since the response at 20°C was of cross resistance.

In both the 1988 and 1989 studies by Northover and Chiba it was suggested that the reason for the differences in activity may be from a dual mode of action similar to benomyl (Hammerschlag and Sisler, 1973). As mentioned earlier, the MBC moiety interrupts spindle microtubule assembly and nuclear division of S isolates (Davidse, 1986). Northover and Chiba (1988, 1989) suggested that the alkyl isocyanate moiety might be biologically active against both S and R isolates. It has

been shown that MIC, BIC, and HIC inhibited respiration and fermentation of yeast (Chiba *et al.*, 1987). However, MIC and EIC are considered to be very reactive and are expected to quickly hydrolyze to their respective amine and carbon dioxide. Further studies to determine the stability and possible modes of action of these compounds are required to make any further elucidations.

3. Properties of Benzimidazole Fungicides

3.1 Azoles, Imidazoles and Benzimidazoles

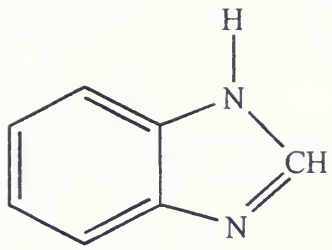
Azoles are five-membered aromatic heterocycles containing two nitrogens, one nitrogen and one oxygen, or one nitrogen and one sulfur.

Imidazole is the azole with an aromatic five-membered heterocycle containing two nitrogen atoms (Figure 4). The nitrogen without the bonded hydrogen atom has a lone pair of electrons in an sp^2 orbital that is not involved in the aromatic system. This lone pair of electrons is considered basic in behavior. The third sp^2 orbital of the other nitrogen is used to bond the hydrogen atom while the lone pair of electrons takes part in the aromatic ring. This nitrogen is not very basic in nature (Wade, 1991).

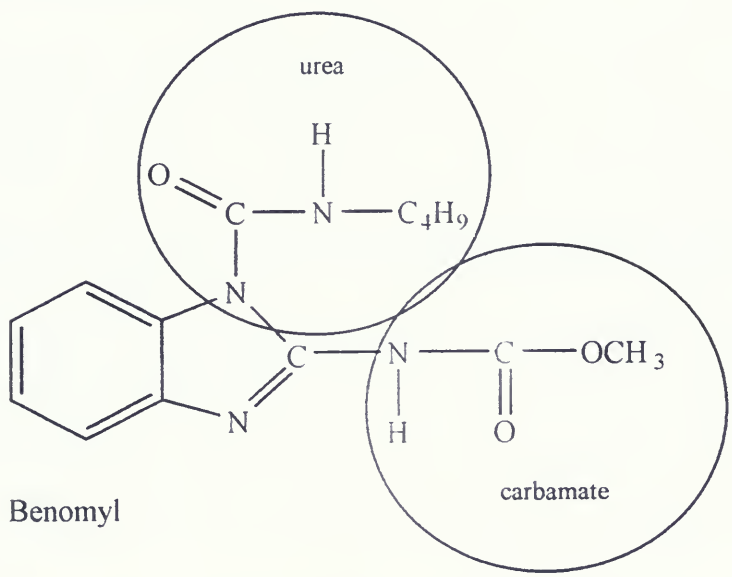
Benzimidazole compounds are simply imidazoles with a benzene ring as part of the structure at carbon positions 4 and 5 (Figure 4).

3.2 Isocyanates, Carbamates and Ureas

Isocyanates may be prepared by S_N2 displacement of alkyl halides with cyanate ion. This reaction occurs preferentially at the nitrogen end (Streitwieser and Heathcock, 1981):



Benzimidazole



Benomyl

Figure 4: The molecular structures of imidazole and benzimidazole and the fungicide benomyl. Benomyl has been labeled to further identify functional groups that compose the molecule.

$CH_3Br + Na^- NCO^-$ ----------> CH3-N=C=O

methyl isocyanate

These compounds react with water to give N-alkyl carbamic acids that are unstable and spontaneously lose carbon dioxide to give corresponding amines (Streitwieser and Heathcock, 1981):

$CH_3\text{-}N{=}C{=}O + H_2O$ ---> $[CH_3\text{-}NH\text{-}COOH]$ ---> CH_3NH_2 + CO_2

methyl amine carbon dioxide

When reacted with alcohols, isocyanates produce carbamate esters which are also called urethanes (Streitwieser and Heathcock, 1981):

R-NCO + R'OH -----> R-NH-CO-O-R'

isocyanate alcohol carbamate

Combined with amines, isocyanates produce ureas (Streitwieser and Heathcock, 1981):

$R\text{-}NCO + R'NH_2$ ----> R-NH-CO-NH-R'

isocyanate amine urea

4. Chemistry of Benzimidazole Fungicides

4.1 Benomyl Chemistry

Benomyl decomposes (Figure 5) in water (Clemons and Sisler, 1969; Peterson and Edgington, 1969) and organic solvents (Chiba, 1977a; Chiba and Cherniak, 1978; Chiba and Doornbos, 1974) but is stable in concentrated acidic solutions (Singh *et al.*, 1992; Singh *et al.*, 1993) (see Fig 6).

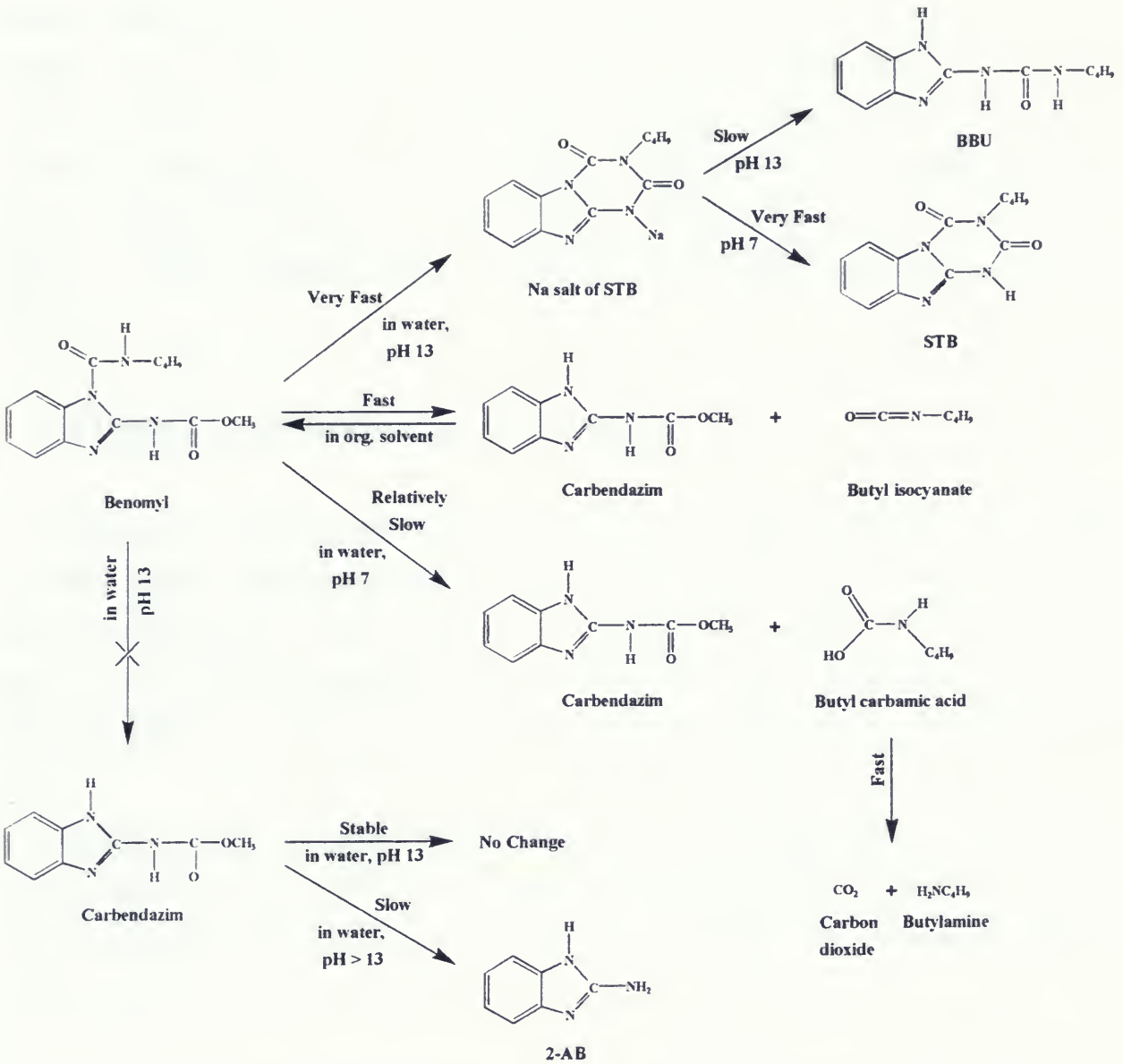


Figure 5 : Degradation of benomyl in aqueous and organic solvents at room temperature (Chiba and Singh, 1986).

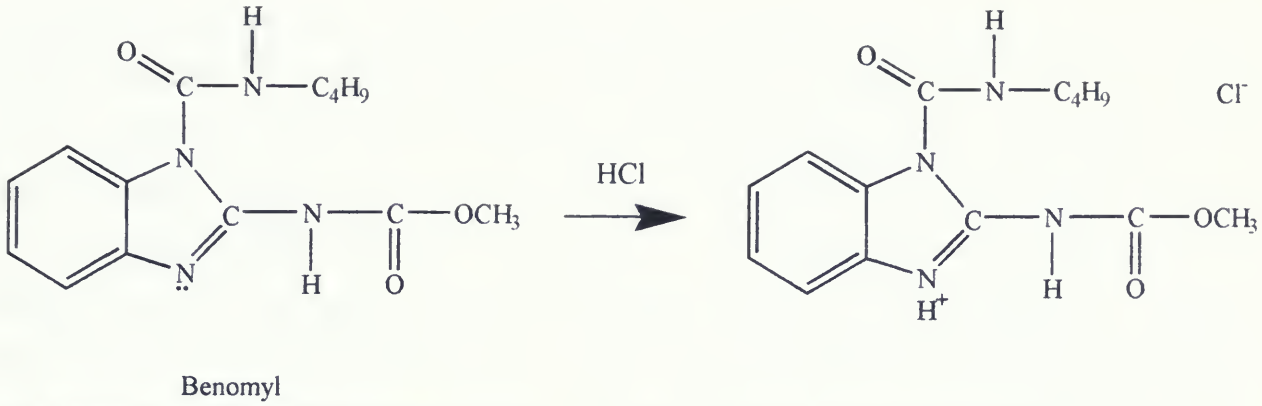
Its rate of degradation to MBC depends on time, temperature, and solvent (Chiba, 1977b) and is relatively slow in water (Baude *et al.*, 1973) or slower than in common organic solvents (Chiba, 1975). Early studies showed that depending on the alkalinity, benomyl can convert to MBC, STB (3-butyl-2,4-dioxo-*s*-triazino[1,2-a]benzimidazole), or BBU (1-(2-benzimidazolyl)-3-*n*-butylurea) (White *et al.*, 1973). Benomyl was reported to have converted to STB in alkali followed by slow conversion to BBU in standing solutions. The quantity of BBU depended on time, temperature, and alkalinity.

4.2 Degradation and Solubility in Aqueous Media

The first kinetic studies of benomyl decomposition were studied by Calmon and Sayag (1976a, 1976b) in aqueous-methanol solutions (50:50, v/v). The observed kinetic rate constant (k_{obs}) values indicated that below pH 2.5 the decomposition of benomyl was inhibited while over the range of pH 2.5 to 7.0 the rate was pH independent (Calmon and Sayag, 1976a). The kinetics of degradation to STB and BBU were studied in alkali solutions and several reaction mechanisms were proposed.

The works done by Calmon and Sayag (1976a,b) show a trend of degradation rates over a range of pH values but these results cannot be used quantitatively to develop analytical methods for benomyl determination since the experiments were performed using buffer-methanol solutions and not pure water.

In 1990, Singh *et al.* reported the first systematic study on the kinetics of the conversion of benomyl to MBC in aqueous solutions without organic solvent. The experiments were carried out using distilled water and aqueous solutions of pH 1 to 7 at room temperature (21 ± 1 °C). Similar values for the first order rate constants were found in the pH 2-7 range ($k = 2.5$ to $3.7 \times 10^{-5} s^{-1}$) but a significant decrease was observed at pH 1 ($k = 1.0 \times 10^{-5} s^{-1}$) where an increase in solubility was also observed. The authors were in agreement with suggestions made by Calmon and Sayag, 1976a) that benomyl is stabilized by protonation of the nitrogen in the benzimidazole group (Figure 6)



Benomyl

Figure 6: The stabilization of benomyl by the protonation of the nitrogen in the benzimidazole group under acidic conditions.

under acidic conditions (pH <2).

These results prompted Singh *et al.* to study the preparation of stable stock solutions of benomyl with an acidic medium (Singh *et al.*, 1992; Singh *et al.*, 1993) where results were in contrast with previous reports (Pyysalo, 1977; Spittler *et al.*, 1984). Between 0.1 and 0.001 M HCl solutions the MBC-BIC half lives were 16 to 5.3 hours (k = 1.2 X 10^{-5} s^{-1} and 3.6 X 10^{-5} s^{-1}). At acidic concentrations of 1.0 and 2.5 molar a sharp increase was observed in the half lives to 2.5 X 10^{2} hrs (k = 7.7 X 10^{-7} s^{-1}) and 1.5 X 10^{3} hrs (k = 1.3 X 10^{-7} s^{-1}) respectively. At 5.0 and 10.0 M nitric acid concentrations benomyl was found to be stable (Singh *et al.*, 1992).

Additional work conducted at the Federal Ministry of Agriculture and Agri-Food Canada Pest Management Research Centre by Aboaen (1993) determined the decomposition kinetic rates of MBC-MIC in various aqueous and organic solvents. The half life of MBC-MIC when dissolved in water was 7.4 hours (k = 2.6 X 10^{-5} s^{-1}) while in nitric acid (pH 1.3) it was 14.8 hours (k = 1.3 X 10^{-5} s^{-1}).

The stability of the new benomyl homologues was studied by Northover and Chiba (1989) to determine the effects of temperature upon the rate of in vitro degradation. Methyl, ethyl, and hexyl

isocyanate homologues of benomyl were prepared and compared with benomyl at concentrations of 178, 17.8 and 1.78 μM in water (pH 6.2). The compounds were less stable at 10 and 25°C than at 1°C. Suspensions of 178 μM had half life values of 27 to 42 days at 1°C while suspensions of lower concentrations were increasingly unstable. The half lives for 178 μM mixtures were 2.7 to 7.7 times greater than at 1.78 μM concentrations. This was expected by the authors since the compounds have low aqueous solubilities, possibly similar to that of benomyl. At highly concentrated suspensions most of the parent compound exists as particulate, insoluble in a saturated solution and therefore chemically stable. It was supposed that only the parent molecules that entered the solution could degrade and that the rate of degradation would have been influenced by the solubility of the compounds in aqueous suspensions. In the preparation of the samples dextrose and Tween 20 were used as a grinding aid and surfactant respectively. These additives may have had some effect on the solubility and degradation rates of the fungicides.

In 1985 Singh and Chiba investigated the solubility of benomyl between the pH values of 1 to 13 and identified the corresponding degradation compounds and their quantities in an effort to accurately understand the behaviour of benomyl in water. Previous to this study the only value reported for the solubility of benomyl was 3.8 ppm at 20°C and pH 7 (Austin *et al.*, 1976). Singh and Chiba observed low solubility values for benomyl within the pH range of 3 to 10 (1.8 to 4.0 μg/mL) and higher values at the extreme pH values of 1 and $\geq$11 (18.2 and 8.8 mg/mL respectively). At pH 13 no benomyl was detected due to conversion to STB.

5. Methods of Analysis

5.1 Determination of Benomyl and Degradation Products

The extensive use and suspected carcinogenicity of benomyl and its degradation products makes their determination in water, soil extracts, and crops vital. In addition, scientific studies of efficacy and modes of action require accurate analytical methods of analysis.

Since the new fungicide analogues are similar in structure to benomyl it is possible that these compounds exhibit similar physical and chemical behaviour. Therefore, previous methods of analysis for benomyl might be applicable to the analysis of the new benzimidazole fungicide analogues. Early methods of analysis are reviewed by Slade (1975) and Gorbach (1980).

Probably most popular were methods which determined benomyl as carbendazim (Austin and Briggs, 1976; Kirkland, 1973; Kirkland *et al.*, 1973). Benomyl was not directly determined due to the many problems associated with its instability in organic and aqueous solvents. In addition, MBC is also fungitoxic and the fungitoxicity of benomyl is thought to be due to the presence of carbendazim (Clemons and Sisler, 1971). Although it has been widely used in the past this method is not acceptable in principle since MBC is a natural degradation product of benomyl. The MBC produced during the sample preparation cannot be distinguished from the MBC that was present in the sample and analysis can lead to overestimating benomyl content. In certain areas MBC is registered for use as a fungicide on some fruits and vegetables and determining MBC as benomyl would give positive errors. Furthermore, the fungitoxicity of carbendazim is different from benomyl (Hall, 1980; Koller *et al.*, 1982) so the total quantity expressed as MBC or benomyl cannot be used to assess the toxicity or biological activity of fungicides present in a test sample (Chiba and Singh, 1986).

A review of chromatographic methods of benomyl analysis is given by Singh and Chiba (1993a).

5.2 Thin Layer and Gas Chromatography

As stated by Singh and Chiba (1993a) thin layer chromatography (TLC) can be used as a simple method for the separation of benomyl as MBC from coextractives from water, wettable powder (WP) formulations, and crops and for quantitative determination of benomyl as MBC. Although this method has been used in the past (White and Kilgore, 1972, Baker *et al.*, 1973) these studies are limited as mentioned above from using MBC as an indication of benomyl quantities

which results in positive error. TLC has been used to directly separate benomyl from its degradation products (von Stryk, 1972) and to determine intact concentrations of benomyl and MBC in crops by converting the parent compound to BBU (Baude *et al.*, 1973).

Gas Chromatography has also been used to determine MBC and benomyl as MBC (Rouchaud and Decallone, 1974; Pyysalo, 1977; Cline, 1981). This method suffers from the limitations of determining benomyl as MBC and also requires the additional labour of derivatizing MBC before analysis by GC.

5.3 High Performance Liquid Chromatography

High Performance Liquid Chromatography (HPLC) is probably the most common technique used for the analysis of benomyl and its degradation products. These compounds are suitable for LC analysis because their polarity, low volatility, and thermolability make them unlikely candidates for gas chromatography-mass spectrometry (GC-MS) methods. An added advantage of LC techniques for such compounds is that analytical procedures which are often required for GC analysis to increase a compound's thermolability or volatility are minimized.

Liquid chromatographic detection of pesticides is normally carried out using ultaviolet (UV) or electrochemical detection. From the review by Singh and Chiba (1993) it becomes apparent that UV detection is most popular for benomyl and its degradation compounds. During the analysis of pesticides these methods may not provide the selectivity and/or sensitivity for target compounds in complex (e.g., environmental) samples that mass spectrometry detection can give (Lamoree *et al.*, 1993). Furthermore, the legal implications related to environmental data may make the coupling of LC with MS an important technique for the future (Crescenzi, 1995).

Singh and Chiba (1993a) have divided methods of benomyl determination by HPLC into 6 categories and are briefly discussed in the following sections.

1. Determination of benomyl as MBC after its quantitative conversion to MBC.

The problems with this method pertain to overestimation of benomyl quantities in samples and interpretations for toxicological data in pathological studies and have been discussed in Section 5.1 above.

2. Determination of benomyl after its stabilization in organic solvents by BIC.

Since benomyl degradation to MBC is reversible in most organic solutions (Chiba and Cherniak, 1978) wettable powder formulations were extracted with acetonitrile that contained 3% *n*-butyl isocyanate (BIC) and benomyl was determined directly by RP-HPLC (Stringham and Teubert, 1984; Teubert and Stringham, 1984). The limitations with this method are similar to those for the determination of benomyl as MBC since any carbendazim present in the sample will be converted to the parent compound and a positive error will result. In addition the use of potentially dangerous isocyanates are required during sample preparation.

3. Simultaneous determination of benomyl and MBC after selective conversion of MBC to MBC-PIC (n-propyl isocyanate) and stabilization of benomyl by the addition of excess BIC.

This method is based on the principle that benomyl does not degrade significantly in chloroform at 1°C for at least 75 minutes (Chiba and Veres, 1980). The degradation compound MBC is difficult to extract with chloroform and derivatization is required. In an extraction with 5000 µg *n*-PIC/mL chloroform any MBC in the sample is converted to the easily extracted MBC-PIC while benomyl remains intact. To ensure the stability of benomyl during the extraction 5000 ug *n*-BIC/mL chloroform is added and the sample is analyzed by RP-HPLC. This method's advantages include the simultaneous determination of benomyl and MBC which also allows the determination of the fate of benomyl after its spray on crops. The disadvantages are that extractions at low temperatures and the use of potentially dangerous isocyanates are required.

4. Simultaneous determination of benomyl and MBC after quantitative conversion of benomyl to STB (STB method)

In 1986 Chiba and Singh reported a RP-HPLC method where benomyl was quantitatively

converted to STB with pH = 13 NaOH and determined as STB while any MBC present in the sample was unaffected and determined as MBC. The resolution and peak height of MBC and STB depended not only on the composition and pH of the mobile phase but also the composition of the sample solutions prepared for HPLC analysis. For accurate determinations the standard and sample solution must be prepared with identical compositions to ensure comparable peak shapes and resolutions. The method has been used for the analysis of benomyl and its degradation compounds in aqueous solutions of different pH values (Singh and Chiba, 1985), water and WP formulations (Chiba and Singh, 1986; Singh and Chiba, 1993b), organic solvents (Singh *et al.*, 1990), and pathological studies (Chiba *et al.*, 1987). This simple method was described as reproducible, accurate, and suitable for routine determinations (Singh and Chiba, 1993b) and includes the advantages of an ability to determine benomyl (as STB) and MBC simultaneously without either cold temperatures or derivatizations with potentially dangerous isocyanates.

5. Determination of benomyl and MBC after their respective conversions to BBU and 2-AB

Similar to the STB method, Chiba (1987) reported a method that converts benomyl in strong alkali solution (pH = 13.2) to its degradation compound BBU. Unlike the STB method MBC is quantitatively converted in the higher alkali solution to 2-AB (2-aminobenzimidazole). Using the slightly higher pH condition for conversion of benomyl to BBU, this method has the same advantages as the STB method. A slight disadvantage is that the analysis of BBU and 2-AB may require a longer RP-HPLC run time or greater amounts of organic solvent in the mobile phase than that for STB and MBC (see results from Singh and Chiba, 1985).

6. Direct Determination of Benomyl

Benomyl can be separated in its intact form from its degradation products and other pesticides by HPLC (Austin *et al.*,1976; Cabras *et al.*, 1979; Singh and Chiba, 1985; Chiba and Northover, 1988; Northover and Chiba, 1989; Singh *et al.*, 1990; Marvin *et al.*,1990 & 1991). Although it was possible to study the biological activity of benomyl (Chiba and Northover, 1988; Northover and Chiba, 1989) and the kinetics of benomyl degradations in solutions (Singh *et al.*, 1990), the unavailability of stable concentrated aqueous stock solutions at room temperature made

the direct analysis of benomyl by HPLC impossible until 1992 when Singh *et al.* reported the preparation of stable stock solutions of benomyl in an acidic medium (Singh *et al.*, 1992; Singh *et al.*, 1993). These results were in contrast with previous reports (Pyysalo, 1977; Spittler *et al.*, 1984). An advantage of this method is that it avoids converting benomyl to its degradation compounds but dilutions are needed to meet the recommended pH limit for reversed-phased analytical columns (pH 2-7). Chloride ions from HCl can also damage the stainless steel components of a HPLC system. In addition, longer RP-HPLC run times or greater amounts of organic solvent in the mobile phase may be required for the elution of benomyl compared to its degradation products (see results from Singh and Chiba, 1985). Further work is required to validate this method for use in the extraction and determination of benomyl in real samples.

6. Purpose of this Project

Extensive studies have been initiated to generate enough data to register the methyl homologue (MBC-MIC) of benomyl as the first commercial product through a joint effort between the federal government and industry. The objective of this study, initiated as part of the above project, is to generate fundamental data on the properties of not only MBC-MIC, but also two other homologues, MBC-EIC and MBC-PIC by using benomyl (MBC-BIC) as the reference compound. These data will include the solubility and degradation rates of the new benomyl analogues in aqueous media over a range of pH values. Comparisons will be made between different pH conditions and with the use of surfactants. This information will be vital in interpreting biological data, design of commercial formulations and practices in the field. In addition, structure activity relationships between the analogues and their properties will be explored.

EXPERIMENTAL

1. Instrumentation

1.1 HPLC Apparatus

Two HPLC systems were used. A Hewlett Packard HP-1090 unit equipped with a 79835A solvent delivery system, a 79846A autoinjector, a 79847A autosampler set for 20 µL injections, a 1040A diode-array UV absorption detector and HP Chemstation software was used for the confirmation of the authenticities of the compounds. For all other studies, a Spectra Physics SP-8000 chromatographic system equipped with a SP 8880 Autosampler set for 80 µL injections, SP 8800 Ternary pump, SP 8490 UV/Vis detector at 280 nm, and an integrator with WOW (Winner On Windows) software was used.

Reverse phase (C_{18}) analytical columns and precolumns were employed for the analysis of the samples. An Upchurch 0.2 µm titanium frit was placed in line before the analytical column. All other detailed operational parameters are described in relevant experimental sections.

1.2 Mass Spectrometer

Mass spectroscopic analyses were performed with a Kratos Concept IS double-focusing E/B configuration type machine The source was operated in EI mode at a temperature of 180 °C with 70 eV potential. The mass spectrometer was interfaced to a Kratos DART speed data acquisition and control system linked to software running on a Sparcstation 10 computer.

1.3 General

A Horiba F-13 pH meter (Kyoto, Japan) was used with a three point calibration for all measurements of pH. Buffers of pH 4, 7, and 10 (BDH) were used for the calibrations.

For the solubilization of samples two methods were used. A Cavitator Ultrasonic Cleaner bath (Mettler Electronics Corp., Anaheim, CA) was used during the degradation studies and a home made tissue blending homogenizer was a unit assembled at Agriculture and Agri-Food Canada (Vineland Station, Ont.) was used during the solubility studies. The blending speed of the homogenizer was controlled using a rheostat set at 45.

To keep the samples at constant temperature during homogenization a water bath was employed. The water bath was a combination of a Braun Frigomix U-1 cooling system and Thermomix BU heating system (Torrance, CA). The temperature of the water was 24 ± 1 °C.

Syringes were of gas-tight design from Hamilton (Reno, Nevada). Needles for drawing samples were of 21 gauge while delivering filtered samples were done with 12 gauge needles.

The melting point apparatus (serial # BU 0016) assembled by the Brock University Chemistry Department (St. Catharines, Ont.) had the following configuration: 115 V, 60 Hz, a current draw of 750 MA, and a 1A external fuse. For all compounds the temperature control was set at 15 with the boost switch on from 100 to 240 °C. At 240 °C the temperature control was raised to 30 until 290 °C. Samples were packed about 1 cm in height into capillary tubes.

2. Materials

2.1 Chemicals and Solvents

The HPLC mobile phase solutions used in this study were acetonitrile of HPLC grade from Caledon Laboratories, Ltd. (Georgetown, Ont.), reverse osmosis water and buffer solutions. Mobile phase solutions were filtered and vacuum degassed using 0.45 um nylon filter paper from Chromatographic Specialties Inc. (Brockville, Ont) and continuously sparged by a stream of helium gas.

Reverse osmosis water was prepared at Agriculture and Agri-Food Canada, Pest Management Research Centre, Vineland Station, Ontario. Throughout this study water refers to reverse osmosis water unless otherwise noted.

Additional reagents and solvents used in the experiments are listed below:

- Propyl isocyanate (PIC) and butyl isocyanate (BIC) were obtained from Aldrich Chemical Company Inc. (Milwaukee, Wis.) And Eastman Kodak Co. (Rochester, NY), respectively.
- Disodium hydrogen phosphate (Na_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4) were of HPLC grade from Fisher Scientific (Nepean, Ont.).
- HPLC grade methanol (MeOH), chloroform and distilled in glass hexane used in the synthesis were also from Caledon Laboratories Ltd.
- Nitric acid (73%) and hydrochloric acid (37%) were reagent grade from Caledon Laboratories, Ltd.
- Sodium Hydroxide (reagent grade), Tween 20 and Tween 80 were obtained from Fisher Scientific (Unionville, Ont.).
- Bacto-Dextrose was obtained from DIFCO Laboratories (Detroit, Mich.)

2.2 Buffer Solutions

Phosphate buffer solutions were prepared from Na_2HPO_4 and KH_2PO_4. Two separate 0.067 M stock solutions were prepared from approximately 19.0 g Na_2HPO_4 dissolved in 2 L water and from approximately 18.2 g KH_2PO_4 dissolved in 2 L water. Two separate 0.001 M stock solutions were prepared from approximately 0.142 g Na_2HPO_4 dissolved in 1 L water and from approximately 0.136 g KH_2PO_4 dissolved in 1 L water. All stock solutions were filtered and vacuum degassed using 0.45 um nylon filter paper from Chromatographic Specialties Inc. (Brockville, Ont) and stored in the dark to prevent micro-organism contamination.

Mobile phase buffer solutions of pH 6 and 7 was prepared at 0.067 M using the Na_2HPO_4 and

KH_2PO_4 stock solutions (10:90 and 60:40, v/v, respectively). The buffer solutions were adjusted to the desired pH value by monitoring with the pH meter.

Solutions for sample preparations between pH 1- 13 were prepared as follows: pH 1-4 with HNO_3 by serial dilutions from 1 M HNO_3, pH 6 with 0.001 M Na_2HPO_4 and KH_2PO_4 (85:15, v/v), pH 6 with Tween 20 at 0.05 mg/mL, pH 8 with 0.001 M Na_2HPO_4 and KH_2PO_4 (95:5 v/v), pH 8 with Tween 20 at 0.05 mg/mL, and pH 10-13 with aqueous NaOH by serial dilutions from 1 M NaOH.

2.3 Fungicide Standards

- MBC analytical standard was from the Agriculture and Agri-Food Canada Pest Management Research Centre (Vineland Station, Ont.).
- MBC-MIC obtained from Sanex (Mississauga, Ont.) and Wilson Laboratories Inc. (Dundas, Ont.) were used; their purity was 98% and 87% respectively.
- MBC-EIC was obtained from the Agriculture and Agri-Food Canada Pest Management Research Centre (Vineland Station, Ont.). The purity of this material was 99%.
- MBC-PIC and MBC-BIC were obtained from the Agriculture and Agri-Food Canada Pest Management Research Centre (Vineland Station, Ont.). These samples contained high levels of MBC contamination (>50%).

3. Experiments

3.1 Preparation of Fungicide Standards

3.1.1 Synthesis of STM and MBU

Both methods for STM (3-methyl-2,4-dioxo-*s*-triazino[1,2-a]benzimidazole) and MBU (1-(2-benzimidazolyl)-3-*n*-methylurea) synthesis were derived from those used by Singh and Chiba (1985)

to synthesize STB and BBU.

STM

To 1 g MBC-MIC, 0.01 M NaOH was added until dissolved. The solution was filtered through a No. 1 Whatman filter paper and then neutralized with 37% hydrochloric acid and brought to pH 1. The time from the addition of NaOH to the acidification was 20 minutes. The precipitate that formed was filtered with a sintered glass filter and washed with 0.1 M hydrochloric acid, water, water/methanol (50:50, v/v) and water a second time. The compound was vacuum dried at room temperature. A sample was saturated in 50:50 (v/v) acetonitrile and water and analyzed for its purity by HPLC using the MBC-MIC mobile phase program (see Experimental section 3.2.3 for detail). In addition, a sample was analyzed by mass spectroscopy.

MBU

To 65 mg MBC-MIC, 300 mL 1 M NaOH was added and stirred for 1 hour with gentle heating under nitrogen gas. The solution was allowed to stand for 3 days before neutralizing and further lowering the pH to 1 with 37% hydrochloric acid. When no precipitate formed, 0.15 M NaOH was added to pH 8 and then adjusted to pH 6 with HCl to obtain the precipitate. The compound was vacuum dried at room temperature. A sample saturated in methanol and filtered was analyzed for its purity by HPLC using the MBC-MIC method (see Experimental section 3.2.3 for detail). In addition, a sample was analyzed by mass spectroscopy.

3.1.2 Preparation of Standard Solutions of Fungicides and Selected Degradation Compounds

The following standard solutions were prepared:

- A combined standard of 9.5 µg/mL MBU and 14.6 µg/mL MBC was prepared in 20% methanol and 0.001 M pH 6 phosphate buffer. Another standard was prepared with the same concentrations of MBU and MBC with 10 µg/mL STM.

- A combined STM, MBU and MBC standard was prepared in pH 2 nitric acid (0.01 M) at 1.3, 3.6, and 3.6 µg/mL, respectively.
- MBC was prepared at 4 µg/mL in an water/methanol solution (80:20 v/v).
- STM saturated in pH 2 solution was prepared by ultrasonicating 0.01 M nitric acid with excess STM for 15 minutes and collecting the clear solution from filtering the mixture with a Gelman GHP Acrodisc GF 0.45 um filter (25 mm dia.) obtained from Gelman Sciences (Montreal, Que.).
- Standard stock solutions of MBC-MIC, MBC-EIC, MBC-PIC and MBC-BIC were prepared at 1250 µg/mL with 5M HNO_3 and diluted with 5M HNO_3 for individual experiments as defined in relevant experimental sections
- A standard stock solution of MBC was prepared at 250 µg/mL with methanol and diluted with methanol for individual experiments as defined in relevant experimental sections.

3.1.3 Purification of MBC-PIC and MBC-BIC

This method was derived from Chiba and Northover (1988) for the synthesis of benomyl homologues. Samples of MBC-PIC and MBC-BIC were added to separate beakers of chloroform at 1 g sample per 20 mL chloroform. After mixing well, 10 mL of the corresponding isocyanate was added and stirred to dissolve the mixture for 10 minutes. To totally dissolve, chloroform was added slowly (20 to 40 mL more). Hexane containing corresponding isocyanate (10%, v/v) was added gradually (up to 400 mL) to precipitate the desired compound. Suspensions were filtered using a sintered glass filter and the precipitate was washed with the isocyanate/hexane solvent. Compounds were vacuum dried at room temperature.

Standards of MBC-PIC (6.47 µg/mL) and MBC-BIC (2.01 µg/mL) were prepared in 5 M HNO_3. To meet the pH requirements of the analytical column (pH 2-7), standards were prepared for HPLC analysis with the following components: 0.5 mL benomyl homologue standard in 5 M nitric acid, 1 mL MBC standard in methanol, 10 mL Na_2HPO_4, and 13.5 mL of water. Analyses were performed by HPLC using the mobile phase programs described in Experimental section 3.2.3.

3.1.4 Purity Analysis of MBC-MIC and MBC-EIC

Standard solutions of Wilson MBC-MIC (6.47 µg/mL), Sanex MBC-MIC (25 µg/mL) and MBC-EIC (10.02 µg/mL) were prepared in 5 M HNO_3. To meet the pH requirements of the analytical column (pH 2-7), standards were prepared for HPLC analysis as described in Experimental section 3.1.2 and analyzed as described in section 3.2.3.

3.2 Development of RP-HPLC Methods

3.2.1 Use of Phosphate Buffer

a) Effect on MBC

An aqueous/methanol solution (80:20 v/v) of MBC (4 µg/mL) was used as a standard. Analysis were performed using the Spherisorb ODS2 (C-18) 5 um, 250 X 4.6 mm analytical column with a 30 X 4.6 mm guard column of the same material. The following isocratic mobile phase compositions were run with a flow rate of 0.5 mL/minute to compare the effect of buffer concentration on peak shape and retention time:

A. 45% ACN, 55% H_2O
B. 45% ACN, 55% 0.067 M pH 6 phosphate buffer

b) Effect on MBC and MBU

Aqueous buffer/methanol solutions (80:20, v/v) of MBU (9.5 µg/mL) and MBC (14.6 µg/mL) in 0.001 M pH 6 and 8 phosphate buffer solutions were used as standards. Analysis were performed using the analytical columns in a) listed above to improve the resolution of the MBC and MBU peaks.

The experiment compared the resolution of the peaks in pH 6 sample solvent to that in pH 8

sample solvent under the following chromatographic program with pH 6 phosphate buffer (0.067 M) in the mobile phase and a flow rate of 0.5 mL/minute:

Time (min)	0	10	15	17	25
%ACN	30	50	50	30	30
%H_2O	60	40	40	60	60
% Buffer	10	10	10	10	10

c) Effect on STM

The STM saturated in 0.01 M nitric acid was used as a standard. Analyses were performed using the analytical columns in a) listed above. The following gradient mobile phase programs were run with different buffer compositions and a flow rate of 0.5 mL/minute:

A. 0.0067 M, pH 7 phosphate buffer

Time (min)	0	2.8	10	20	25	35
%ACN	25	25	60	60	25	25
%H_2O	65	65	30	30	65	65
% Buffer	10	10	10	10	10	10

B. 0.0067 M, pH 7 phosphate buffer

Time (min)	0	2.8	10	20	25	35
%ACN	25	25	60	60	25	25
%H_2O	35	35	0	0	35	35
% Buffer	40	40	40	40	40	40

C. 0.067 M, pH 7 phosphate buffer

Time (min)	0	2.8	10	20	25	35
%ACN	25	25	60	60	25	25

$\%H_2O$	65	65	30	30	65	65
% Buffer	10	10	10	10	10	10

3.2.2 Column Comparisons

The performance of three analytical columns were investigated using a mobile phase at a flow rate of 0.5 mL/minute under a variety of gradient mobile phase programs. The analytical columns used in the comparisons were from Phenomenex (Torrence, CA):

1) Spherisorb ODS2 (C-18) 5 um, 250 X 4.6 mm with a 30 X 4.6 mm guard column of the same material.
2) Prodigy ODS2 (C-18) 5 um, 250 X 4.6 mm with a Spherisorb ODS2 (C-18) 5 um, 30 X 4.6 mm guard column.
3) Phenosphere ODS2 (C-18) 5 um, 250 X 4.6 mm with a 30 X 4.6 mm guard column of the same material.

For comparison (A) of the Spherisorb and Prodigy columns, the MBU and MBC (9.5 and 14.6 µg/mL, respectively) standard in pH 6 phosphate buffer (0.001 M) and methanol (80:20, v/v) was used. For comparison (B) of the Spherisorb and Phenosphere columns the STM, MBU and MBC (1.3, 3.6, and 3.6 µg/mL, respectively) standard in pH 2 nitric acid (0.01 M) was used.

The following gradient mobile phases were used in the respective comparisons:

A. 0.067 M, pH 6 phosphate buffer

Time (min)	0	10	15	17	25
%ACN	30	50	50	30	30
$\%H_2O$	60	40	40	60	60
% Buffer	10	10	10	10	10

B. 0.067 M, pH 7 phosphate buffer

Time (min)	0	2.8	10	15	16	30
%ACN	25	25	60	60	25	25
%H_2O	65	65	30	30	65	65
% Buffer	10	10	10	10	10	10

Each of the three columns were used for the analysis of all the samples and results obtained were compared to decide which column should be used throughout the remaining studies of this thesis. The criteria used were peak shape, theoretical number of plates and resolutions.

3.2.3 Gradient Mobile Phase Programming for Determination of Benomyl Homologues and their Degradation Compounds

A gradient mobile phase program was developed to separate and quantify the benomyl homologues and their respective degradation compounds. These methods were used for the purity determination of the benomyl homologue standards and the degradation in aqueous media studies. A Spherisorb ODS2 (C-18) 5 um, 250 X 4.6 mm analytical column was employed with a 30 X 4.6 mm guard column of the same material. The mobile phase was run at a flow rate of 1 mL/minute under the following gradient programs:

For MBC-MIC (sometimes MBC-EIC also)

Time (min)	0	10	15	20	30
%ACN	22	60	60	22	22
%H2O	68	30	30	68	68
% Buffer	10	10	10	10	10

For MBC-EIC

Time (min)	0	7	25	30	40
%ACN	25	65	65	26	26

%H2O	64	25	25	64	64
% Buffer	10	10	10	10	10

For MBC-PIC (sometimes MBC-BIC also)

Time (min)	0	4.5	9	10	14.9	15	25
%ACN	30	55	80	80	30	30	30
%H2O	60	45	20	20	70	60	60
% Buffer	10	0	0	0	0	10	10

For MBC-BIC

Time (min)	0	4.5	9	15	20	30	35	45
%ACN	40	60	80	80	40	40	40	40
%H2O	50	40	20	20	60	60	50	50
% Buffer	10	0	0	0	0	0	10	10

For the solubility studies the column length and time of analysis and column equilibration was reduced. A Spherisorb ODS2 (C-18) 5 um, 150 X 4.6 mm analytical column was employed with a 30 X 4.6 mm guard column of the same material. The mobile phase was run at a flow rate of 1 mL/minute under the following gradient programs:

For MBC-MIC (sometimes MBC-EIC also)

Time (min)	0	10	15	20
%ACN	22	60	22	22
%H2O	68	30	68	68
% Buffer	10	10	10	10

For MBC-EIC

Time (min)	0	6	8.5	12	20
%ACN	26	65	65	26	26

%H2O	64	25	25	64	64
% Buffer	10	10	10	10	10

For MBC-PIC (sometimes MBC-BIC also)

Time (min)	0	4.5	9	10	14.9	15	20
%ACN	30	55	80	80	30	30	30
%H2O	60	45	20	20	70	60	60
% Buffer	10	0	0	0	0	10	10

For MBC-BIC

Time (min)	0	4.5	9	10	14.9	15	20
%ACN	40	60	80	80	40	40	40
%H2O	50	40	20	20	60	50	50
% Buffer	10	0	0	0	0	10	10

3.3 Degradation of Benomyl and Homologues in Aqueous Media

3.3.1 Preparation of Samples

Each 5 mg fungicide sample was ground in a mortar with pestle and transferred to 50 mL Erlenmeyer flasks with 25 mL of solvent. When surfactant Tween 20 was used, fungicide samples were mixed with 0.5 g dextrose (20 mg/mL), ground and transferred to 50 mL Erlenmeyer flasks with 25 mL of solvent. Suspensions were ultrasonicated for 15 minutes. Aliquots were drawn with a syringe and then filtered using a Gelman GHP Acrodisc GF 0.45 um filter (25 mm dia.). Filters were primed with 5 mL of sample solution before pooling the remaining filtrate into a 20 mL scintillation vial. Samples were analyzed by HPLC. Those samples prepared between pH 2-8 were analyzed directly. Those prepared at pH 10 to 13 were first neutralized (2 mL) with an equivalent number of moles of nitric acid (2 mL) in a volumetric flask (25 mL) and topped to the mark with 0.067 M pH 7 phosphate buffer before HPLC analysis. Acidic standards in 5 M nitric acid (0.5 mL)

were combined in a volumetric flask (25 mL) with MBC in methanol (1 mL), 0.2875 M Na_2HPO_4 (10 mL), and water (13.5 mL). Neutralized samples were again filtered using Gelman syringe filters directly to a 2 mL HPLC vial and analyzed by HPLC. Each analysis used a new sample stored in a Teflon sealed HPLC vial at 21 ± 1 °C until essentially all the parent compound was decomposed or at least the time for its peak height to reduce in half twice over (the equivalent to two half lives.)

3.3.2 Data Analysis

Each homologue and its degradation compound was analyzed by measuring its peak height. The decrease in the peak height of the parent compound was plotted against time to calculate the kinetic rate constant (k) and the half life ($t_{1/2}$) according to the equations
where C_1 is the homologue peak height

$$k = \frac{2.303}{\Delta t} \log \frac{C_1}{C_2} \qquad t_{1/2} = \frac{\ln(0.5)}{|k|}$$

at time t_1, C_2 is the homologue peak height at t_2, and $\Delta t = t_2 - t_1$.

3.4 Solubility of Benomyl and Homologues in Aqueous Media

3.4.1 Method Development

a) MBC Peak Shape Versus % Methanol in Sample Solution

MBC standards (4 μg/mL) in various percentages of methanol (v/v) were prepared by diluting 1 mL MBC (100 μg/mL) in methanol with varying volumes of methanol and water. Triplicate samples from each standard were transferred to individual HPLC vials and analyzed by HPLC to check the percentage of methanol which gives reasonable MBC peak shapes.

A 30 mm LC-DB8 reverse phase analytical column was employed under an isocratic mobile

phase of 15 % acetonitrile and 85% water run at a flow rate of 1 mL/minute.

b) Neutralization of Nitric Acid with Na_2HPO_4

To 1 mL 5 M HNO_3 a solution of 0.125 M Na_2HPO_4 was added slowly with continuous gentle stirring. The pH was recorded using a pH meter in order to determine the amount of Na_2HPO_4 required to neutralize the acid to an acceptable pH range for analysis using conventional reverse-phase HPLC analytical columns (pH 2-7).

3.4.2 Preparation and Analysis of Standard Solutions for Calibration Curves

Standards of MBC-MIC, MBC-EIC, MBC-PIC and MBC-BIC were prepared at 1250 µg/mL and diluted to desired concentrations with 5M HNO_3. A standard stock solution of MBC was prepared at 250 µg/mL and aliquots were diluted with methanol to obtain the desired concentrations.

The above two standard components were combined in a 25 mL volumetric flask to achieve a pH of 2.5 for HPLC analysis as follows. For the standard containing MBC-MIC and MBC, 0.5 mL MBC-MIC in 5M HNO_3, 1 mL MBC in methanol and 10 mL 0.2875 M Na_2HPO_4 were taken and made up the volume with water (13.5 mL). Other standards containing other benomyl homologues and MBC were also prepared accordingly.

3.4.3 Preparation and Analysis of Sample Solutions

Each 10-mg benomyl homologue sample (except at pH 1, 12, and 13 where 150-mg samples were used) was ground in a mortar with pestle and transferred to a 50 mL Erlenmeyer flask with 25 mL of solvent. When surfactant Tween 20 was used, fungicide samples were mixed with 0.5 g dextrose (20 mg/mL), ground and transferred to 50 mL Erlenmeyer flasks with 25 mL of solvent. Suspensions were blended for 10 minutes (except at pH 12 and 13 where only 5 minutes blending was allowed) and kept at 24 ± 1 °C by partially submerging the Erlenmeyer flask in the water bath. At a room temperature of 24 ± 1 °C aliquots were drawn with a syringe and samples filtered using

Gelman GHP Acrodisc GF 0.45 um filters (25 and 13 mm dia.). Each filter was initially primed with 5 and 2 mL of sample respective to filter diameter before aliquoting filtered sample. For pH 1 to 10 filtered samples were pooled into individual 20 mL scintillation vials to ensure homogeneity. Aliquots of 10 mL were taken with volumetric pipettes to add the sample to the standard preparation solution (see below) and water was added to the mark. At pH 11-13 suspensions were filtered directly to the mark of the volumetric flasks containing the standard preparation solution by submerging the syringe needle under the surface of the neutralizing liquid. The standard preparation solution in each 25 mL flask contained 0.5 mL HNO_3, 1 mL methanol, 10 mL Na_2HPO_4 and an amount of water to reach an appropriate dilution of the sample. Neutralized samples were filtered again using Gelman syringe filters directly to a 2 mL HPLC vial and analyzed by HPLC. Each analysis was done in triplicate. The analysis was repeated after 30 minutes of blending to ensure that the analyte was completely saturated using the method previously described.

3.4.4 Data Analysis

The concentration of a benomyl homologue in aqueous solution was determined by using a standard calibration curve (Appendix 2) based on the peak areas. Benomyl homologue standards were corrected by subtracting the amount of MBC impurity already present in the standard. Concentrations of MBC were also determined by using a standard calibration curve.

3.5 Melting Point Determination Method for Benomyl and Homologues

Each compound was tested for their melting points using the temperature programs previously described (Experimental section 1.3). The temperature, duration of heating, and visual observations were recorded for each.

RESULTS AND DISCUSSION

1. Preparation for Fungicide Standards

1.1 STM and MBU Synthesis and Yield

The alkali degradation product of MBC-MIC, 3-methyl-2,4-dioxo-*s*-triazino[1,2-*a*]benzimidazole (STM, the methyl equivalent of the benomyl degradation compound STB) appeared to be completely pure by HPLC analysis since no other peaks were found in the chromatogram (Figure 7) and the mass spectrum (Figure 8) gave a major molecular ion at m/z 216.

The yield of STM was 83% (0.96 g). Some loss likely occurred during recrystallization at pH 1. Furthermore, some loss of STM would have occurred during the washing with water and methanol/water (50:50, v/v).

A concentration of 0.01 M NaOH instead of 0.1 M NaOH (Singh and Chiba, 1985) was used for synthesis of STM as a precaution to prevent the formation of MBU (1-(2-benzimidazolyl)-3-*n*-methylurea, the methyl equivalent of the benomyl degradation compound BBU). Further studies revealed that no MBU was formed in 0.01 M NaOH during the time required to complete this procedure (Section 3.2).

The degradation product of MBC-MIC under stronger alkali conditions (0.1 M NaOH) was found to be MBU which appeared completely pure since no other peaks were found in the HPLC chromatogram, and the mass spectrum (Figure 8) gave a significant molecular ion for MBU at m/z 190. The yield was 47% (24 mg). The low yield was most likely due to the loss of the compound during recrystallization with pH 6 buffer.

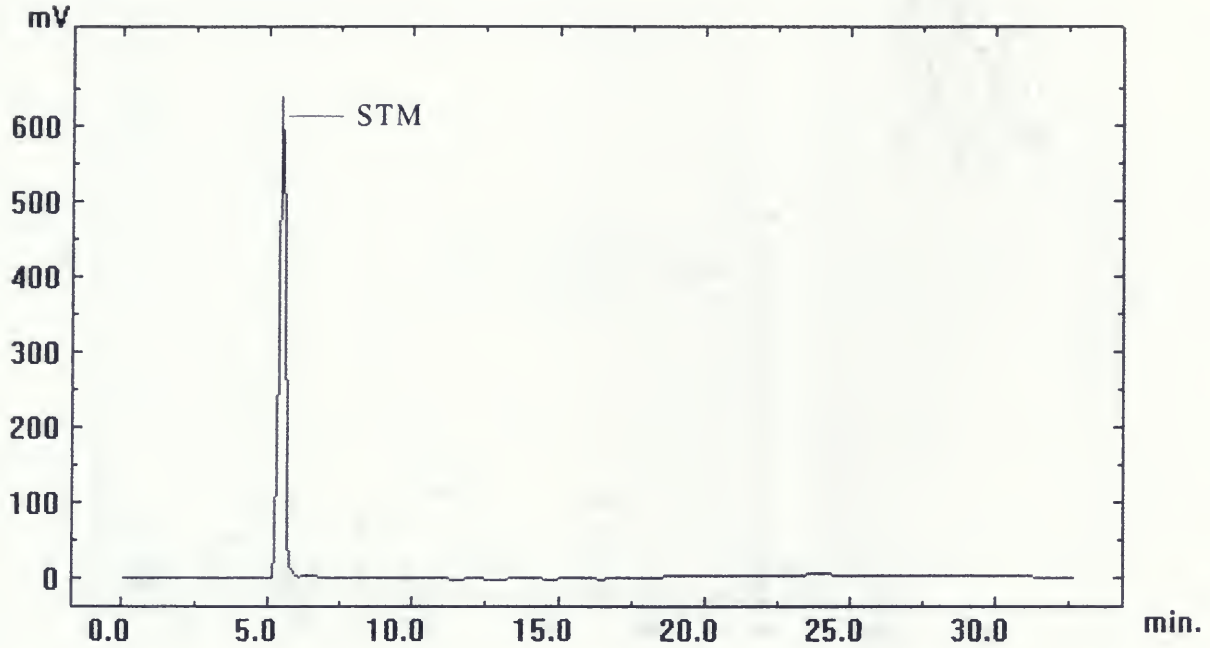


Figure 7: HPLC Chromatogram of STM . The solution was prepared by saturating the synthesized STM in methanol and filtering.

Future work to determine the solubility of these degradation compounds in aqueous and organic solvents would be useful for increasing their percent yield from synthesis. This information would also be helpful for establishing methods of analysis and understanding their behaviour in agricultural and biological systems.

1.2 MBC-PIC, MBC-BIC Purification

The composition of the purified MBC-PIC and MBC-BIC was 95% and 91% respectively with the remaining impurity being MBC (Figures 9 and 10). To prevent degradation of any intact fungicide to MBC and to ensure complete formation of the desired compound from any present MBC, the isocyanates were initially added in proportion (10 mL) as if all the starting material was pure MBC. The MBC present after purification was likely formed during the drying process.

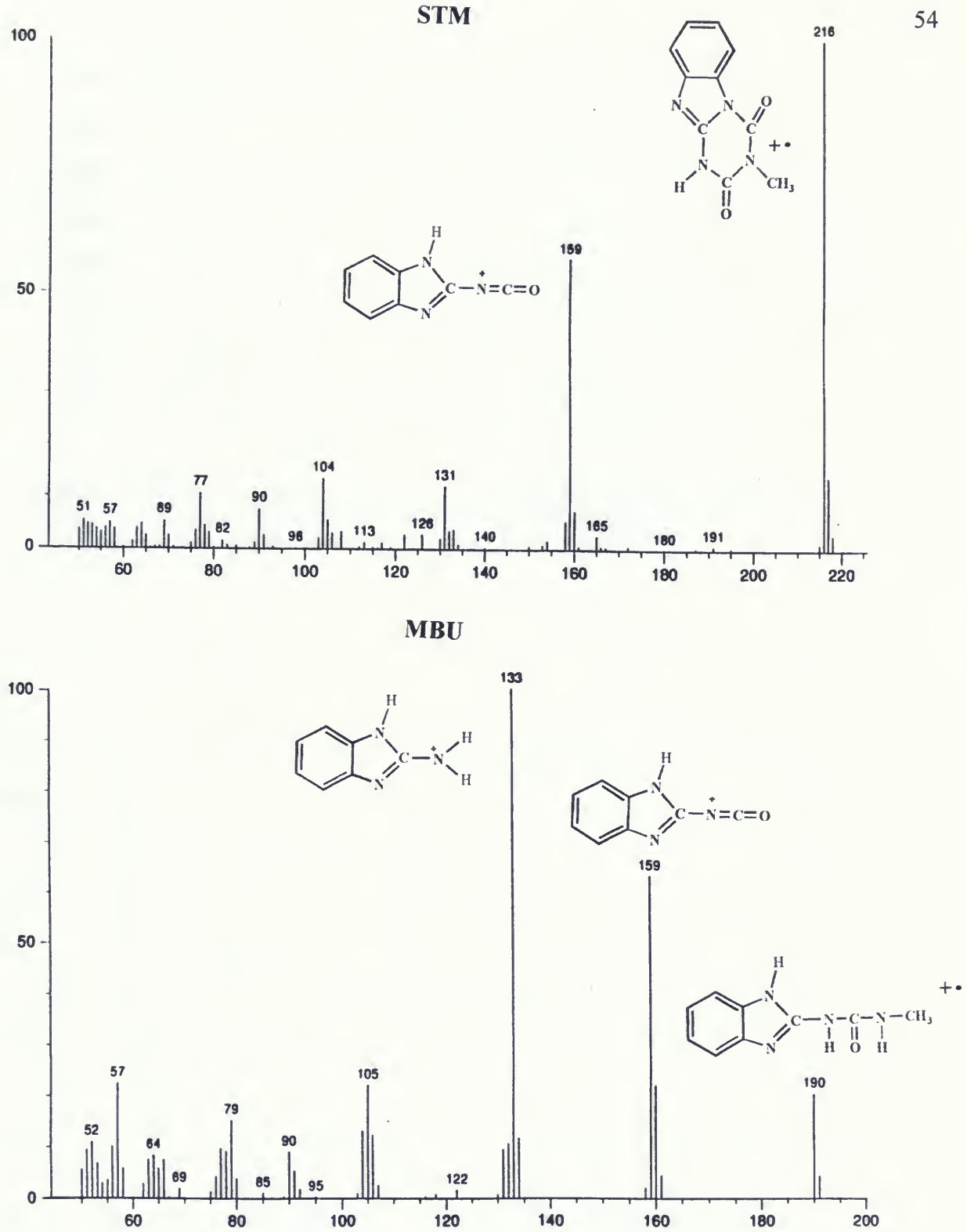


Figure 8: The Mass Spectra for STM and MBU.

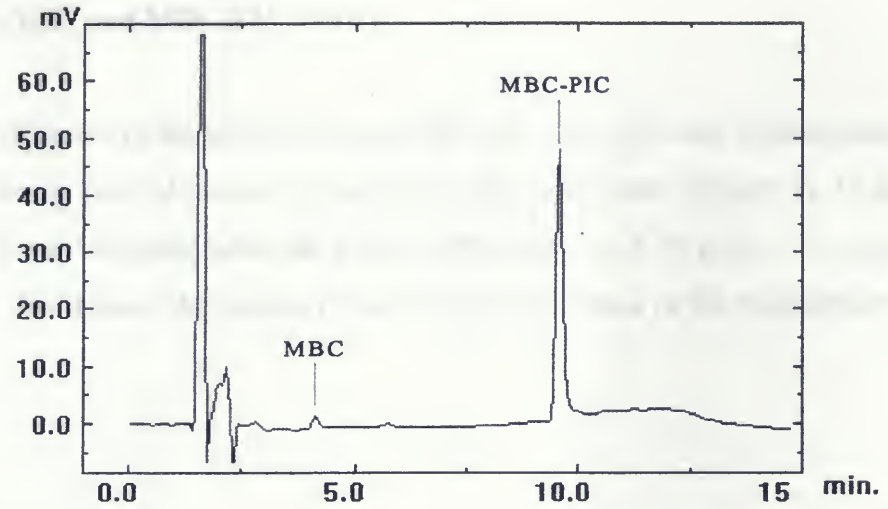


Figure 9: HPLC Chromatogram of MBC-PIC (6.47 µg/mL) in the sample solution as described in section 1.4 of the Experimental. The solution was prepared just before HPLC determination by diluting a 400 µg/mL MBC-PIC stock solution in 5M HNO_3.

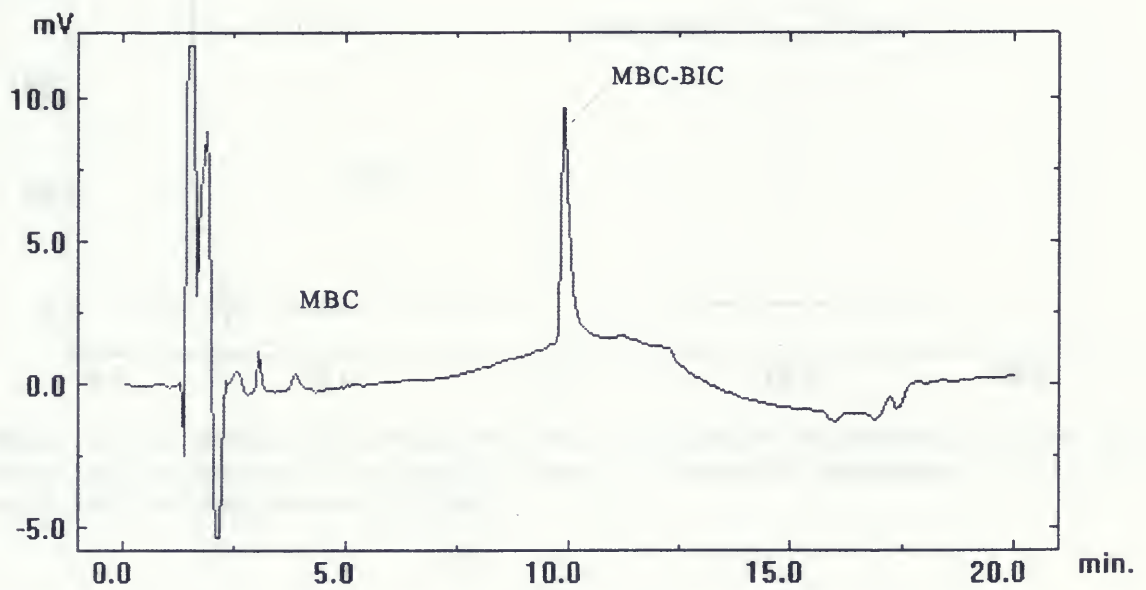


Figure 10: HPLC chromatogram of MBC-BIC (2.01 µg/mL) in the sample solution as described in section 1.4 of the Experimental. The solution was prepared just before HPLC determination by diluting a 1257 µg/mL MBC-BIC stock solution in 5M HNO_3 .

1.3 MBC-MIC and MBC-EIC Purity

No purification of the MBC-MIC and MBC-EIC fungicides was required since these compounds were relatively pure as proven by the HPLC chromatograms (Figures 11,12 and 13). The Sanex MBC-MIC was 98% pure while the Wilson MBC-MIC was 87% pure. The purity of the MBC-EIC was 99%. In all cases the impurity was due to the presence of the degradation compound MBC.

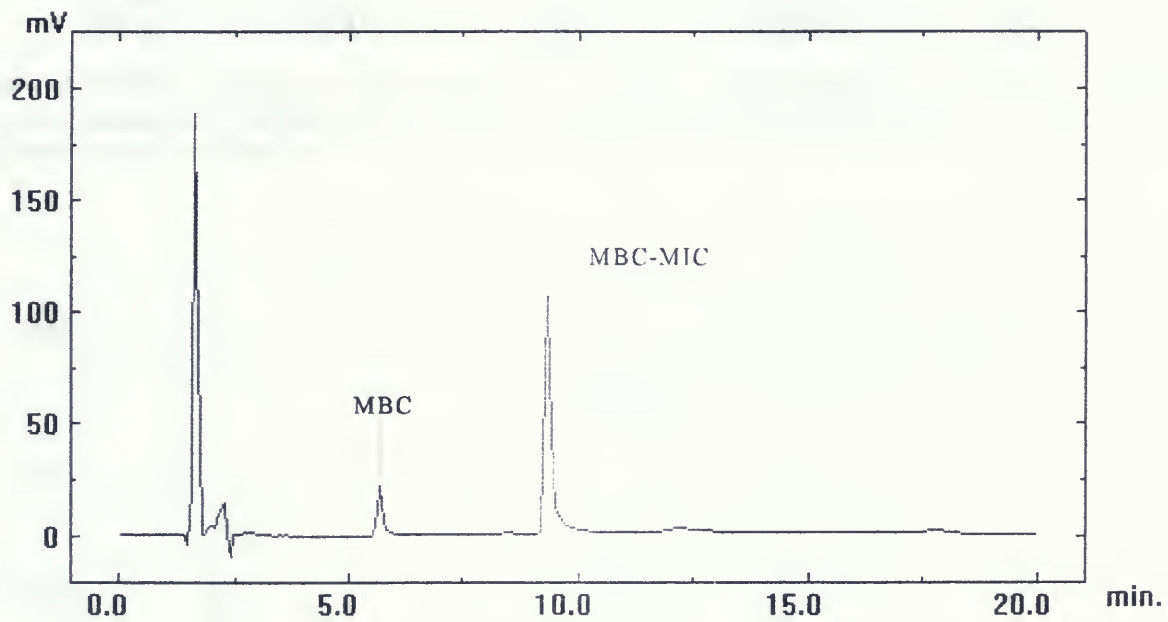


Figure 11: HPLC chromatogram of Wilson MBC-MIC (10.15 µg/mL) in the sample solution as described in section 1.4 of the Experimental. The solution was prepared just before HPLC determination by diluting a 500 µg/mL MBC-MIC stock solution in 5M HNO_3.

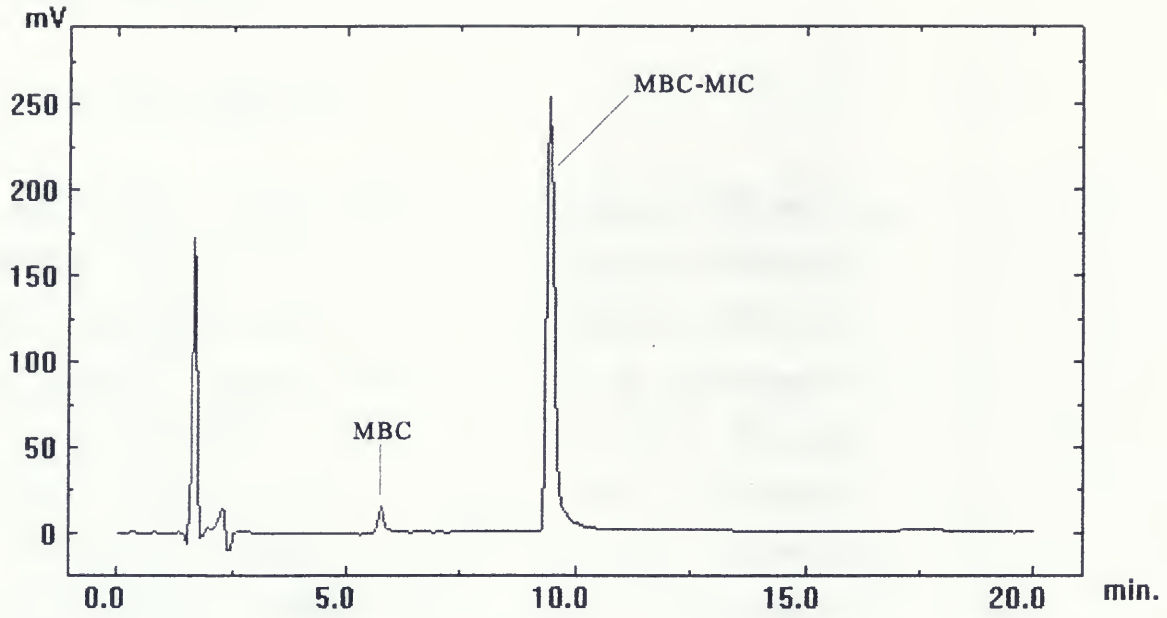


Figure 12: HPLC chromatogram of Sanex MBC-MIC (25 µg/mL) in the sample solution as described in section 1.4 of the Experimental. The solution was prepared just before HPLC determination by diluting a 1240 µg/mL MBC-MIC stock solution in 5M HNO_3.

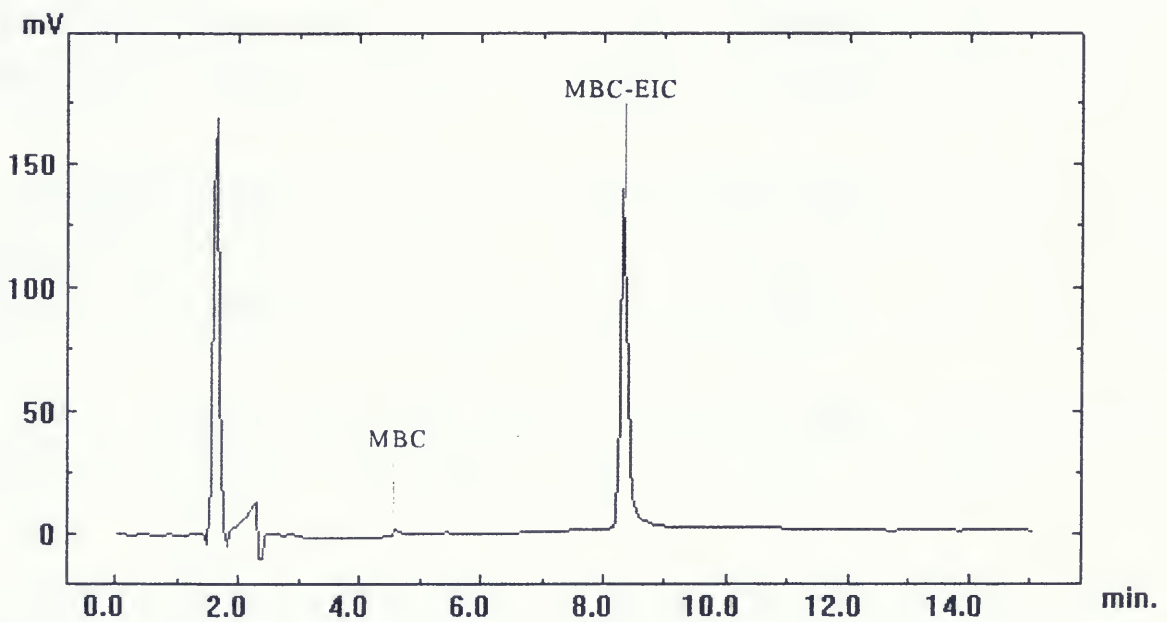


Figure 13: HPLC chromatogram of MIC-EIC (10.02 µg/mL) in the sample solution as described in section 1.4 of the Experimental. The solution was prepared just before HPLC determination by diluting a 500 µg/mL MBC-EIC stock solution in 5M HNO_3.

1.4 Characterization of Compounds

1.4.1 Mass Spectra

Mass Spectra were obtained for the newly synthesized MBC-MIC degradation compounds STM and MBU and also the benomyl degradation compounds STB and BBU for comparison. The MS results gave molecular ions for STM and MBU (Figure 8), STB and BBU at m/z 216, 190, 258, and 232, respectively. The general fragmentation trend for all the compounds is to give an intense peak at m/z 159 that signifies the loss of CH_3OH from MBC. The fragmentation for MBU and BBU provides an intense peak at m/z 133 that may signify a 2-AB structure. The mass spectra obtained for STB and BBU compared well with the solid probe EI spectra found in the literature (Singh *et al.*, 1993) although an additional intense peak at m/z 216 was present in the results from this study.

1.4.2 Retention Times and UV Spectra

The retention times for the benomyl homologues and their degradation compounds are given in Table 2. These values are only relative between a parent compound and its respective degradation compounds since different mobile phase gradient programs were used for each homologue.

In comparison to MBC, all of the primary alkali degradation compounds STM, STE, STP, and STB elute first. When comparing MBC to the secondary alkali degradation compounds, only MBU elutes first. The compounds EBU, PBU and BBU elute after MBC.

The UV spectra for MBC-MIC, MBC-EIC, MBC-PIC, MBC-BIC, and their respective degradation compounds generally show strong absorptions between 275 and 300 nm.

Similarities between UV spectra were found by comparing the compound series from MBC-MIC to MBC-BIC, STM to STB, and MBU to BBU (Figures 14-16). The degradation compound common to all of the parent compounds, MBC, most resembled the UV spectrum of MBU; a

reflection of their similar molecular structures.

Table 2: The Retention Times in Minutes of the Benomyl Homologues and their Degradation Products.

Homologue	STX[a]	XBU[b]	MBC	MBC-XIC[c]
MBC-MIC	4.1	6.8	7.1	10.9
MBC-EIC	3.3	5.4	4.6	9.8
MBC-PIC	4.0	6.9	5.1	10.8
MBC-BIC	3.8	7.3	4.3	11.3

[a] STX refers to STM, STE, STP and STB, respectively

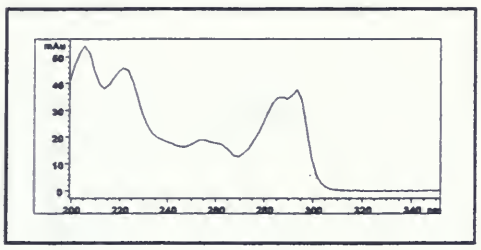
[b] XBU refers to MBU, EBU, PBU and BBU, respectively

[c] MBC-XIC refers to MBC-MIC, MBC-EIC, MBC-PIC and MBC-BIC, respectively

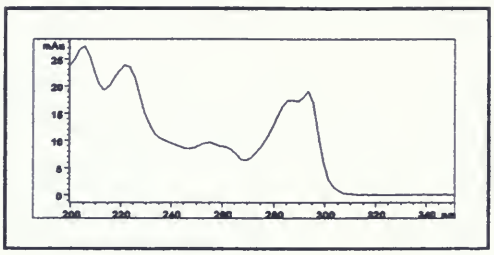
All the parent compounds provided UV spectra with maxima at 287 and 293 nm. The primary alkali degradation compounds STM, STE, STP, and STB gave UV maxima at 277 and 289 nm that were less distinct and broader in range than the maxima given by their parent compounds. The secondary alkali degradation compounds MBU, EBU, PBU, and BBU provided a narrower UV maxima range than the primary alkali degradation compounds but the maxima at 281 and 287 nm were less distinct than those for the parent compounds.

The absorption spectra of these compounds were in good agreement with reports by Singh and Chiba (1985) for benomyl and its respective degradation compounds.

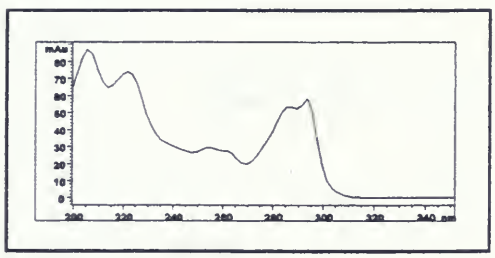
MBC-MIC



MBC-EIC



MBC-PIC



MBC-BIC

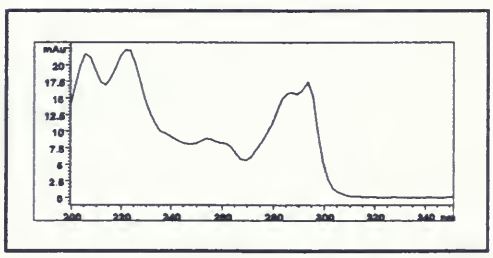
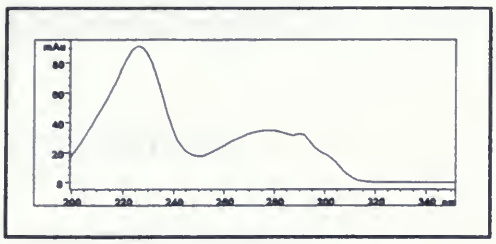
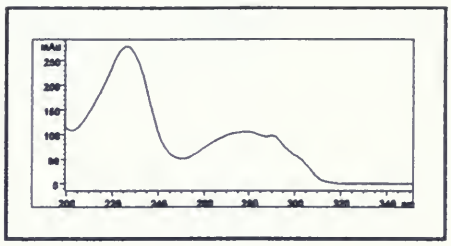


Figure 14: The UV spectra for the benomyl homologues in pH 7 buffer with 10 % methanol.

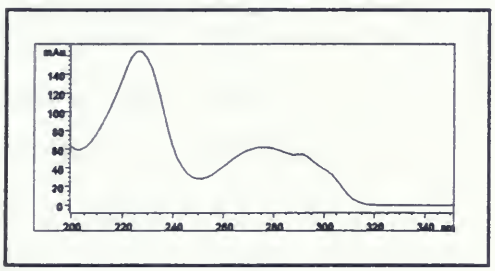
STM



STE



STP



STB

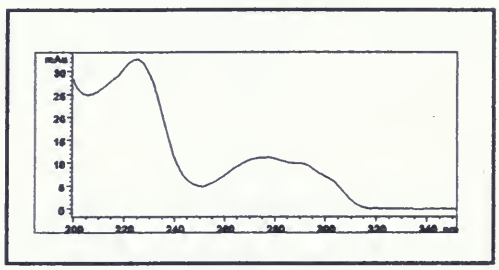
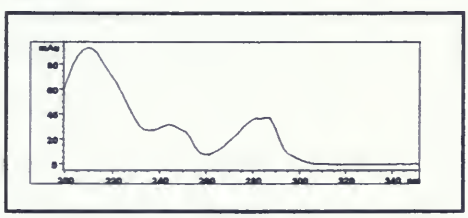
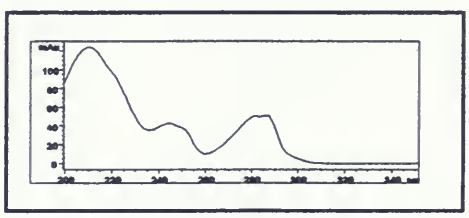


Figure 15: The UV spectra of STM, STE, STP, STB.

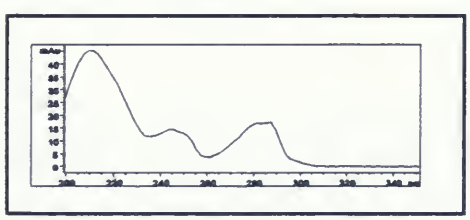
EBU



MBU



PBU



BBU

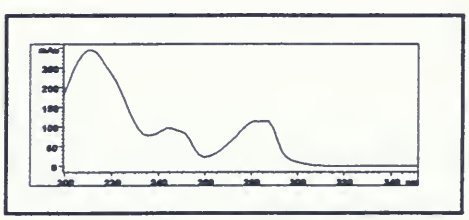


Figure 16: The UV spectra for MBU, EBU, PBU and BBU

2. Column Comparisons and Method Developments

2.1 Use of Buffer

2.1.1 Effect on MBC

In an isocratic mobile phase of 45% acetonitrile and 55% water the MBC (4 µg/mL in water/methanol, 80:20, v/v) peak was broad. By replacing the water with 0.067 M, pH 6, phosphate buffer there was a dramatic decrease in retention time from 4.85 to 3.47 minutes (see Figure 17). The MBC k' value decreased from 1.74 to 1.23 while the N_{eff} value increased from 255 to 1668. Since the excessive use of phosphate buffers can damage conventional RP-HPLC analytical columns (Claessens *et al.*, 1996), a mobile phase buffer concentration was reduced to 6.7 mM; the concentration that had been used in earlier benomyl studies (Singh and Chiba, 1985; Singh *et al.*, 1990).

2.1.2 Effect on MBC and MBU

When compared, the resolution of MBC and MBU was better at pH 6 then at pH 8. In particular, the MBC N_{eff} value increased from 1787 to 1994 and the resolution between the peaks increased from 1.79 to 1.82 at pH 8. These results encouraged the use of a mobile phase buffer that was alkali in nature. Since the recommended operating pH limits of the column are between 2-7, a 0.067 M phosphate buffer at pH 7 was used throughout the remainder of the experiments.

2.1.3 Effect on STM

The peak shape and retention time of STM were significantly influenced by the concentration of pH 7 buffer used as part of the mobile phase. The retention time of STM became longer when the percentage of buffer in the mobile phase increased. Also the peak shape became better

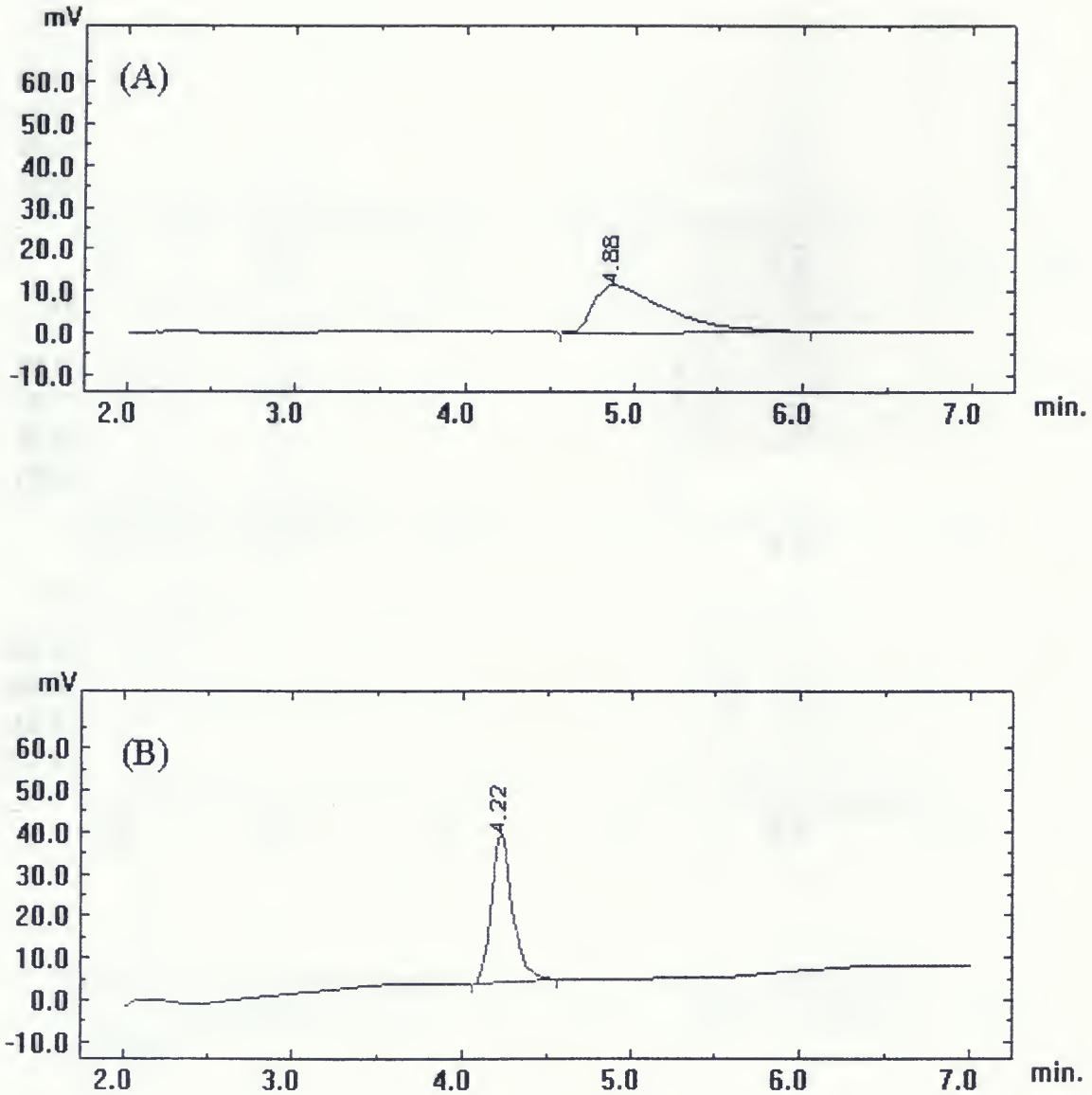


Figure 17: The HPLC chromatograms showing MBC peak shape from mobile phases with (A) no buffer present and (B) with pH 6 phosphate buffer present.

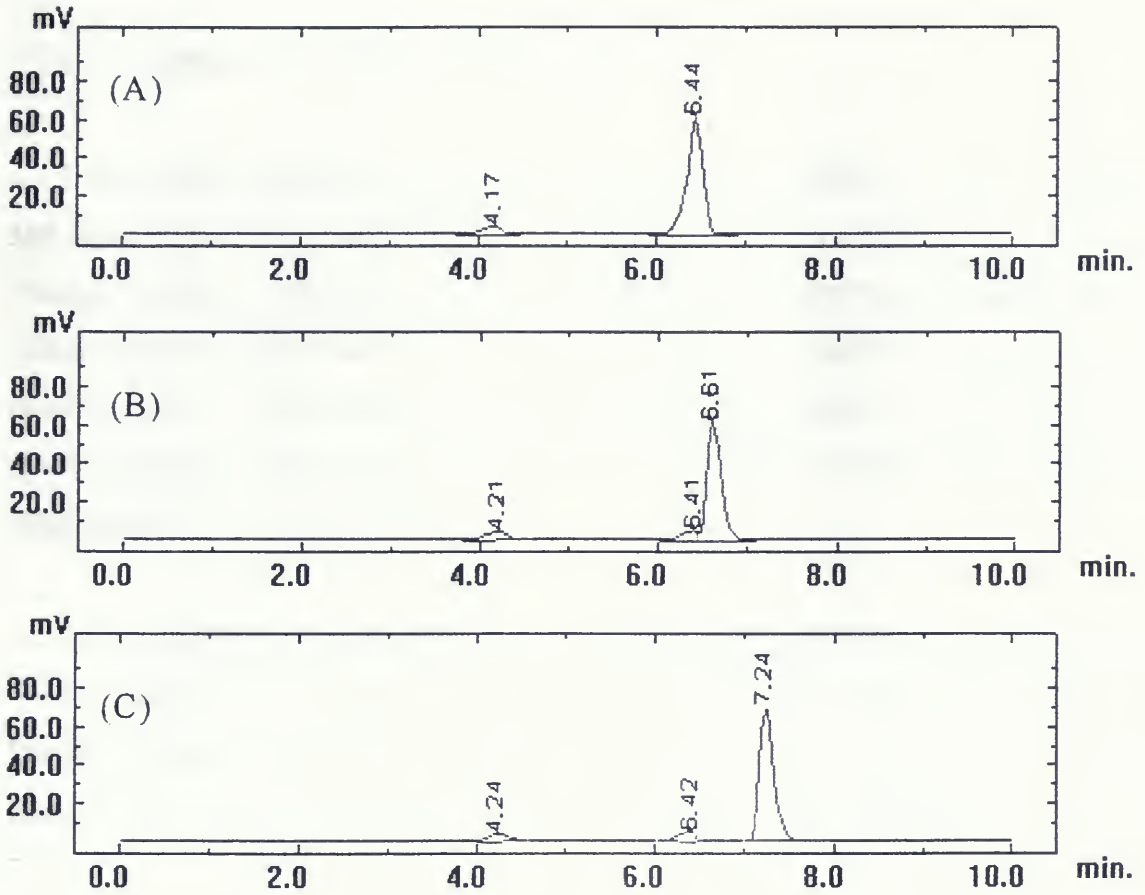


Figure 18: The HPLC chromatograms of STM and the solvent front. STM is eluted at 6.44, 6.61 and 7.24 minutes in mobile phases with (A) 0.67, (B)1.7, and (C) 6.7 mM of pH 7 buffer, respectively.

with higher concentrations of buffer. Similar results were obtained by Chiba and Singh (1986) for the butyl analogue STB. However, with lower concentrations of buffer STM was eluted too fast and the resolution of its peak from the solvent front became difficult. In order to separate the STM peak from the solvent front (Figure 18) without reducing the initial acetonitrile concentration in the mobile phase, the minimum buffer concentration required in the mobile phase was 6.7 mM (10% 0.067 M, v/v). Under these conditions the k' value was 0.13 and the Neff value was 70. Reducing the initial acetonitrile concentration in the mobile phase to improve the column performance for STM was not desired since this action would increase the retention times of later eluting analytes.

2.2 Spherisorb vs. Prodigy and Phenosphere

Under identical gradient mobile phase conditions the resolution of a sample containing MBC and MBU in methanol/water (20:80, v/v) was better with the Spherisorb column than with the Prodigy column. Although the Prodigy column produced sharper peaks and higher N_{eff} values (Table 3) and a resolution value of 1.07, the peak separation was poorer compared to the Spherisorb column (Figure 19) The higher resolution of the Spherisorb column was desired in the degradation studies in case the detection of a small quantity of one analyte was interfered by the presence of a large quantity of the other analyte.

After a gradient mobile phase had been developed for the analysis of benomyl homologues and their degradation compounds, the Spherisorb column was once again compared with the Phenosphere analytical column. The Phenosphere column gave poorer peak shapes and resolution between MBU and MBC was poorer, too, than those given by the Spherisorb material (Table 3.)

Table 3: The Capacity Ratio (k'), Effective Number of Plates (N_{eff}) and Resolution (R_s) values for MBU and MBC Using Three HPLC Analytical Columns.

Analytical Column	MBU		MBC		
	k'	N_{eff}	k'	N_{eff}	R_s
Prodigy	0.79	3460	0.85	4040	1.07
Spherisorb	0.69	690	0.85	1990	1.82
Phenosphere	0.98	1060	1.04	5380	0.58
Spherisorb	0.96	4270	1.05	8270	1.64

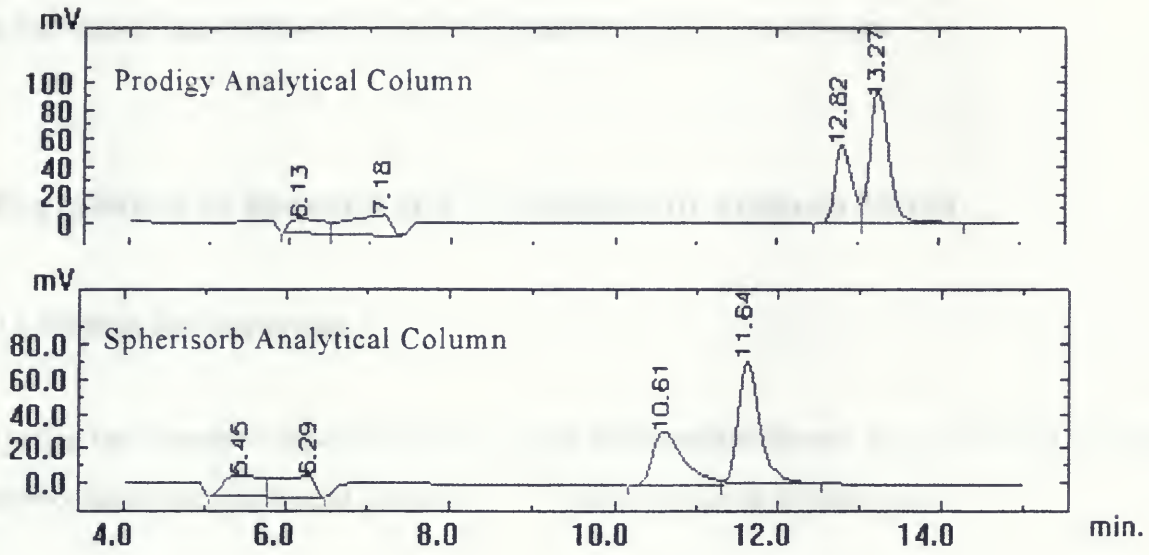


Figure 19: The HPLC chromatograms of the resolution between MBU and MBC using the Prodigy and Spherisorb analytical columns.

From the comparisons to both the Prodigy and Phenosphere analytical columns Spherisorb was found to provide the best separation between MBC and MBU with acceptable peak shapes. The Spherisorb analytical column was used for all the experiments throughout this study.

2.3 Further Gradient Mobile Phase Developments

The aim of the method developments was to determine time efficient methods for each homologue. For the chromatography of the parent compounds and their respective degradation compounds the programs listed in the Experimental section 3.2.3 provide suitable methods for 250 X 4.6 mm and 150 X 4.6 mm 5 um Spherisorb ODS2 columns, respectively. The 250 X 4.6 column was used for determining degradation compounds but a shorter column of 150 X 4.6 mm was suitable for everyday analysis since it provided reasonable separation of the compounds with shorter analysis and column equilibration times of under 20 minutes. For MBC-PIC and MBC-BIC the percentage of acetonitrile required in the mobile phase was much higher than that for MBC-MIC and MBC-EIC. To help prevent any precipitation of buffer salts onto the column, the buffer was removed

from the mobile phase before acetonitrile levels reached their maximum.

3. Degradation of Benomyl and Homologues in Aqueous Media

3.1 Method Developments

During the course of this study the following three critical factors were identified in order to accurately determine the rate of degradation of benomyl and its homologues.

3.1.1 Priming Syringe Filters

Each filter should be primed with a suitable amount of sample before filtering a portion for analysis. This is mainly because the filter initially acted as a chromatographic column in a way that the first portion of filtrate did not contain the same proportions of analyte as the remainder of the filtrate. It is probable that some analytes were retained on the filter during the earliest part of filtration. In order to ensure consistent results, 25 and 13 mm syringe filters were primed with 5 and 2 mL, respectively, of sample solution before aliqouts were taken for analysis.

3.1.2 Samples in Sealed HPLC Vials

Samples should be kept in sealed vials until the time of analysis since exposure to an open atmosphere affected the rates of degradation. Further discussion will take place in section 3.3 of the Results and Discussion for experiments at pH 4.

3.1.3 Temperature Control

The maintenance of constant temperature was crucial since the half life at 25 °C was as much as 57% shorter than that at 21 °C (see Table 4) in 0.001 M pH 8 phosphate buffer.

Table 4: Homologue Half Lives in Hours at 21 and 25 °C

Compound	MBC-MIC	MBC-EIC	MBC-PIC
21 °C	0.7	2.3	4.7
25 °C	0.4	1.0	2.4
$\frac{t_{1/2}(25°C)}{t_{1/2}(21°C)}$	57%	43%	51%

3.1.4 Reproducibility of Method

The reproducibility of the method used for the half life study was investigated by preparing three replicate samples of MBC-MIC in pH 3 dilute nitric acid. The sample mean for half life value was 7.79 hours while the sample standard deviation (SD) was 0.01 hours and the relative standard deviation (RSD) was 0.15% (Table 5). The reproducibility experiment was run over a single day with 3 samples prepared on the same morning. This result is really impressive, but the actual errors occurred during the study was expected much higher than the above. This is mainly because the degradation results for all four homologues over the range of pH values were obtained over a period of several months. It is rather impractical to expect this kind of reproducibility over a period of this length.

Table 5: The Half Life Sample Mean, Sample Standard Deviation, and Relative Standard Deviation for MBC-MIC in pH 3 Nitric Acid.

Sample	1	2	3	Mean	SD	RSD
$t_{1/2}$(hrs)	7.79	7.80	7.78	7.79	0.01	0.15%

3.2 Results: Degradation Compounds and Half Lives

3.2.1 a) Degradation Compounds of the Benomyl Homologues

All benomyl homologues, MBC, STM, MBU, STB, and BBU were identified by HPLC retention time comparisons to available analytical standards. The degradation compounds STE, STP, EBU and PBU were not synthesized as analytical standards but were identified by retention time and UV spectra comparisons to the other known compounds. The rates of formation of the degradation compounds were not determined since in many cases their solubility in the solutions reached saturation or exceeded the maximum detection limit before enough data could be acquired.

Chromatograms for MBC-EIC in pH 6, and 12 are used to show typical degradation patterns for the benomyl homologues. (Figure 20)

MBC-MIC

In HNO_3 solution at pH 1 to 3, MBC-MIC quantitatively converted to MBC. At pH 4 and 6 , an unidentified peak in addition to MBC was observed at or very close to the retention time of STM. Only MBC was expected as a degradation compound in solutions of pH < 8 since MBC-BIC has not been reported to degrade to any other compounds in this range. Further discussion for this unidentified compound is given in section 3.3 of the Results and Discussion. At pH 8 STM was the major degradation compound while only low levels of MBC were formed. At pH 10 STM was the main degradation compound while MBC was formed only in trace amounts. No MBC was formed from MBC-MIC at pH 11 and 12 since the parent compound was quantitatively converted to STM. At pH 12 MBU was present after a period of 24 hours. In NaOH solution of pH 13.6 quantitative conversion to STM, then MBU, occurred within an hour.

MBC-EIC

At pH 1 to 3 MBC-EIC quantitatively converted to MBC. At pH 4 and 6 (Figure 20), an

unidentified peak in addition to MBC was observed at or very close to the retention time of STE although the concentration of the former was low as was the case with MBC-MIC. Further discussion about this unknown compound is given in section 3.3 of the Results and Discussion. At pH 8 the major degradation compound was STE while levels of MBC were formed in lower amounts (Figure 20). At pH 10 STE was the main degradation compound while MBC was only formed in trace amounts. No MBC was formed from MBC-EIC at pH 11 and 12 (Figure 20) since the parent compound was quantitatively converted to STE. At pH 12 EBU was present after a period of 24 hours. In NaOH solution of pH 13.6 quantitative conversion to STE, then EBU, occurred within two hours.

MBC-PIC

At pH 1 to 3 MBC-PIC quantitatively converted to MBC. At pH 4 and 6, an unidentified peak in addition to MBC was observed at or very close to the retention time of STP although the concentration of the former was only a trace. Further discussion about this unknown compound is given in section 3.3 of the Results and Discussion. At pH 8 STP was the major degradation compound while MBC was formed at lower levels. At pH 10 STP was the main degradation compound while MBC was formed only in trace amounts. No MBC was formed from MBC-PIC at pH 11 and 12 since the parent compound was quantitatively converted to STP. At pH 12 PBU was present after a period of 24 hours. In NaOH solution at pH 13.6 quantitative conversion to STP, then PBU, occurred within 2.5 hours.

MBC-BIC

From pH 1 to 6 MBC-BIC quantitatively converted to MBC. At pH 8 MBC was still the dominant degradation compound but STB was formed at a low concentration. At pH 10 STB was the main degradation compound while MBC was formed only in trace amounts. No MBC was formed from MBC-BIC at pH 11 and 12 since the parent compound was quantitatively converted to STB. At pH 12 BBU was present after a period of 24 hours. In NaOH solution at pH 13.6 quantitative conversion to STB, then BBU, occurred within three hours.

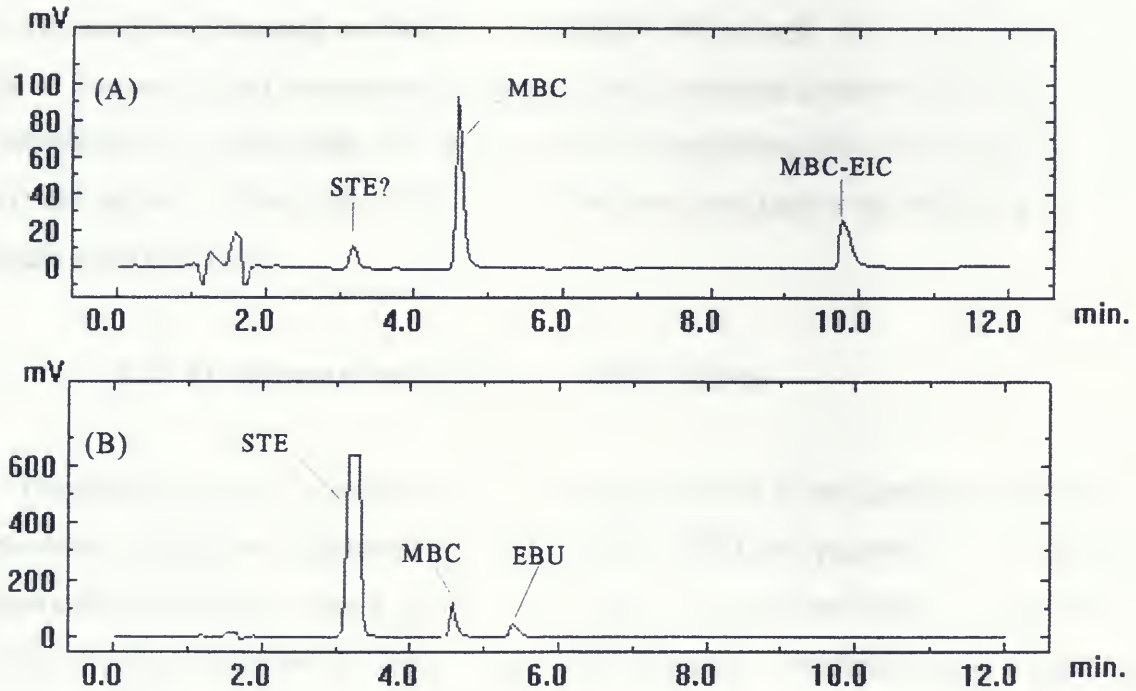


Figure 20: The HPLC chromatograms for the degradation of MBC-EIC in (A) pH 6 after 20 hours, and (B) pH 12 after two days. The STE response in (B) exceeded the maximum detection limit.

In aqueous buffer solutions the quantitative conversion of benomyl to MBC alone in pH 1-7, and to both MBC and STB in pH 8 was observed which is in agreement with earlier reports made by Singh and Chiba (1985) and Singh *et al.* (1990). Similar degradation was expected during this thesis study for the methyl, ethyl, and propyl homologues of benomyl but the formation of unknown STM-like compounds occurred with MBC-MIC between pH values 4-6 along with MBC. The finding of this compound was not consistent, but the presence of STM-like compounds (expressed as STX in the following) was observed with MBC-EIC and MBC-PIC as well. These may have been the same type of compounds as the unidentified degradation compounds observed by Northover and Chiba (1989)for MBC-MIC and MBC-EIC in water (pH 6.2) containing dextrose (20 mg/mL) and Tween 20 (0.05 mg/mL). Further discussion of this unknown compound is included in section 3.3 of the Results and Discussion.

3.2.1 b) Structure and Character Relationships

The different rates of formation between the unknown STX-like compounds and between the first alkali degradation compounds (STM, STE, STP, STB) are apparent from the degradation of parent compounds at pH 6 and 8, respectively. As the size of the molecule increases from methyl to butyl the rate of formation of these compounds decreased. The formation of these compounds was unexpected since MBC-BIC was never reported to degrade to STB at values pH < 8. Furthermore, the rate of formation for the final alkali compounds (MBU, EBU, PBU, and BBU) also appears to become slower as the size of the molecule increases from methyl to butyl.

3.2.2 a) Half Lives

Estimations for those values in alkali solutions are listed in Table 6 since the degradation of the homologues in alkali solutions greater than pH 10 was too rapid to obtain enough measurements for rate determinations. All values for the half lives of the benomyl homologues in acid and aqueous buffers are summarized in Table 7.

The degradation reactions followed first order kinetics since the plots of log (peak height of homologue) versus time provided linear relationships (Figure 21). An exception was observed at pH 4 where linear plots were not obtained (section 3.3).

MBC-MIC

MBC-MIC was stable in 5M HNO_3 for at least 1 month (Figure 22) but unstable in diluted concentrations to pH 2 and 3 with half lives of 7.8 and 8.2 hours respectively. At pH 6 the half life became shorter when Tween 20 surfactant was added into the solution (7.5 and 5.1 hour half lives respectively). A similar trend was found at pH 8 with faster degradation occurring with the presence of Tween 20 (1.2 and 0.7 hours half lives respectively). These results will substantiate the previous finding of Northover and Chiba (1989)

MBC-EIC

In 5M HNO_3 MBC-EIC was stable for at least 2 weeks. The half life at pH 6 was 8.6 hours but the addition of Tween 20 surfactant reduced this value to 5.3 hours as in the case for MBC-MIC. At pH 8 the half life was the shortest (2.3 hours) with no significant difference with the addition of Tween 20 surfactant.

MBC-PIC

MBC-PIC was stable in 5M HNO_3 for at least 2 weeks but not in dilute solutions at pH 2 and 3 (half lives of 3.1 and 3.6 hours, respectively). At pH 6 the half life was longer (5.1 hours) but reduced with the addition of Tween 20 surfactant (4.7 hours). At pH 8 the half life was the shortest (2.8 hours) and increased with the addition of Tween 20 (4.7 hours).

MBC-BIC

In 5M HNO_3 MBC-BIC was stable for at least 3 weeks. From pH 2 to 6 the half lives became longer (6.7 to 10.1 hours) but the addition of Tween 20 surfactant reduced this value (5.8 hours) at pH 6 as in the case for MBC-MIC. At pH 8 the half life was the shortest (2.8 hours) and increased with the addition of Tween 20 (7.0 hours).

Table 6: Degradation of Benomyl Homologues in Alkali Solutions of Sodium Hydroxide.

pH	Information	MBC-MIC	MBC-EIC	MBC-PIC	MBC-BIC
10	Rate (1/s)	-1.75E-04	8.29E-05	-1.55E-04	Fast
	Half Life (hr)[a]	1.1	2.3	1.2	-
	MBC formed	yes	yes	yes	yes
	STX[b] formed	yes	yes	yes	yes
	XBU[c] formed	no	no	no	no
11	Rate (1/s)	fast	fast	fast	fast
	Half Life (hr)	-	-	-	-
	MBC formed	no	no	no	no
	STX formed	yes	yes	yes	yes
	XBU formed	no	no	no	no
12	Rate (1/s)	fast	fast	fast	fast
	Half Life (hr)	-	-	-	-
	MBC formed	no	no	no	no
	STX formed	yes	yes	yes	yes
	XBU formed[d]	slow	slow	slow	slow
13.6	Rate (1/s)	fast	fast	fast	fast
	Half Life (hr)	-	-	-	-
	MBC formed	no	no	no	no
	STX formed	yes	yes	yes	yes
	XBU formed	complete > 42 min	complete > 1.5 hrs	complete >2.5 hrs	complete > 3 hrs

[a] Estimations or no values were given since insufficient data were available to determine the degradation rates and half lives. [b] STX refers to the primary alkali degradation compounds (STM, STE, STP, STB) for the corresponding parent compounds. [c] XBU refers to the secondary alkali degradation compounds (MBU, EBU, PBU, BBU) for the corresponding parent compounds. [d] Small amounts of XBU compounds had formed after 24 hours.

Table 7: The half lives (hours) for the benomyl homologues at 22 ± 1 °C in aqueous solutions of different pH values.

pH	MBC-MIC	MBC-EIC	MBC-PIC	MBC-BIC
5 M HNO_3	stable	stable	stable	stable
2	7.8	3.1	3.7	6.7
3	8.2	3.6	4.5	7.0
4	2.2	4.4	4.7	6.4
6	7.5	8.6	5.1	10.1
6TD[a]	5.1	5.3	4.7	5.8
8	1.2	2.3	2.8	2.8
8TD	0.7	2.3	4.7	7.0

[a]TD refers to the addition of Tween 20 surfactant and dextrose.

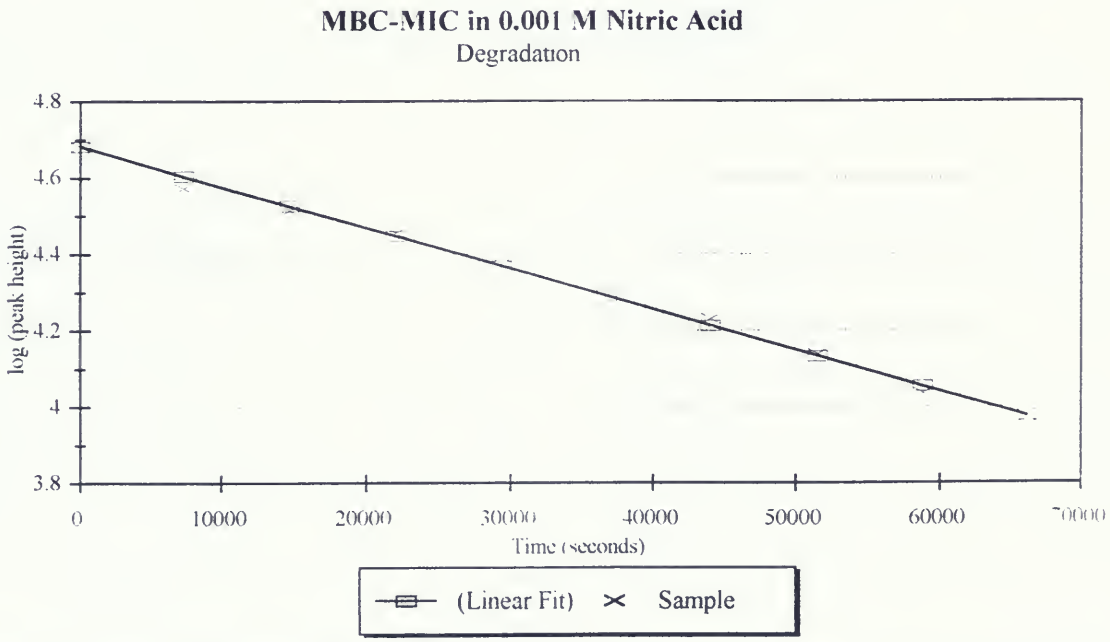


Figure 21: The plot of log (MBC-MIC peak height) versus time showing the degradation as linear first order kinetics.

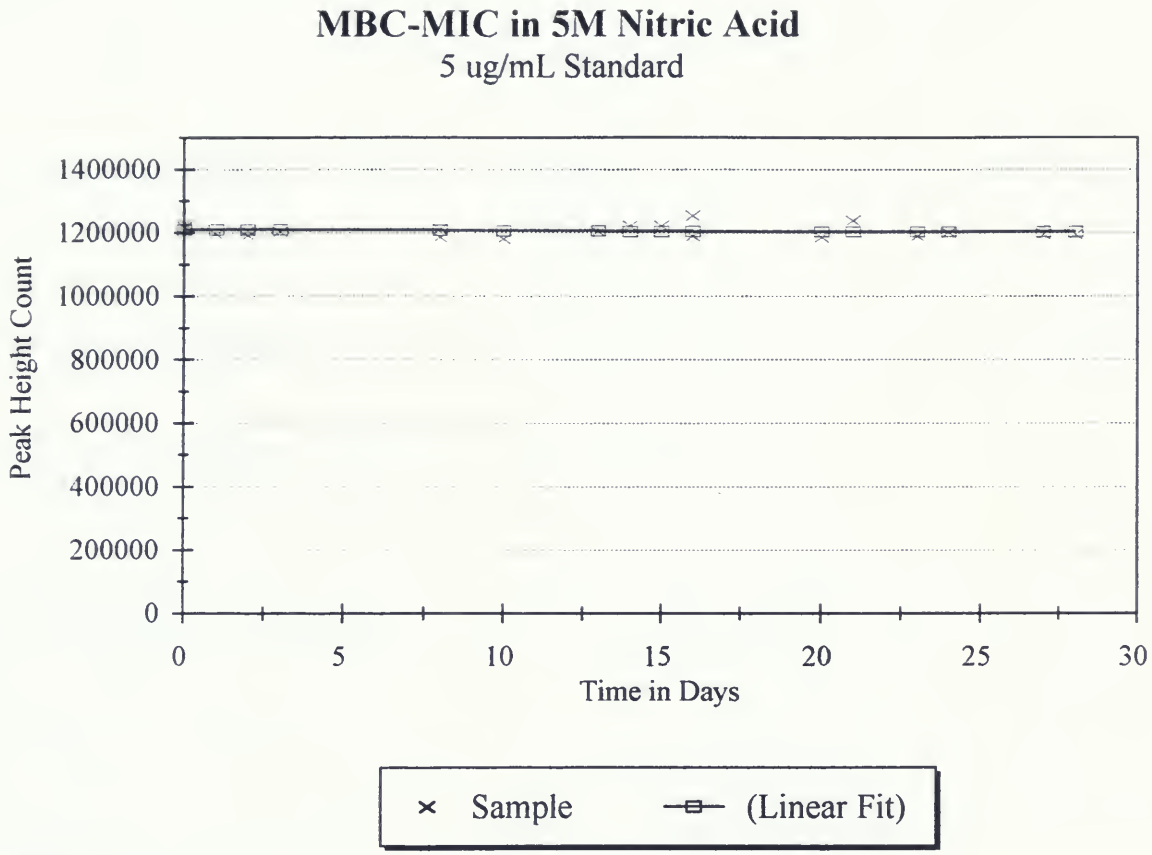


Figure 22: Plot of peak height versus time for MBC-MIC in 5 M nitric acid.

With the exception of 5M HNO_3, the half lives were longest at pH 6 for MBC-EIC, MBC-PIC, and MBC-BIC. The stability of the benomyl homologues in 5 M nitric acid was in agreement with results from Singh *et al.* (1992) where benomyl was found to be stable in 5 M hydrochloric acid. This stability is likely a result of protonation at the nitrogen of the benzimidazole group in the presence of acid (Calmon and Sayag, 1976; Singh *et al.*, 1990; Singh *et al.*, 1992). It was suggested by Singh *et al.* (1990) that the protonation of benomyl in acid prevents the hydrogen bonding between the ring nitrogen and the proton of the amine group in the butyl-carbamoyle moiety. This action would stabilize benomyl against intramolecular catalysis which was reported as the potential mechanism of benomyl degradation to MBC (Calmon and Sayag, 1976). Generally, the half lives of these compounds increased from pH 2 to 6. MBC-MIC had the longest half lives at pH 3, then pH 2 and pH 6. In all comparisons the half lives at pH 6 dropped with the addition of Tween 20

surfactant. However, at pH 8, the effect of Tween 20 was not consistent; the half life of MBC-MIC was reduced, MBC-EIC was unchanged, while MBC-PIC and MBC-BIC actually increased.

The inconsistent changes in MBC-BIC half life values of 6-10 hours between pH 2 to 7 are in agreement with studies of benomyl in aqueous buffers by Singh *et al.* (1990) where $t_{1/2}$ values of 5-8 hours between pH values of 2 and 7 were reported. Singh *et al.* (1990) observed that their reactions seems to have little dependence on the ionic strength and buffer components for a particular pH.

3.2.2 b) Structure and Property Relationships

In general, half lives of MBC-MIC, MBC-EIC, MBC-PIC, and MBC-BIC increased with molecular size but some exceptions are found in the results (Table 7). At pH 2 and 3 MBC-MIC was more stable than any of the other homologues. At pH 6 with and without Tween 20 the half life of MBC-PIC was the lowest of all the homologues. A similar result was observed by Northover and Chiba (1989) for 1.78 mM suspensions of MBC-MIC, MBC-EIC and MBC-BIC at 10 °C. In their study, MBC-EIC had shorter half life values than both MBC-MIC and MBC-BIC.

An increase in half life with molecular size was followed at pH 4 and at pH 8 with and without Tween 20 surfactant.

3.2.3 Significance of Degradation Compounds and Half Lives

The stability of all the homologues in concentrated acid is important for the development of concentrated stable stock solutions of known concentration. These standards may remain intact for a number of months or longer if stored at low temperatures. In addition the use of concentrated acid may be of use for the extraction and determination of intact benomyl homologues from environmental samples. The knowledge of the relationship between pH and stability is important for the design of commercial formulations, the understanding of the fungicidal mode of action , and the efficient use in the field. For instance, water in an agricultural setting may be alkali in nature (Chiba, 1979) and the ratio of MBC to STM that can form may vary significantly with pH value as

shown in the studies with pH 6 and 8 buffer solutions. The formation of one or the other degradation compound may effect the fungicidal efficiency since MBC is an active fungicide itself while STM has no significant activity (Chiba and Northover, unpublished results). Furthermore, understanding the degradation is useful for the synthesis of pure degradation compounds (i.e., MBC, STB, BBU in alkali) or perhaps the formation of the parent compound (i.e., MBC-BIC from MBC and BIC in concentrated acid).

Future work could consider the stability of benomyl homologues with different surfactant concentrations, and changes in temperature. As a future technique for extractions from environmental samples, the use of concentrated acid could be explored.

3.3 Experiments at pH 4 HNO_3

The degradation of all the compounds in pH 4 HNO_3 was not completely linear by first order kinetics. The most linear response for the plot of log (MBC-MIC peak area) versus time was obtained with multiple HPLC injections obtained from a single vial
with a resealable septum. In experiments that obtained samples from a single non-resealable vial or multiple sealed vials the chromatograms for successive analyses (Figure 24) provided inconsistent results with respect to degradation of MBC-MIC to MBC and an unidentified compound that was eluted with a retention time where STM was expected. STM was not expected to form at this pH since STB, the benomyl analogue, had formed only under alkaline
conditions. Changes were made to the way that the samples were stored before analysis. Using a single non-resealable vial or multiple sealed vials made little or no difference to the linearity of the rate curve. Samples from a single resealable vial gave more linearity to the degradation curve (Figure 23) but not enough to identify the rate as first order kinetics. Using different water sources (reverse osmosis water from the Research Centre at Vineland Station and

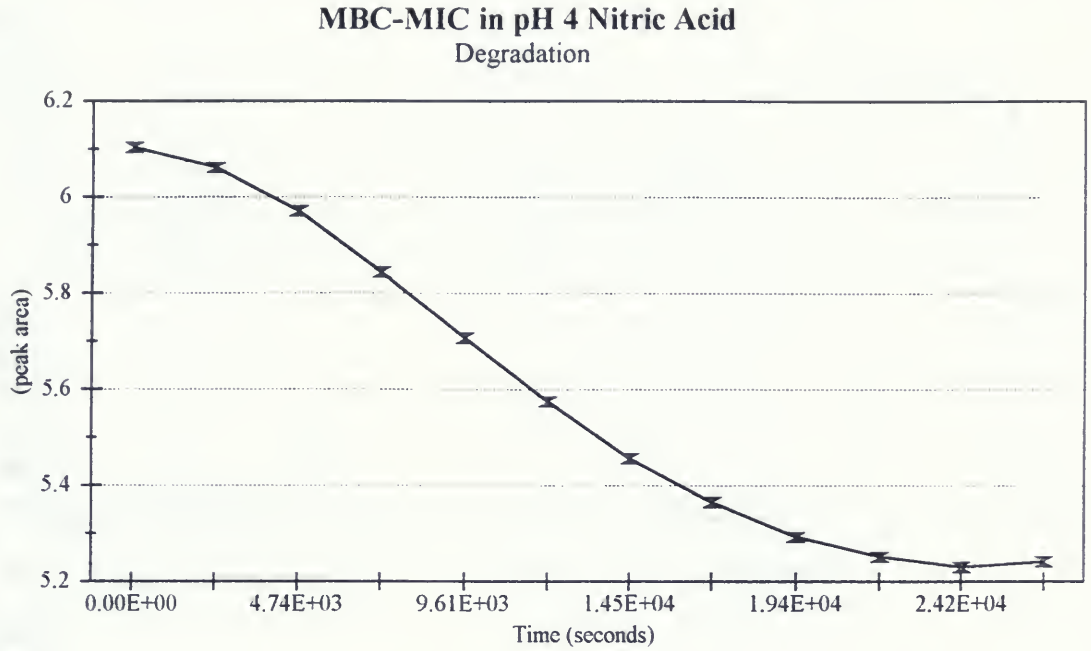


Figure 23: The non linear plot of log (MBC-MIC peak area) versus time in pH 4 nitric acid.

distilled/deionized water from Brock University) made no difference. No other reports were found in the literature of similar kinetic behaviour for benomyl or any of its homologues.

In an attempt to identify the unknown compound that behaved like STM a sample was submitted for MS analysis at Brock University. The only sample available was from an actual HPLC vial used in one of the degradation rate experiments since no conditions were known to obtain a pure sample. The sample was chosen on the basis that the unknown compound that eluted at the same retention time as STM was the major degradation compound in that vial. The MS results indicated a molecular ion of the same mass as STM but the fragmentation pattern was very different from an STM standard (Figure 25) and there was a fair amount of interference observed. In order to obtain the true mass spectrum for the unknown compound GC-MS would be required to eliminate sample interferences but time restrictions on this project and the availability of the GC-MS apparatus made this pursuit unfeasible.

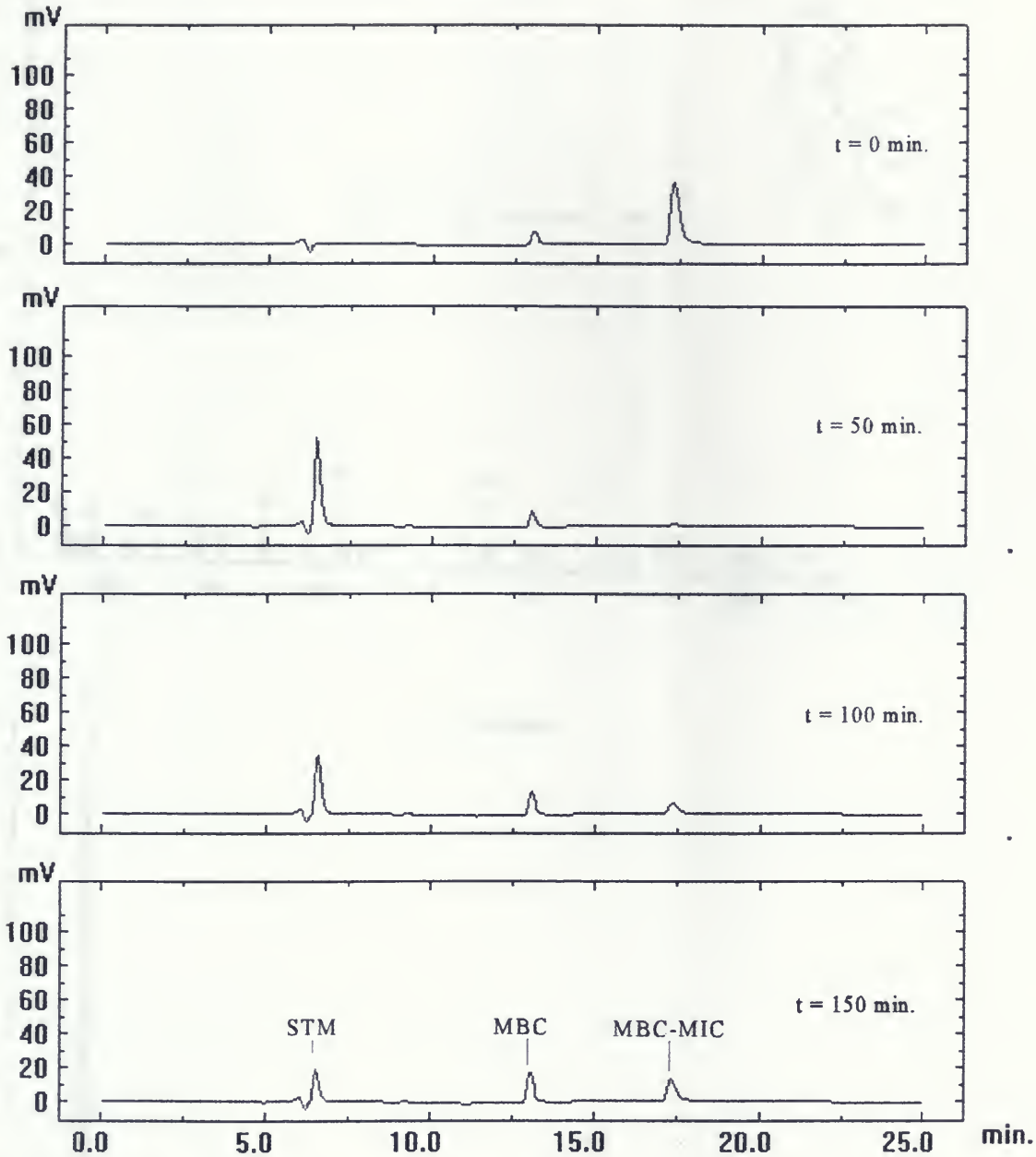


Figure 24: The HPLC chromatograms of MBC-MIC degradation in separate vials at pH 4 nitric acid at times 0, 50, 100 and 150 minutes.

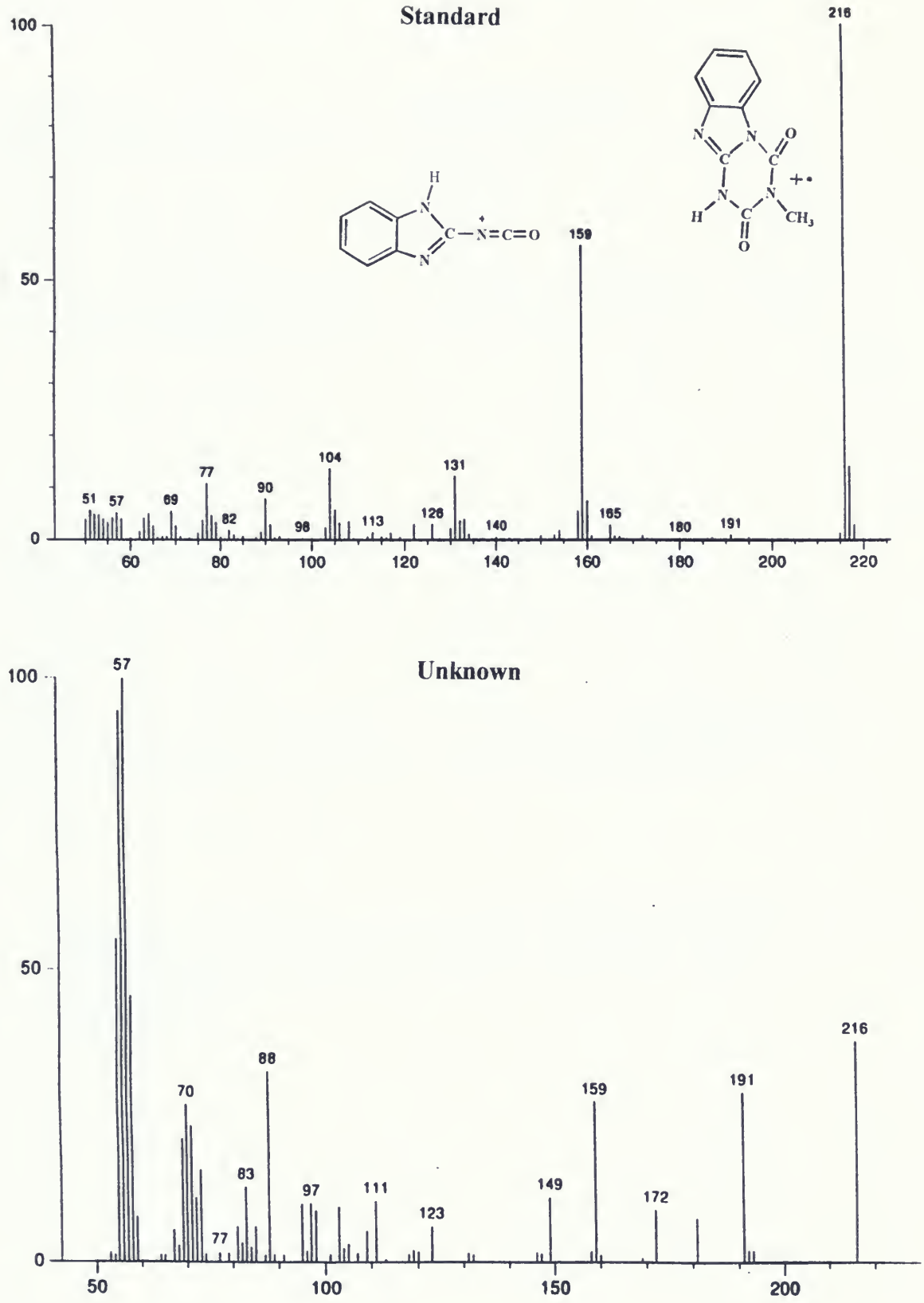
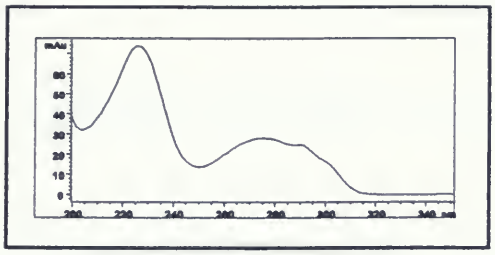
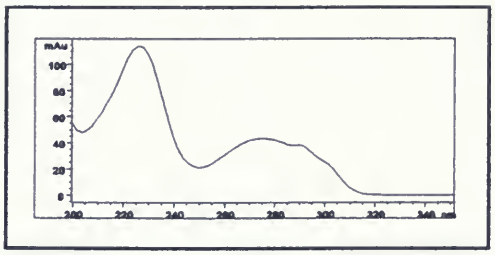


Figure 25: The mass spectra for a standard sample of STM and the unknown compound formed from MBC-MIC degradation in pH 4 nitric acid.

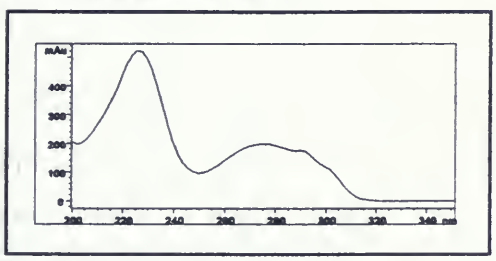
pH 4 Nitric Acid



pH 6 Phosphate Buffer



pH 8 Phosphate Buffer



pH 11 NaOH

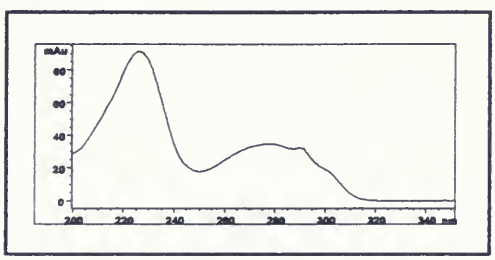


Figure 26: The UV spectra for the peak that elutes at the retention time expected for STM. The samples were obtained from the degradation of MBC-MIC in solutions of pH 4, 6, 8 and 11.

4. Solubility of Benomyl and Homologues

4.1 Standard and Sample Preparation

Throughout this study standards and samples were kept constant as described by Vukmanic and Chiba (1989). MBC standards were prepared in methanol and benomyl homologue standards were prepared in 5 M nitric acid. Standards containing a benomyl homologue and MBC were combined and diluted with buffer and water to pH 7 for the determination of the calibration curves. Benomyl homologue samples used to determine their solubility were prepared using an identical solvent composition as the standards.

To determine what percentage of methanol in the solution would provide reasonable peak shapes, the peak height of MBC was plotted against increasing percentages of methanol in MBC aqueous sample solutions (Figure 29). Increasing the amount of methanol in solution broadened the MBC peak shape. At 4%, the peak heights were relatively sharp and practical concentrations were attained in the preparation of standards.

Contrary to previously published papers (Pyysalo, 1977; Spittler *et al.*, 1984) benomyl is stable in concentrated acidic media (Singh *et al.*, 1992) and standard aqueous solutions of desired concentrations can be prepared and are stable over long periods of time. In order to meet the standardized criterion set for this study using a reversed-phase HPLC analytical column (pH 2-7) the parent compound standard in 5 M HNO_3 was neutralized. For every 1 mL 5M HNO_3, 5.75×10^{-3} moles Na_2HPO_4 was required for neutralization. The weak base Na_2HPO_4 was chosen since it simulated the buffered mobile phase and, more important, neutralized the sample to an acceptable pH level where small differences in the volume of base between samples would not alter the sample pH significantly (Figure 28). For the preparation of standards and samples to meet the required conditions 10 mL 0.2875 M Na_2HPO_4 was adequate to bring 0.5 mL of 5 M HNO_3 to pH 2.5.

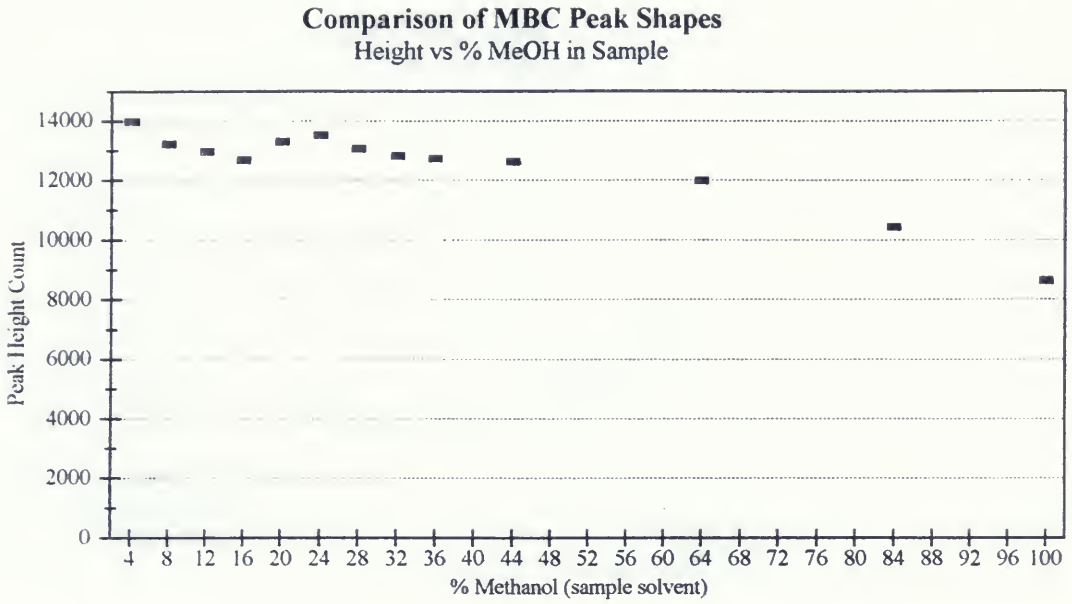


Figure 27: The peak height count for MBC (4 µg/mL) with various percentages of methanol in the sample solvent.

4.2 Results of Benomyl Homologue Solubility in Aqueous Media

Solubility values for all benomyl homologues are given in Table 8. All benomyl homologues had low solubility between pH 3 to 10. The highest values for each were at pH 1 and 12. No experiments were conducted at pH 13 due to the rapid conversion of parent compounds to corresponding degradation compounds (e.g., STM, MBU). Solubility limits were not determined in concentrated nitric acid.

MBC-MIC

MBC-MIC was most soluble in pH 1 HNO_3 (82 µg/mL). With an increase to pH 2 the solubility dropped (16.3 µg/mL) and was at its lowest between pH 3-10 (10.2 to 10.5 µg/mL). At pH 11 the solubility increased again (11.9 µg/mL) and reached a high point again at pH 12 (39.1 µg/mL).

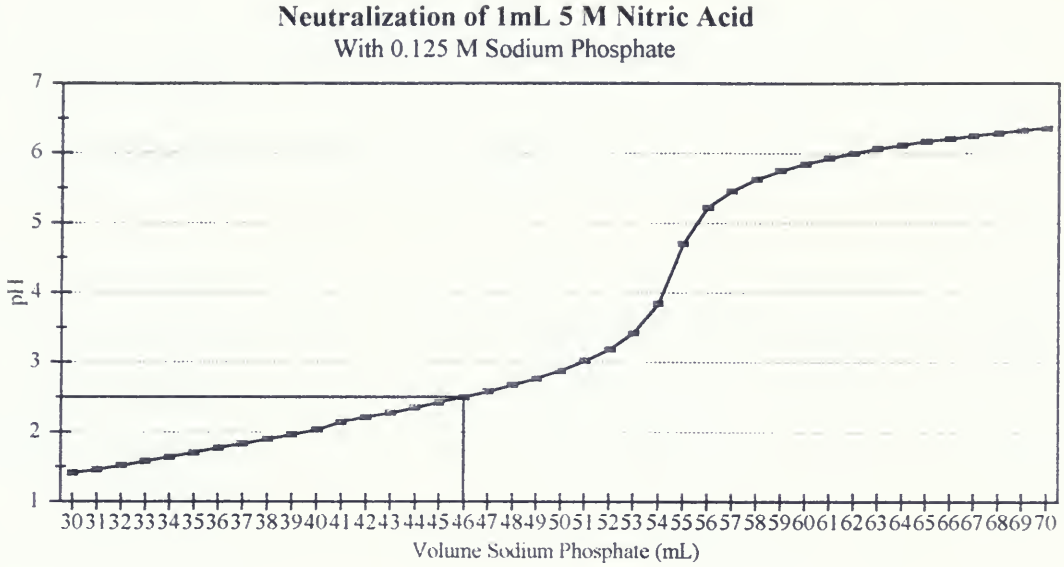


Figure 28: The neutralization of 1 mL, 5 M nitric acid to pH 2.5 required 46 mL, 0.125 M Na_2HPO_4 (5.75 X 10^{-3} moles).

Table 8: The Solubility (µg/mL) of Benomyl Homologues in aqueous Solutions of Different pH Values at 24 ± 1 °C.

pH	MBC-MIC	MBC-EIC	MBC-PIC	MBC-BIC
1	82	66	77	14
2	16.3	10.1	12.7	1.6
3	10.5	6.2	7.1	0.8
4	10.3	5.8	6.2	0.7
6	10.4	5.6	6.0	1.0
6 TD	10.2	6.5	6.6	1.0
8	10.2	5.8	5.5	0.6
8 TD	10.4	6.6	5.9	1.0
10	10.4	6.7	6.5	0.7
11	11.9	7.5	33	-
12	39.1	396	214	123

[a]TD refers to the addition of Tween 20 surfactant and dextrose.

MBC-EIC

MBC-EIC was very soluble in pH 1 HNO_3 (66 µg/mL). With an increase to pH 2 the solubility dropped (10.1 µg/mL) and was at its lowest between pH 3-10 (5.6 to 6.7 µg/mL). At pH 11 the solubility increased (7.5 µg/mL) and reached a high point at pH 12 (396 µg/mL).

MBC-PIC

MBC-PIC was very soluble in pH 1 HNO_3 (77 µg/mL). With a change in pH to pH 2 the solubility dropped (12.7 µg/mL) and was at its lowest between pH 3-10 (5.5 to 7.1 µg/mL). At pH 11 the solubility increased again (33 µg/mL) and reached a maximum at pH 12 (214 µg/mL).

MBC-BIC

MBC-BIC was relatively soluble in pH 1 HNO_3 (14 µg/mL). With an increase to pH 2 the solubility dropped (1.6 µg/mL) and was at its lowest between pH 3-10 (0.6 to 1.0 µg/mL). At pH 11 the solubility was not determined since reproducible results could not be obtained. The highest solubility occurred at pH 12 (123 µg/mL).

In comparison to those results from Singh and Chiba (1985), the solubility of benomyl in this study was generally lower between pH 1 to 10. This may be an effect of the formulation of the commercial product Benlate that was used as a source of benomyl in the Singh and Chiba study. In contrast, the solubility of MBC-BIC at pH 12 in this study was found to be much higher at 123 µg/mL whereas Singh and Chiba determined a solubility of 4.5 µg/mL. Benomyl solubility will be influenced by differences in pH, ionic strength and temperature of solvents. In the study by Singh and Chiba a pH 12 buffer with 0.05 M Na_2HPO_4 (162 mL) and 0.1 M NaOH (88 mL) was used at a temperature described as "below 25 °C" while this thesis study used 0.01 M NaOH at 24 ± 1 °C. Since the increase in solubility with an increase in pH appears to have an exponential relationship, small differences between the pH of the solvents used in the studies could be responsible for large difference in the solubility of benomyl.

Another reason for the difference is probably due to the difference in the analytical procedures

used in the two methods. In this study the extra solid was filtered after 5 minutes blending and the filtrate was neutralized immediately; thus the total time the fungicide was exposed to the alkali solution was substantially shorter than that in Singh and Chiba. In the case of Singh and Chiba the exposure time to alkali was over 20 minutes.

For all the homologue solubilities between pH 2-8, the percent standard deviation was between 0.9 and 5.0 percent. In the pH range 10-12 the standard deviation was between 0.01 and 13.79 percent. Although the precision is poor under alkaline conditions, the results give a good indication of the compounds solubility.

In concentrated acid, standards of MBC-MIC, MBC-EIC, MBC-PIC and MBC-BIC have been prepared at concentrations of over 1000 µg/mL almost instantaneously. The explanation for the high solubility is probably related (Singh *et al.*, 1992) to the suggestion for benomyl's stability in acidic media first made by Calmon and Sayag (1976). In high concentrations of acidic media, benomyl may be easily protonated (Figure 6) and become very soluble. In weakly acidic or neutral solutions benomyl may be very difficult to dissolve due to the lack of protonation. Reports by Singh *et al.*, (1992) describe dissolving benomyl up to 10000 mg L in 5 M and higher concentrations of HCl almost instantaneously.

With the exception of 5M HNO_3, the solubilities were greatest at pH 1 and 12. Generally, the solubility of these compounds decreased at pH 2 and 11 while the lowest values for solubility were between pH 3 and 10. In most cases MBC-MIC had the highest solubility, except at pH 12 where MBC-BIC was the most soluble. In all comparisons at pH 6 and 8 with and without Tween 20 surfactant little or no increase in solubility was observed.

4.2.1 Structure and Property Relationships

In general, solubility of MBC-MIC, MBC-EIC, MBC-PIC and MBC-BIC decreased with increasing molecular size, but some exceptions were found in the results. Most notable are the

results for MBC-PIC. Expected to have a lower solubility than its smaller analogue MBC-EIC, MBC-PIC was actually more soluble at a pH range of pH 1-6 and at 11, suggesting that solubility may depend on more than just the carbon chain length between the ethyl and propyl groups. Furthermore, the solubility of MBC-EIC, MBC-PIC and MBC-BIC in solution at pH 12 was much greater than that for MBC-MIC. This might suggest that increased solubility of the homologues does not depend only on the smaller size of the molecule. A more probable explanation is that the rate of degradation for MBC-MIC at pH 12 during sample preparation is much faster than that for MBC-BIC and the reaction reduces the accuracy and value for MBC-MIC solubility.

4.2.2 Significance of Solubility and Method

The solubility of all the homologues in concentrated acid is important for the development of stable stock solutions of known concentration. Standards were made above 1000 µg/mL and others have reported dissolved concentrations up to 10000 µg/mL (Singh *et al.*, 1992). The knowledge of the relationship between pH and solubility is essential for the design of commercial formulations, efficient use in the field, and the understanding of the fungicidal mode of action. For example, the use of surfactants to increase the solubility of benomyl in water may not be practical at those concentrations used in previous studies (Chiba and Northover, 1988; Northover and Chiba, 1989). Understanding the solubility is useful for the synthesis of pure degradation compounds (i.e., MBC, STB, BBU in alkali) or perhaps the formation of the parent compound (i.e., MBC-BIC from MBC and BIC in concentrated acid). Furthermore, knowing the solubility of the compounds in a variety of media is critical for the development of new analytical methods and extraction procedures from environmental samples.

The method for determining benomyl homologue solubility does not convert the parent compound to its degradation products. Degradation of the parent compound between filtering and HPLC analysis is minimized by preparing the sample within a short time frame (5 minutes). This method is especially useful under conditions where benomyl homologues degrade quickly (e.g., pH > 10). The analysis of the benomyl homologues in this manner avoids overestimations that can

occur by using the conversion to STB or MBC methods since MBC or STX like compounds may already exist in samples as natural degradation products.

Future work should consider the effect of benomyl homologue solubility with varying surfactant concentrations, and changes with temperature.

5. Melting Points

5.1 Decomposition Before Melting

Decomposition occurred before a melting point could be determined for MBC-MIC, MBC-EIC, MBC-PIC and MBC-BIC. Visible changes began between 230 and 250 °C (temperatures reached between 10 and 15 minutes) when each sample started to shrink in size, slowly twisted into a spiral, and gradually turn dark brown before reaching a liquid stage at 290 °C (final temperature reached in 22 minutes)

5.2 Composition of Sample at Various Temperatures

Extra work from the original thesis outline was conducted in an effort to determine the composition of MBC-MIC samples at increasing temperatures with 20 minute exposure. Samples were measured with exposure to the atmosphere and samples that were sealed into a closed system. The method used was not statistically reproducible and time restrictions limited further attempts for improvement. With these conditions taken into account the method and data has been put into Appendix I. The information obtained from HPLC analyses revealed that some degradation to MBC had occurred at 100 °C and little difference between samples exposed to and sealed from the atmosphere (open and closed systems respectively). Closed systems were obtained by sealing the open end of the capillary tube with a flame. At 150 °C greater degradation to MBC took place in both open and closed systems while some signs of what eluted at the same retention time as STM

appeared in the closed system. By 200 ℃ greater degradation had occurred from parent compounds to STM and MBC in both open and closed systems. In addition, the closed system appeared to form a small amount of compound that eluted at the same retention time as MBU.

5.3 Future Work

Further studies should be conducted on the stability of benomyl homologues at various temperatures. This work would be critical for efficient field use and formulation design for reasonable shelf lives. Work should also be concentrated on the stability of STM, STE, STP and STB at elevated temperatures for their use as an analyte in GC experiments for the determination of benomyl homologues.

SUMMARY AND CONCLUSIONS

1. Column Selection and Method Improvement

The best column tested in this study was the Phenomenex 5 um Spherisorb ODS(2) 250 X 4.6 mm analytical column. For practical routine analyses the shorter 150 X 4.6 mm column was sufficient while the longer column with greater separation ability might be best when searching for unknown compounds. A significant improvement was made in RP-HPLC gradient systems, which enabled to complete a total analysis within 20 minutes. Furthermore, another significant improvement was made in the sample preparation procedure for the solubility and kinetics studies so that RP-HPLC can be achieved without converting the parent compounds to STX type compounds. Thus the results obtained are very accurate and have helped to elucidate the behaviour of benomyl and its homologues in alkaline solutions.

2. Degradation of Benomyl Homologues in Aqueous Media

Stable standard stock solutions were prepared in 5 M HNO_3. These standards remained stable for at least 2 weeks and in the case of MBC-MIC at least 1 month. In the pH range of 1-8, the longest half lives for all the benomyl homologues (5.1 to 10.1 hours) generally occurred at pH 6 without the addition of Tween 20 surfactant or dextrose. MBC-MIC was an exception and the longest half life was observed at pH 3 and 2 (8.2 and 7.8 hours respectively). For all the benomyl homologues an increase in the rate of degradation occurred at pH 6 with the addition of Tween 20. Meanwhile, there was no marked difference in the half life of MBC-EIC at pH 8, regardless of the presence of surfactant, although MBC-PIC and MBC-BIC were actually more stable with the addition of the surfactant. In general, the stability of the benomyl homologues increased with the increase of the molecular size.

3. Solubility of Benomyl Homologues in Aqueous Media

The solubility of benomyl homologues was greater at either strongly acidic or alkaline conditions (e.g., pH 1 or 12 where the solubilities ranged between 14-396 µg/mL). The lowest solubility was observed between pH 3 to 10 (0.6 to 10.5 µg/mL from MBC-BIC to MBC-MIC, respectively). In all comparisons at pH 6 and 8 with and without Tween 20 surfactant little or no increase in solubility was observed. Generally, the solubility of the compounds decreased with molecular size with the exception of MBC-PIC which was more soluble than MBC-EIC. The results suggest that solubility may depend on more than just the carbon chain length of the molecule.

4. Melting Points of Benomyl Homologues

All of the benomyl homologues decomposed before melting. This process was observed between 230 and 250 °C, and was completed by 290 °C. Extra studies revealed that degradation did occur at lower temperatures (100, 150, 200 °C) and suggest that the formation of MBC, STM and MBU increased with temperature.

5 Conclusions

The studies within this thesis have generated an important portion of data required for the registration of the methyl homologue (MBC-MIC) of benomyl (MBC-BIC) as a commercial product. Fundamental data on the properties of not only MBC-MIC, but also two other homologues, MBC-EIC and MBC-PIC were determined by using benomyl (MBC-BIC) as the reference compound.

Several methods developed during this study contributed significantly for the determination of solubility and the rate of decomposition of parent compounds in alkaline solutions. These methods enabled to minimize the analytical errors which were substantially larger previously.

It is expected that the results of this study will contribute to the development of formulations as commercial products. Several notable aspects of the results are the influence of surfactant, effect of pH on the stability and solubility of these compounds, and their findings will be incorporated for the production of commercial formulations. It is also expected that the findings will help to understand the mode of action, interpretation of biological data, and improvement of application methods in the field. Furthermore, the understanding of these properties can be useful for the improvement of synthetic methods (e.g., to increase yields) for pure degradation compounds (i.e., MBC, STB, BBU in alkali).

Future work to determine the solubility of these degradation compounds in aqueous and organic solvents would be useful for increasing their percent yield when synthesizing. This information would also be helpful for establishing methods of analysis and understanding their behaviour in agricultural and biological systems. Other areas of interest are studying the solubility and degradation of benomyl homologues with varying surfactant concentrations and temperatures. New methods could be explored using concentrated acid for the extraction of benomyl homologues or their synthesis (e.g., MBC-BIC from MBC and BIC in 5 M nitric acid). Finally, the determination of unknown compounds which were recognized during the degradation of MBC-MIC, MBC-EIC and MBC-PIC at pH 4 and 6 is worthwhile to pursue, particularly by an efficient GC-MS system.

APPENDIX 1: Degradation of MBC-MIC at Elevated Temperatures

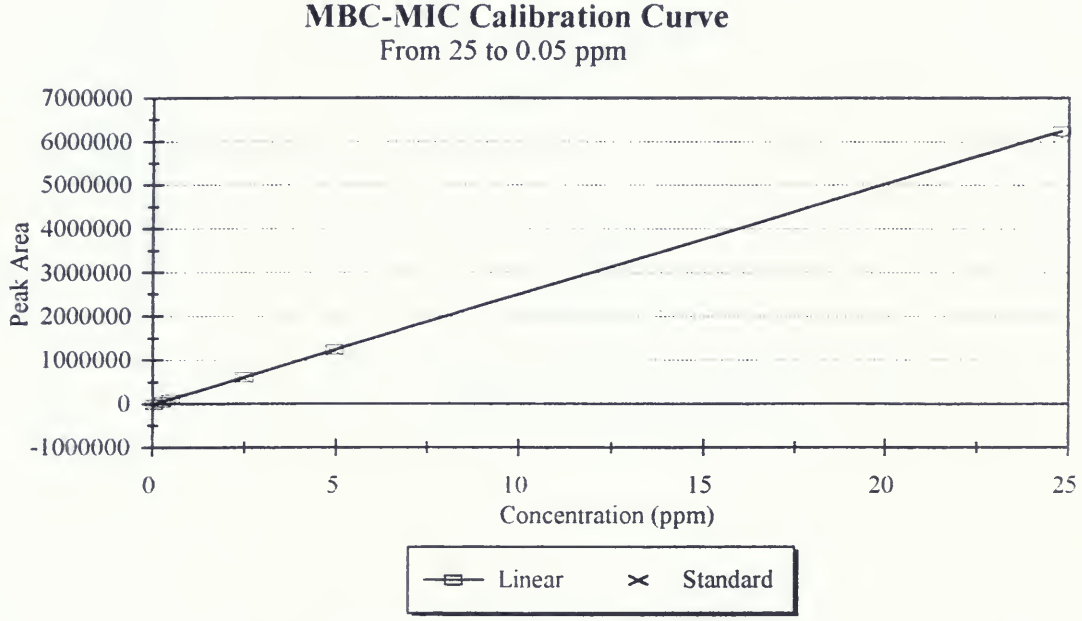
Method

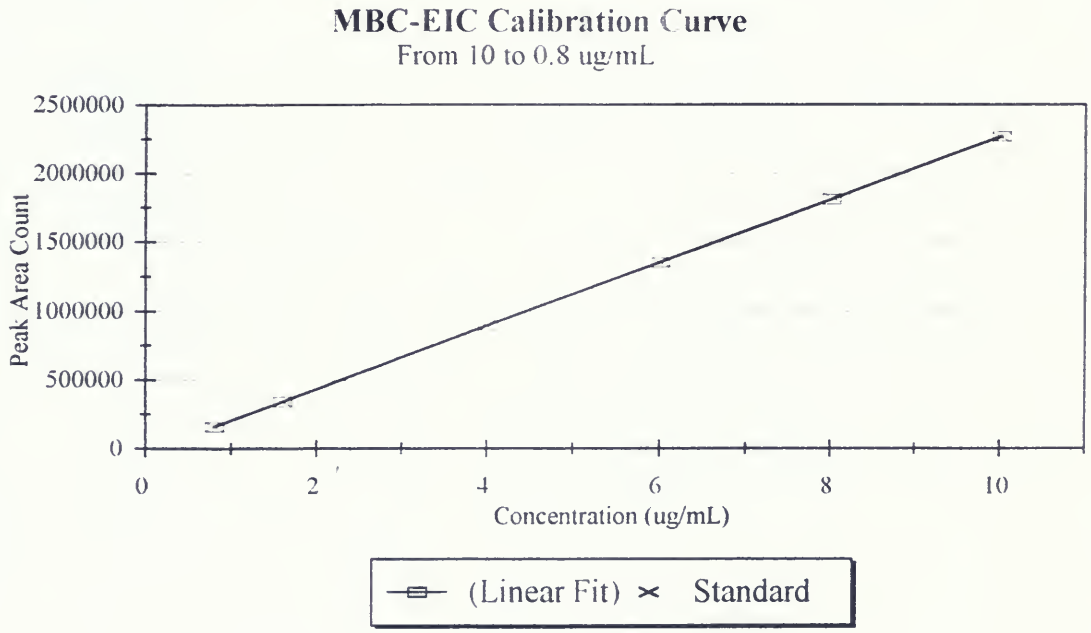
Samples of MBC-MIC were ground in a mortar with a pestle and packed into capillary tubes to about 0.5 cm in height. Each sample was exposed to temperatures of 100, 150, and 200 °C for a period of 20 minutes. After cooling, the samples in the capillary tubes were crushed in a clean mortar with a pestle, dissolved in 1.0 M HNO_3 and mixed for two minutes. A 0.5 mL aliquot was pipetted and transferred to a 50 mL volumetric flask, and diluted to the mark with water. An HPLC sample was prepared by filtering some sample to an HPLC vial using a 0.45 um Gelman GHP Acrodisc GF syringe filter (0.13 mm dia.). Peak Area percentages were compared for an indication of the degree of degradation that had occurred with each sample.

Table 9: Control Peak Height Percentages for MBC-MIC and the MBC, MBU, and STM degradation products.

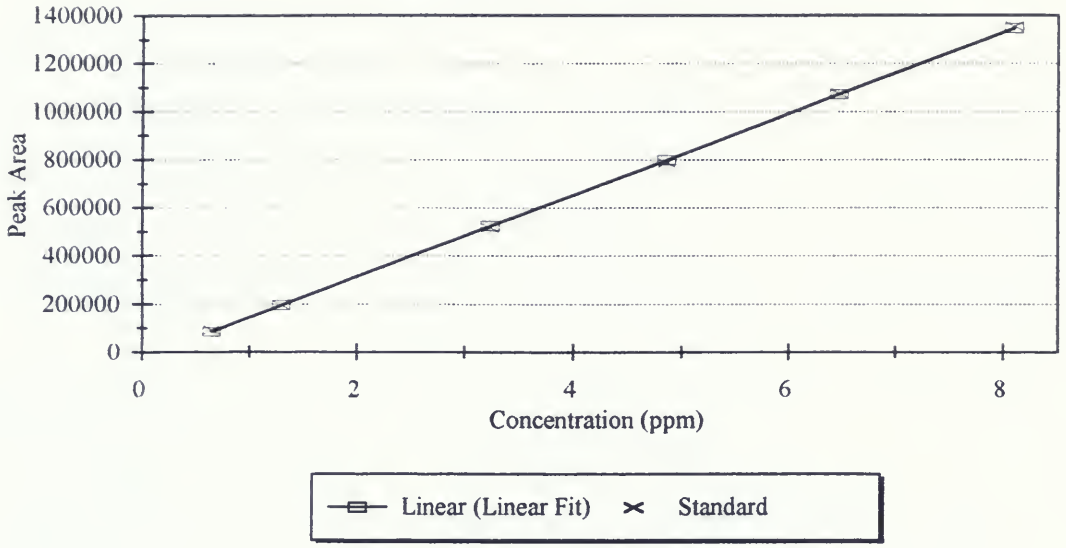
Temp	Data	STM		MBU		MBC		MBC-MIC	
deg C	Type	Open	Closed	Open	Closed	Open	Closed	Open	Closed
	Area	0	-	0	-	21974	-	1348386	-
Control	%	0	-	0	-	1.604	-	98.396	-
	Height	0	-	0	-	771	-	22026	-
	%	0	-	0	-	3.382	-	96.618	-
	Area	0	0	0	0	49874	23093	999969	1154972
100	%	0	0	0	0	4.751	1.916	95.249	95.806
	Height	0	0	0	0	1335	566	13292	12242
	%	0	0	0	0	9.127	4.077	90.873	88.173
	Area	0	197187	0	0	752540	764474	331746	270266
150	%	0	16.006	0	0	69.404	62.055	30.596	21.938
	Height	0	3833	0	0	16528	17288	1781	1547
	%	0	16.909	0	0	90.273	76.266	9.727	6.825
	Area	61312	345716	0	17177	626986	353154	501183	456054
200	%	5.155	29.495	0	1.465	52.711	30.13	42.135	38.909
	Height	1482	6461	0	539	13190	7294	1958	1944
	%	8.912	39.789	0	3.319	79.314	44.919	11.774	11.972

APPENDIX 2: Calibration Curves for MBC-MIC, MBC-EIC, MBC-PIC and MBC-BIC Solubility

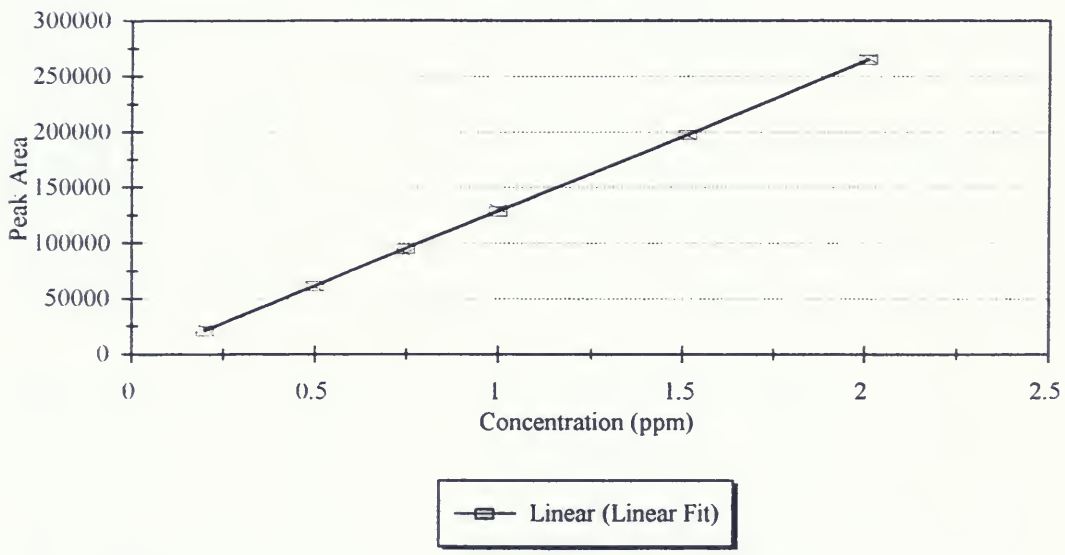




MBC-PIC Calibration Curve
From 8.1 TO 0.6 ppm



MBC-BIC Calibration Curve
From 2.5 to 0.2 ppm



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