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IDENTIFICATION OF MUTAGENIC BY-PRODUCTS FROM AQUATIC HUMIC CHLORINATION

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CONTENTS

Page

LIST OF TABLES v

LIST OF FIGURES vii

ACKNOWLEDGMENTS ix

EXECUTIVE SUMMARY xi

1. INTRODUCTION 1

2. EXPERIMENTAL METHODS 5
   - Water Samples and Chlorination Procedure 5
   - Procedure for Isolation and Concentration of Mutagens 5
   - Synthesis 6
   - Derivatization Procedure and Conditions of GC/MS Analyses 9
   - Nuclear Magnetic Resonance Spectroscopy 15
   - Fractionation of Mutagenic Material 15
   - Mutagenicity Testing Procedure 15

3. RESULTS AND DISCUSSION 19
   - Mutagenicity of the MX Analogues 19
   - Quantitative Analyses of the MX Analogues in Extracts of Chlorinated Water 22
   - Fractionation of Extracts of Chlorinated Fulvic Acids 28

4. CONCLUSIONS 37

5. RECOMMENDATIONS 39

REFERENCES 41

LIST OF ABBREVIATIONS AND ACRONYMS 45

TABLES

		Page
2.1	Derivatization Procedure and Gas Chromatography Conditions for SIM-Mode Gas Chromatography/Mass Spectrometry Determination of Mutagens in Extracts	12
2.2	Ion Peaks Used and Relative Peak Area Ratios Found for SIM-Mode Gas Chromatography/Mass Spectrometry Analyses of MX and MX Analogues in Standard Solutions and in Chlorinated Water	13
3.1	Mutagenic Potencies of MX and the MX Analogues	20
3.2	Results of Gas Chromatography Analyses of ox-MX	22
3.3	Concentration and Mutagenicity Contribution of MX and MX Analogues in Extracts of Chlorinated Water	25
3.4	Concentration of ox-MX and red-MX in Unspiked and Spiked Samples of Chlorinated Drinking Water	27
3.5	Mutagenic Activity and MX and EMX Concentrations in Aqueous Solutions of Chlorinated Fulvic Acids	29
3.6	Mutagenicity, MX and EMX Concentrations, and MX Mutagenicity Contribution in Extracts of Fulvic Acid Obtained at Various pH Conditions	31
3.7	Mutagenicity and MX Concentration in Fractions of Fulvic Acid	32

FIGURES

		Page
2.1	Mass Spectrum of ox-MX Anhydride	7
2.2	Mass Spectrum of ox-EMX	8
2.3	Mass Spectrum of red-MX	10
2.4	Mass Spectrum of Compound Formed During Reaction of EMX With $NaBH_4$	11
2.5	Procedure for Purification and Fractionation of Mutagenic Compounds Present in the Extract of the Chlorinated Aqueous Solution of Fulvic Acids	16
3.1	Mutagenic Response of ox-MX in Strain TA 100 (-S9)	21
3.2	Mutagenic Response of red-MX in Strain TA 100 (-S9)	21
3.3	Mutagenic Response of ox-EMX in Strain TA 100 (-S9)	21
3.4	Mass Spectrum of Methylated ox-MX	23
3.5	Mass Spectrum of Methylated ox-EMX	24
3.6	GC Separation of Methylated ox-EMX and Methylated ox-MX on a DB-5/60-m Fused Silica Capillary Column	27
3.7	Examples of SIM Chromatograms of red-MX Ions Generated During Analyses of Underivatized Extract of Chlorinated Humic Water	28
3.8	Contribution of MX to the Total Mutagenic Activity of Chlorinated Aqueous Fulvic Acid Solutions	30
3.9	C_6 Column HPLC Fractionation of Extract of Chlorinated Aqueous Solution of Fulvic Acids	33
3.10	Contribution of MX to the Total Mutagenic Activity of Sample H Subfractions	34
3.11	C_{18} Column Separation of the Organic Material in the C_6 Column Fraction H3	35

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EXECUTIVE SUMMARY

Drinking water is disinfected primarily to destroy and eliminate the pathogenic organisms responsible for waterborne disease. Commonly used disinfectants are chlorine, chlorine dioxide, chloramines, and ozone. Chlorine is most often used because it is toxic to many microorganisms and is cheaper than most other disinfectants. However, the chemical reactivity of chlorine with natural humic substances results in the formation of halogenated by-products, or disinfection by-products (DBP), such as chloroform, other trihalomethanes, and other nonvolatile organohalides. The purpose of this study was to chlorinate water containing natural humic materials, fractionate the nonvolatile DBPs produced, and identify the compounds most responsible for the mutagenic activity of the DBP fractions. The investigators were especially interested in determining if compounds similar to the known mutagen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) were present. The percentage of total chlorinated sample mutagenicity represented by each identified product was also determined.

MX has never been observed without the presence of its geometric isomer, (E)-2-chloro-3-(dichloromethyl)-4-oxobutenoic acid (EMX), the mutagenic potency of which is less than 10 percent of MX. Because each of these molecules contains an acid and an aldehyde group, it was postulated that the oxidizing conditions of chlorination could result in the formation of the diacid of each molecule and that if these compounds represent fragments of the original humic structure, reduced forms of each might be precursors of MX and EMX and might also be present in chlorination mixtures. Thus, an analytical search was performed for MX, EMX, and their oxidized and reduced forms in chlorination mixtures of humic extracts, natural humic waters, and actual drinking water samples.

Pure samples of the oxidized and reduced forms of each isomer (except reduced-EMX) were successfully synthesized and characterized by nuclear magnetic resonance (NMR) and gas chromatography/mass spectrometry (GC/MS) (see Table 2.1, p. 12). In addition, the mutagenic potency of each pure compound was determined (see Table 3.1, p. 20) with the Ames histidine reversion assay with strains TA 98, 100, and 102.

Relatively concentrated humic solutions were prepared from solid humic extracts in order to maximize the concentration of products after chlorination. The solutions were chlorinated and the postulated compounds were sought by selected ion monitoring GC/MS. All compounds (except reduced-EMX) were found and quantified. The experiments were then repeated with natural humic waters, which were lower in humic concentration, and with actual drinking water with the same results (see Table 3.3, p. 25). It was found that lower pH and higher chlorine/carbon (Cl_2/C) dosages increased both the total concentration of products and the total mutagenicity (see Table 3.5, p. 29). The concentrations of EMX and the postulated products were equal to or slightly higher than the MX concentration under all chlorination conditions, except for the concentration of oxidized-EMX, which was found to be 20 to 40 times higher (Table 3.3). However, the contribution of EMX and the postulated compounds to the total chlorinated sample mutagenicity was small relative to the contribution of MX.

Interestingly, the absolute amount of MX produced by chlorination increased with increasing Cl_2/C ratio only at low pH although the percentage contribution of MX to total mutagenicity was greater at neutral pH (Table 3.5).

CHAPTER 1

INTRODUCTION

Drinking water is disinfected primarily to destroy and eliminate pathogenic organisms responsible for waterborne disease. Commonly used disinfectants are chlorine, chlorine dioxide, chloramine, and ozone (Faust and Aly 1983). Chlorine is most often used because it is toxic to many microorganisms and is less expensive than most other disinfectants (Barnes and Wilson 1983). However, the chemical reactivity of chlorine with dissolved organic matter results in the formation of halogenated by-products (Johnson et al. 1982; Miller and Uden 1983; Oyler et al. 1983; Bull 1982). The United States Environmental Protection Agency (USEPA) plans to strengthen regulation of disinfection by-products (DBPs) in the early 1990s.

The production of DBPs from chlorination of drinking water was not recognized until 1974, when Rook demonstrated that chlorination of water containing natural humic substances produced chloroform and other trihalomethanes (Rook 1974). However, it was soon discovered that most of the chlorine that is bound to organic compounds resides in the nonvolatile fraction of the organic material (Glaze et al. 1979). In general, the nonvolatile organohalides are more difficult to identify than are the trihalomethanes, partially because the nonvolatiles are more polar and because they are present as complex mixtures of individual compounds in very small concentrations.

Concern with potential human health risks associated with drinking water has been heightened by the widespread recognition in recent years of mutagenic activity exhibited by nonvolatile products (Simmon and Tardiff 1976; Glatz et al. 1978; Loper 1980; Cheh et al. 1980; Foster 1984; van der Gaag et al. 1982; Grabow et al. 1981; Kool et al. 1982; Nestmann et al. 1979; Athanasiou and Kyrtopoulos 1983; Dolara et al. 1981). In addition, recent epidemiological studies have suggested that the risk of bladder cancer rises with the intake of chlorinated tap water (Cantor et al. 1987).

Although a number of mutagenic compounds have been detected in chlorinated drinking and humic water, the only major mutagen that has been identified is the compound 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX), which has been shown to generate 5,600 net rev/nmol (revertants per nanomole) in the Ames assay with strain TA 100 (Kronberg et al. 1988). For comparison, the most active of the trihalomethanes, chlorodibromomethane, generates approximately 0.004 net rev/nmol (Simmon et al. 1977).

In most of the chlorinated waters studied so far, MX has contributed approximately 30 percent of the total mutagenicity of the sample, while its geometric isomer, (E)-2-chloro-3-(dichloromethyl)-4-oxobutenoic acid (EMX), contributed a few percent or less (Kronberg et al. 1988). In order to more fully

understand the impact of mutagens in drinking water, the chemical identity of the compounds responsible for major portions of the residual activity should be determined. Previous studies have indicated that as much as 90 percent of the mutagenicity in drinking water is attributable to nonvolatile compounds with acid properties and that major mutagens are susceptible to attack by nucleophiles (Kronberg et al. 1986; Holmbom and Kronberg 1988).

Information on the genotoxicity and mutagenicity of drinking water samples has been obtained, to date, primarily by the use of the Salmonella/microsome mutagenicity (Ames) assay (Kool et al. 1983; Meier and Bull 1985; Meier 1988). However, findings from mammalian and nonmammalian eukaryotic assays for genotoxicity provide evidence that the genotoxic activity of the organic fraction of concentrated drinking water samples is not restricted to bacterial mutagenicity (Meier 1988). Ames and McCann (1981) estimated an 83 percent correlation between the ability of chemicals to induce mutations in bacterial assays and the induction of cancer in long-term animal tests. The Ames assay utilizes Salmonella typhimurium strains that carry mutations in the genes that code for enzymes responsible for histidine production (the histidine operon). These strains are therefore unable to synthesize the essential amino acid histidine and are unable to grow in the absence of histidine in the culture media. The assay measures back mutation, wherein the normal enzyme function is restored. The revertant cells, regaining histidine independence, are able to form visible colonies on histidine-free media. Exposure of the Ames Salmonella strains to mutagenic agents induces an increase in the frequency of back mutation (or reversion) that is distinctly above the spontaneous rate. In addition to carrying the histidine mutation, the standard tester strains also contain other mutations that greatly increase their ability to detect mutagens (Maron and Ames 1983).

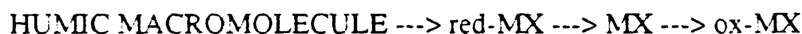
There are a number of different standard tester strains of Salmonella typhimurium that respond to different types of changes in their genetic material. Strain TA 98 detects frameshift mutations, while TA 100 and TA 102 detect base-pair substitution. TA 100 detects mutagens that affect base-pair substitution at the guanine-cytosine (G-C) pairs, while TA 102 detects those mutagens affecting the adenine-thymine (A-T) pairs. It is known that some mutagens react preferentially at the A-T base pair (Levin et al. 1982). In addition, TA 102 can be used to identify mutagens that operate through oxidative mechanisms. It is now recommended that TA 102, in addition to TA 98, be used for all routine screening (Maron and Ames 1983). Based on what is known about the mutagenic responses of MX and EMX, it was felt that strains TA 100, TA 98, and TA 102 were sufficient to detect any mutagenic activity exhibited by the compounds studied in this work.

The MX mutagen is one of the most active mutagens ever tested in the Ames assay. It has been shown that structural arrangements of critical importance for conferring on MX its extreme mutagenicity

are the dichloromethyl group at the 3 position in the open form of MX and the cis arrangement of the $HCCl_2$ and Cl groups around the carbon carbon double bond (Kronberg et al. 1988; Streicher 1987; Ishiguro et al. 1988). The MX analogues with these structural arrangements are the compounds in which the MX aldehyde has been reduced to an alcohol group or oxidized to a carboxyl group.

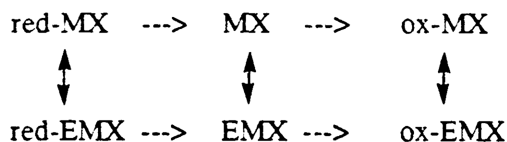
Assuming that MX is an intermediate of oxidation reactions in water, reduced MX (red-MX) could be a precursor to MX, and MX could be a precursor of the oxidized MX (ox-MX). Thus, it is attractive to view these analogues as existing in a red-ox relationship, which can be roughly described as

Presumably, a good oxidant such as chlorine would force this reaction to the right, although the actual equilibrium conditions are unknown at present. In addition, because water without humic macromolecular carbon does not produce MX, one may assume that the carbon precursor units in the humic macromolecule are more reduced than red-MX. Thus, it is possible to write



with the realization that with actual humic and chlorine concentrations, one or more of these products may be unmeasurably small at equilibrium. It is therefore important to establish whether these analogues are present in chlorinated humic waters and to gain some idea of their relative concentrations.

This chemistry is actually more complicated, of course, because of the presence of EMX, the geometric isomer of MX. MX has never been observed without EMX. Thus, the above reactions could also be written for a reduced-EMX ---> EMX ---> oxidized-EMX chain. We already know, however, that MX and EMX isomerize in aqueous solution (Kronberg et al. 1989), and it is, at least theoretically, possible that the reduced and oxidized forms of each compound also isomerize in aqueous solution:



Thus, without further knowledge of the kinetics of these reactions and the equilibrium concentrations of the analogues, it is not possible to write definitive reaction diagrams. At present, the extent of our

knowledge of the structure of the humic precursor material is very limited, and is not even close to the level that might permit us to understand the formation of these products.

The purpose of this study was to chlorinate water containing natural humic materials, fractionate the nonvolatile organohalogens produced, and identify the compounds most responsible for the mutagenic activity of the fractions. In addition, the chemistry of the formation of MX and the effect of the structure of MX on its mutagenicity were investigated using reduced and oxidized forms of MX and its geometric isomer EMX.

CHAPTER 2
EXPERIMENTAL METHODS

WATER SAMPLES AND CHLORINATION PROCEDURE

Fulvic acids, previously extracted by the method of Thurman and Malcolm (Thurman and Malcolm 1981) from a highly colored natural lake (Lake Drummond, southeastern Virginia), were dissolved in distilled water to give a total organic carbon (TOC) content of 2.5 g/L (grams per liter). Part of the water solution was adjusted to pH 7 with phosphate buffer and part was lowered to pH 2 with 4 N HCl. Each of these samples was further divided into four parts and treated with chlorine at Cl_2/TOC weight ratios of 0.5, 1.0, 1.5, and 2.0, respectively.

Natural humic water with a TOC content of 20 mg/L (milligrams per liter) was collected from Lake Savojaervi, located in a marsh region in southwestern Finland. The sample had been chlorinated at pH 7 at a Cl_2/TOC weight ratio of 1 (Kronberg et al. 1988).

During chlorination, the pH of the samples was monitored and readjusted to the preset values when necessary. After a reaction time of 60 hours, the total chlorine residuals in the samples were less than 0.1 mg/L, and the pH values of all samples were lowered to pH 2 by the addition of 4 N HCl.

Four liters of drinking water, derived from surface water with a TOC content of approximately 5 mg/L, were collected from the distribution system of a municipality in North Carolina. The water had been chlorinated at the treatment plant with approximately 5 mg Cl_2/L. The samples were stored for 24 hours in a decanter glass to get rid of most of the residual chlorine. The pH of the sample was adjusted to pH 2 by the addition of 4 N HCl.

PROCEDURE FOR ISOLATION AND CONCENTRATION OF MUTAGENS

Immediately after the pH of the chlorinated humic water and drinking water was lowered, the samples were passed through columns of XAD-4 and XAD-8 resins (1:1 volume mixture). The flow rate was approximately one bed volume/min (20 milliliters per minute). Most of the residual water in the column was then removed by a gentle stream of nitrogen. The adsorbed organics were eluted with three bed volumes of ethyl acetate. The extracts were concentrated, and the final volume was adjusted to 1 mL (milliliter) of ethyl acetate per liter of original water. The extract of chlorinated humic water will, in this report, be referred to as HW and the extract of drinking water as DW.

The chlorinated fulvic acid solutions were extracted with three portions of diethyl ether. The combined extracts were evaporated to dryness and redissolved in ethyl acetate so that 1 mL of ethyl

acetate corresponded to 1 L of water with a TOC content of 20 mg/L. The chlorinated fulvic acid sample that was chlorinated at pH 2 at a Cl_2/TOC ratio of 2 is referred to as sample FA.

SYNTHESIS

Oxidized MX, (Z)-2-chloro-3-(dichloromethyl)-butenedioic acid, was prepared by the oxidation of 40 mg, or 185 umol (micromoles), of MX with 2 mL of fuming nitric acid at 70°C for 24 hours. The reaction mixture was cooled in an ice bath and diluted with 20 mL of ice-cold water and subsequently extracted three times with diethyl ether. The combined ether extract was washed with 0.01 M HCl and then evaporated to dryness. The pure compound (9.1 mg; yield 21 percent) was obtained as white crystals following recrystallization from dichloromethane. The nuclear magnetic resonance spectroscopy (^{1}H NMR) spectrum of the compound showed the resonance signal of the proton in the dichloromethyl group at δ 6.2. The electron impact mass spectrum of the compound is presented in Figure 2.1. The mass spectrum represents the anhydride of ox-MX because the compound loses a molecule of water to form the anhydride when it is heated in the mass spectrometer (MS) inlet probe or the gas chromatograph (GC) injector. An unsuccessful attempt was made to obtain ox-MX by the oxidation of MX with $NaClO_2$ in the presence of resorcinol as a chlorine scavenger. (This procedure did work for the production of ox-EMX from EMX, as described below.)

Oxidized EMX, (E)-2-chloro-3-(dichloromethyl)-butenedioic acid, was prepared by the oxidation of EMX (10 mg; 46.3 umol) with 5.0 mg (56 umol) of $NaClO_2$ and resorcinol as a chlorine scavenger in water at pH 3.5. After a reaction time of 2.5 hours, the pH of the mixture was raised to 4.5, and extraction was carried out with diethyl ether. The ether was discarded, the pH of the water solution was lowered to pH 2, and ether extraction was repeated. The ether extract was washed with 0.01 N HCl and, following evaporation of the ether, the crude product was recrystallized from dichloromethane. Finally, the crystals were washed with CCl_4. The yield of ox-EMX (white crystals) was 20 percent. The ^{1}H NMR resonance signal of the dichloromethyl group was observed at δ 5.6. Figure 2.2 presents the electron impact mass spectrum of ox-EMX. Oxidation of EMX with fuming HNO_3, MnO_2, and $KMnO_4$ did not yield the desired product.

Reduced MX, 3-chloro-4-(dichloromethyl)-2(5H)-furanone, was obtained by the reduction of 20 mg (93 umol) of MX with aluminum isopropoxide (225 umol) in isopropanol (the Meerwein Ponndorf reduction). The reaction temperature was kept at 70°C for 2 hours. The reaction was stopped by the addition of ice and 4 N HCl. The acidified mixture was heated to 50°C for a few minutes and recooled, and the mixture was then extracted three times with diethyl ether. The combined extract was washed with 0.01 N HCl, and, following evaporation of the ether, the crude product was obtained. Purification on

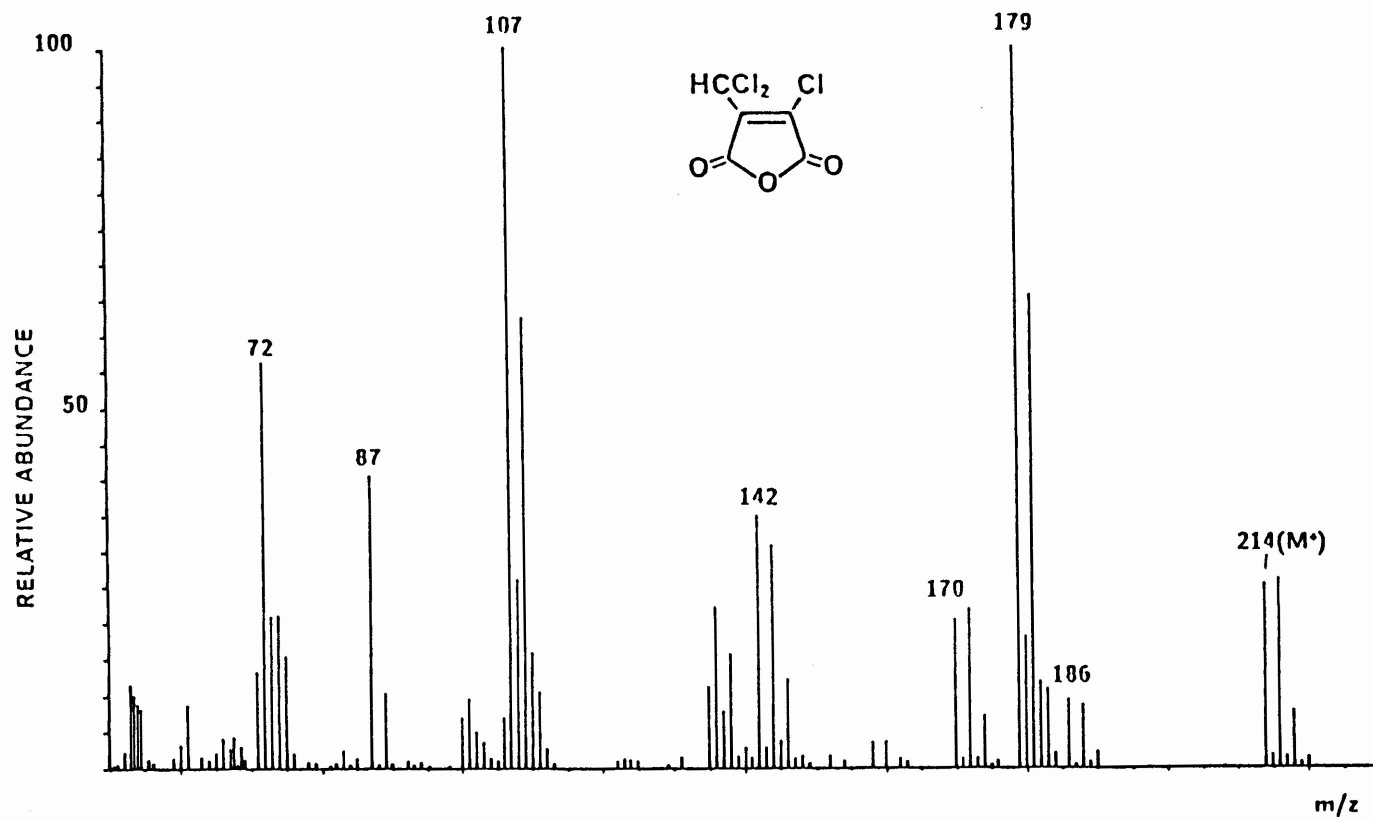


Figure 2.1. Mass Spectrum of ox-MX Anhydride. The ring-closed anhydride is formed in the direct probe inlet system of the mass spectrum by loss of water from the diacid.

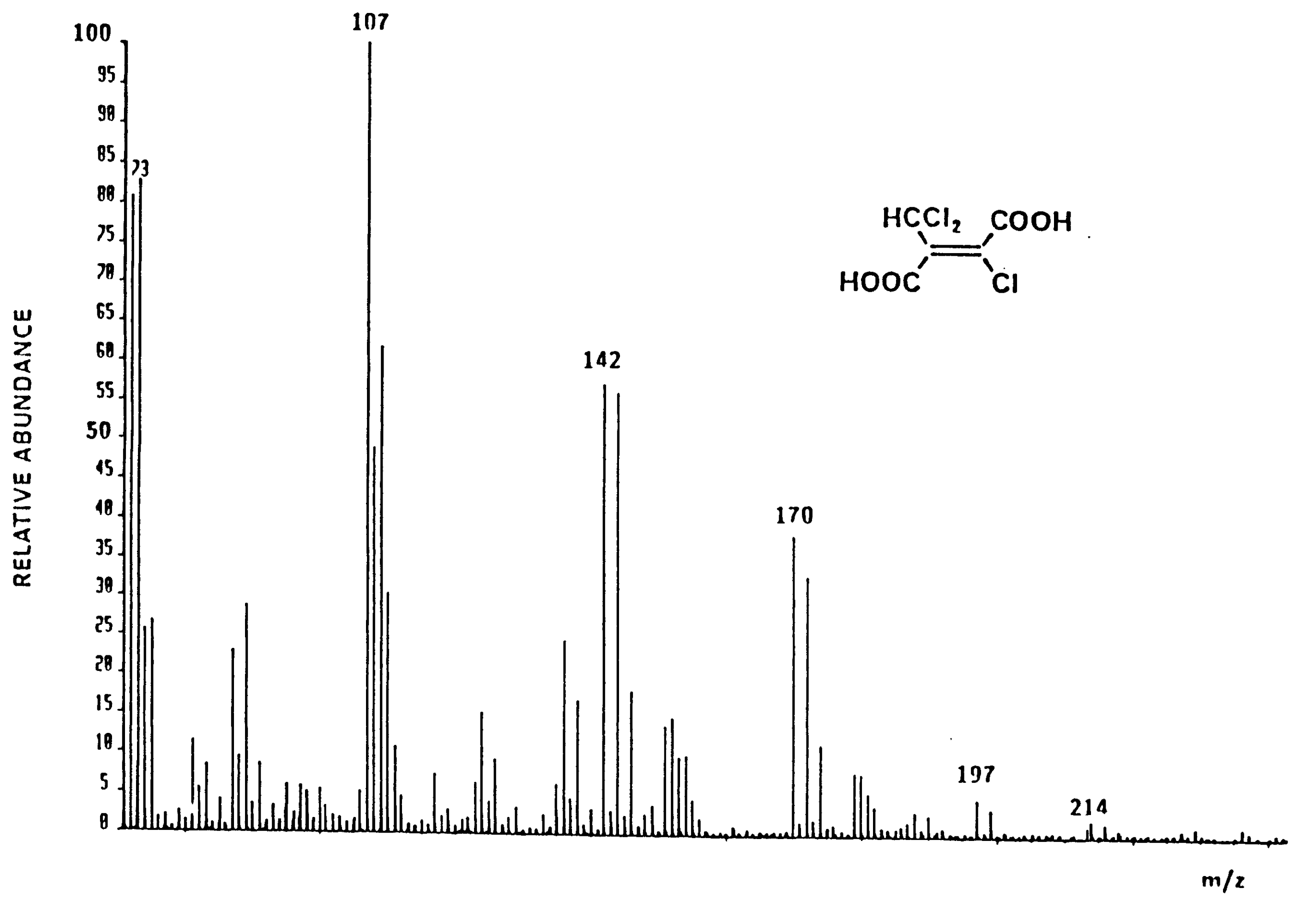


Figure 2.2. Mass Spectrum of ox-EMX

SiO_2 (6 g) with dichloromethane-hexane (1:1) as eluent gave the pure compound (yield 17.3 percent) as a colorless liquid. The ^1H NMR resonance signal of the dichloromethyl group was observed at δ 6.74 (1H) and of the protons in the lactone ring at δ5.16 (2H). The electron impact mass spectrum of red-MX is shown in Figure 2.3.

Reduction of EMX to give (E)-2-chloro-3-(dichloromethyl)- 4-hydroxy-butenoic acid (red-EMX) was attempted with aluminum isopropoxide in isopropanol and with $NaBH_4$ in a mixture of isopropanol and water. However, the results of gas chromatography/mass spectrometry (GC/MS) analyses indicated that neither reaction produced red-EMX. In the reaction mixture of reduction with $NaBH_4$, red-MX was obtained along with a compound that had almost the identical mass spectrum of red-MX with the addition of chlorine ion clusters of masses 121, 123 and 156, 157 (Figure 2.4). At present, the identity of the compound is unknown; it could be an isomer of red-MX.

DERIVATIZATION PROCEDURE AND CONDITIONS OF GC/MS ANALYSES

Prior to derivatization, the samples were evaporated to dryness, and the residues were methylated with 250 uL (microliters) of the methylation agent (Table 2.1). Extracts for selected-ion-monitoring-mode (SIM-mode) GC/MS analyses of MX and EMX were methylated with 2 percent (v/v) H_2SO_4 in methanol for 1 hour at 70°C. The GC analyses of synthesized ox-MX and ox-EMX and the SIM-mode GC/MS determinations of these compounds in the extracts were carried out on samples methylated with 12 percent BF_3 in methanol. The methylation was performed at 70°C for 12 hours. The methylated mixtures were neutralized by the addition of 2 percent aqueous $NaHCO_3$ and extracted twice using hexane (approximately 2 x 250 uL). The combined hexane extracts were concentrated under a stream of nitrogen gas and injected into the GC. Quantitative determination of the analytes was carried out relative to the standard mucobromic acid (MBA) added in known amount to the samples. The analyses of red-MX were carried out on underivatized samples with the use of 2,3-dibromo-2(5H)-furanone (red-MBA) as the standard. Attempts were also made to analyze ox-MX in underivatized samples.

The GC analyses of the reaction mixtures from the syntheses were performed on a Carlo-Erba HRGC 5160 capillary gas chromatograph equipped with a DB-1, 30-m, fused silica capillary column. Separation of ox-MX and ox-EMX was attempted on DB-1/30-m, DB-17/30-m, DB-1701/15-m, SP-2340/30-m, and DB-5/60-m columns.

The GC/MS analyses were performed on a Hewlett-Packard 5890 capillary gas chromatograph interfaced to a VG 70-250SEQ mass spectrometer. The GC conditions for the analyses are given in Table 2.1 For quantitative and qualitative purposes, the mass spectrometer was operated in the SIM mode. Table 2.2 lists the ion peaks that were monitored. The standard SIM routine of the VG 11-250J data

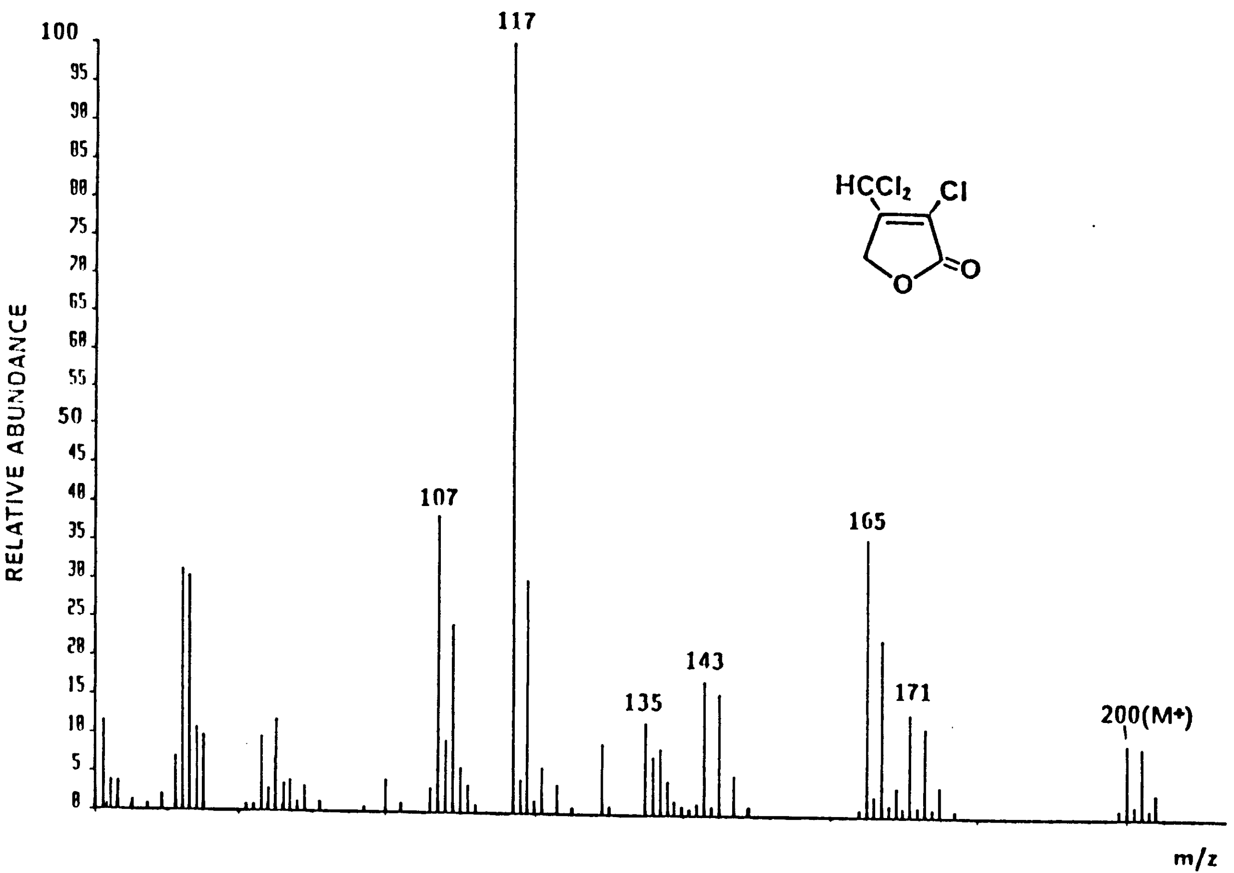


Figure 2.3. Mass Spectrum of red-MX

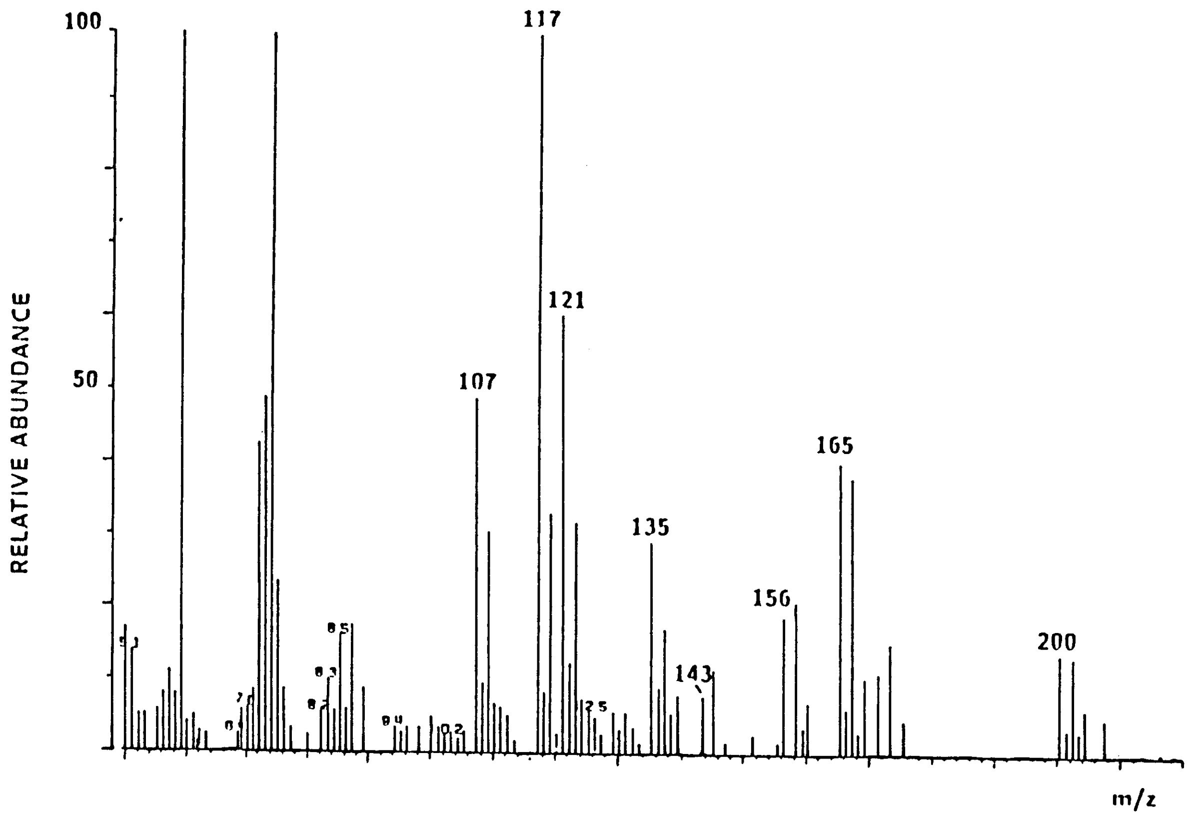


Figure 2.4. Mass Spectrum of Compound Formed During Reaction of EMX With $NaBH_4$

TABLE 2.1

Derivatization Procedure and Gas Chromatography Conditions for SIM-Mode Gas Chromatography/Mass Spectrometry Determination of Mutagens in Extracts

Compound	Internal standard	Column	Temperature program: Initial (^oC)	Hold min	Rate (^oC/min)	Final (^oC)	Hold min	Retention time: Compound	Min	Derivatization agent
MX EMX	MBA	DB-1/30-m	115	3	6	165	1	MBA MX EMX	6:05 7:18 10:15	2% H_2SO_4 in MeOH
red-MX	red-MBA	DB-1/30-m	100	3	6	135	1	red-MBA red-MX	6:57 7:46	None
ox-MX	red-MBA	DB-1/30-m	110	3	6	130	-	red-MBA ox-MX	5:42 4:35	None
ox-MX ox-EMX	MBA	DB-5/60-m	160	3	3	190	1	MBA ox-EMX ox-MX	8:12 10:02 10:07	12% BF_3 in MeOH

TABLE 2.2

Ion Peaks Used and Relative Peak Area Ratios Found for SIM-Mode Gas Chromatography/Mass Spectrometry Analyses of MX and MX Analogues in Standard Solutions and in Chlorinated Water

Compound	Fragmentation	M/z	Response factor*	Relative peak area ratios: Standard	FA	HW	DW	DW spike level 1	DW spike level 2
MX	M-OCH_3	198.9120		0.58	0.43	0.62	0.39		
		200.9091	1.26	1.00	1.00	1.00	1.00		
		202.9061		0.60	0.72	0.60	0.45		
EMX	M-OCH_3	244.9537	3.36	1.00	1.00	1.00	1.00		
		246.9510		0.92	0.88	0.94	0.94		
red-MX	M-Cl	164.9510		1.52	1.49	1.60	2.15**	1.65	
		166.9481		1.00	1.00	1.00	1.00	1.00	
	M-CHO	170.9171		0.62	0.56	0.67	0.64	0.52	
		172.9142		0.59	0.48	0.58	0.58	0.50	
	M^+	199.9199		0.40	**	nm	0.34	0.28	
		201.9169		0.39	**	nm	0.37	0.31	
ox-MX (anhydride)	M-CO_2	169.9093		0.43	0.40	0.37	0.43	0.39	0.46
		171.9063		0.37	0.34	0.36	0.37	0.36	0.43
	M-Cl	178.9303	0.10	1.00	1.00	1.00	1.00	1.00	1.00
		180.9973		0.74	0.62	0.65	0.68	0.70	0.68
	M^+	213.8991		0.19	0.22	0.20	0.20	0.21	0.15
		215.8962		0.16	0.18	0.16	0.25	0.14	0.19

(continues)

TABLE 2.2 (Continued)

Compound	Fragmentation	M/z	Response factor*	Relative peak area ratios: Standard	FA	HW	DW	DW spike level 1	DW spike level 2
ox-MX (methylated)	M-$COOCH_3$	200.9277		0.76	3.79#	0.24#	1.16#	0.78	0.78
		202.9247		0.65	2.79#	0.30#	0.62	0.89	0.73
	M-Cl	224.9722		1.18	0.42	0.60	1.75#	1.09	0.98
		226.9692		0.58	0.19	0.35	0.46	0.57	0.64
	M-CH_3OH	227.9148		0.97	1.03	1.23	1.01	0.95	0.98
		228.9226		1.08	1.41	0.96	0.69	1.16	1.07
	M-OCH_3	229.9118	0.60	1.00	1.00	1.00	1.00	1.00	1.00
		230.9197		1.01	1.57	1.13	**	0.88	0.86
ox-EMX	M-$COOCH_3$	200.9277		0.96	1.03	1.11	1.10		
		202.9247	0.50	1.00	1.00	1.00	1.00		
	M-OCH_3	224.9722		0.87	0.70	0.71	0.79		
		226.9692		0.39	0.43	0.48	0.40		
MBA	M-OCH_3	240.8325							
red-MBA	M-Br	160.9239							

nm = not measured

* Response factor = (Acomp x $Ccomp^{-1}$) x (Cstd. x $Astd.^{-1}$) where C = concentration and A = ion peak area ratio

** Interference

\# Interference from ox-EMX

system was used to record and compute the SIM data. The response factors of the analytes were calculated versus the internal standard (Table 2.2). The identification of the analytes in the extracts was based on positive matching of retention times and relative ion peak area ratios.

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

The ^1H NMR spectra were acquired on a Varian XL-400 (400-mega Hertz) spectrometer (Varian Associates, Palo Alto, CA).

FRACTIONATION OF MUTAGENIC MATERIAL

Aliquots of the aqueous solution of fulvic acids chlorinated at pH 2 to a Cl_2/TOC ratio of 2 were adjusted to pH 6.0, 5.5, 5.0, 4.5, 4.0, 3.5, and 2.0 (= extract FA), and each solution was extracted with ether. The mutagenic activity and the concentration of MX was determined in each fraction.

The extract FA was fractionated by reverse-phase high pressure liquid chromatography (RP-HPLC) methods as depicted in Figure 2.5. Fractionation on a C_6 analytical column (Phase Sep, Spherisorb $5C_6$, 4.5 x 250 mm) was followed by further fractionation on a C_{18} analytical column (Alltech, Econosphere $5C_{18}$, 4.5 x 250 mm). The C_6 column was isocratically eluted with 20 percent acetonitrile (ACN) in 0.1 M phosphate buffer at pH 6.0. The C_{18} column was eluted with a stepwise gradient program of ACN and 0.1 M phosphate buffer at pH 6.0.

Collected fractions were acidified to pH 2.0 and repeatedly extracted using diethyl ether. The combined ether extracts were evaporated to dryness, and the residue was redissolved in ethyl acetate.

MUTAGENICITY TESTING PROCEDURE

The bacterial mutagenicities of red-MX, ox-MX, and ox-EMX were tested with the constructed Salmonella typhimurium strains TA 100, TA 98, and TA 102, according to the standard plate incorporation procedure of Maron and Ames (Maron and Ames 1983). The strains were obtained from Bruce Ames at the University of California, Berkeley. The genotypes of these strains are as follows:

TA 100: his G46, rfa, Δ uvrB, pKM 101
TA 98: his D3052, rfa, ΔuvrB, pKM 101
TA 102: his G428, rfa, pKM 101, pAQ1

The strains were kept in storage at -70°C; master plates were prepared from them and kept at 4°C. The presence of genetic markers, the spontaneous reversion rates, and the positive control responses were

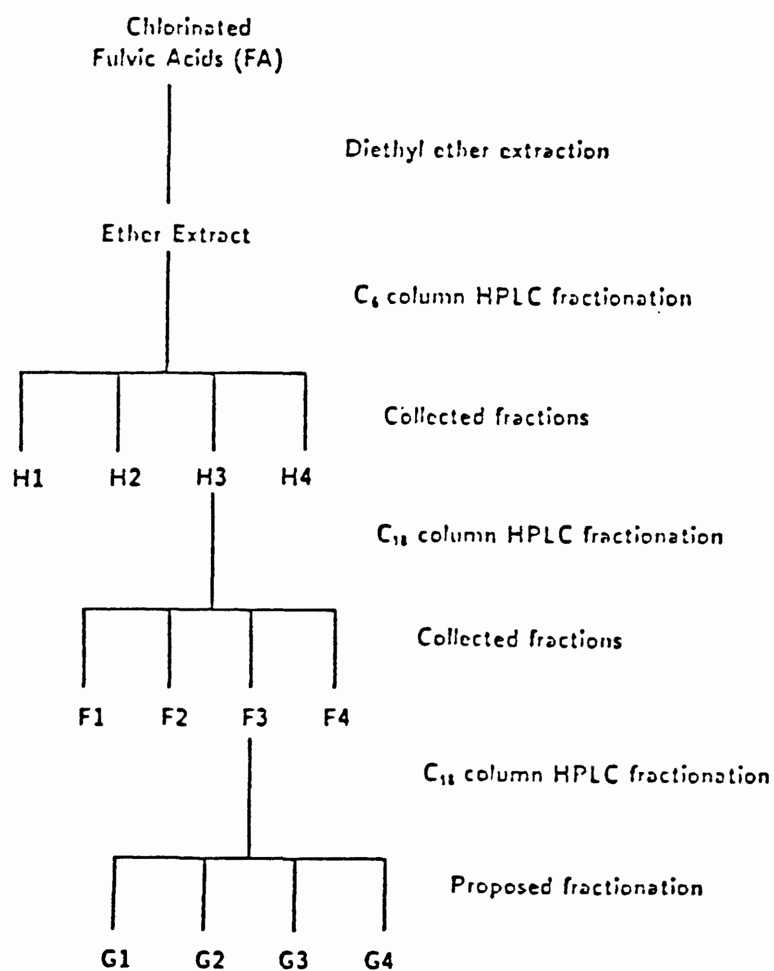


Figure 2.5. Procedure for Purification and Fractionation of Mutagenic Compounds Present in the Extract of the Chlorinated Aqueous Solution of Fulvic Acids

verified for each master plate before it was used to grow overnight cultures of the strain. The positive control and spontaneous responses were also tested with every experiment. The positive control chemicals and the amount added per plate were as follows:

TA 100 (-S9): 1.5 ug sodium azide (Aldrich, Milwaukee, WI)
TA 100 (+S9): 0.5 ug 2-anthramine (Sigma, St. Louis, MO)
TA 98 (-S9): 3.0 ug 2-nitrofluorene (Aldrich)
TA 98 (+S9): 0.5 ug 2-anthramine (Sigma)
TA 102 (-S9): 6.0 ug Daunomycin (Fluka Chemical Corp., Ronkokoma, NY)
TA 102 (+S9): 30 ug 1,8-dihydroxyanthraquinone (Danthron) (Sigma)

The effect of exogenous xenobiotic metabolizing enzymes on the mutagenicity of the MX analogues was tested using Aroclor 1254-induced rat liver homogenate fraction, S9 (Moltox Inc., College Park, MD). The S9 was added at 0.3 mg protein/plate.

The synthesized compounds and the extracts were stored in ethyl acetate that, at the time of testing, was evaporated under a stream of nitrogen or helium. The residues were then redissolved in the test solvent, dimethylsulfoxide (DMSO). All experiments were done with a minimum of four doses using duplicate plates per dose. Each experiment was repeated at least once on a separate day. A linear dose response above the background rate of spontaneous reversion was taken as indicative of positive mutagenicity. The mutagenic potency was determined from the slope of the line fitted by linear regression on the data points. The result was accepted only if the correlation factor, r, was equal to or greater than 0.90. All of the doses tested fell within the initial linear portion of the dose response curve, with r almost always being greater than 0.97.

Only strain TA 100 (without metabolic activation) was used to test the mutagenicity of the chromatographic fractions, since it has been found to be the most responsive strain for testing extracts of chlorinated water and for MX and EMX (Kronberg et al. 1985 and this study).

CHAPTER 3

RESULTS AND DISCUSSION

MUTAGENICITY OF THE MX ANALOGUES

All of the MX and EMX analogues synthesized were found to exhibit mutagenicity (Table 3.1). Figures 3.1, 3.2, and 3.3 show the dose response curves of the pure compounds in TA 100 (data from a representative experiment for each MX analogue). The highest mutagenic response was obtained for tests carried out on strain TA 100 without metabolic activation. Oxidized-MX and oxidized-EMX exhibited 1.4 percent and 2 percent of the mutagenicity of MX and EMX, respectively, and reduced-MX exhibited about 0.5 percent of the mutagenicity of MX.

Previously, it was shown that EMX, which has the $CHCl_2$ and Cl groups trans to each other, generates only one tenth of the mutagenicity generated by MX (Kronberg et al. 1988). This finding indicates that the cis configuration of the $CHCl_2$ and Cl substituents in MX is an important structural feature in its potency. The low mutagenicity of ox-MX and red-MX compared to MX suggests that the aldehyde group is also an important structural factor contributing to the potency of MX. Oxidized-EMX has neither the favored cis configuration nor the aldehyde group and, as expected, was found to have the lowest mutagenicity of the compounds studied. This line of reasoning suggests that red-EMX, had we been able to synthesize it, would have had an even lower potency.

The positive response of the MX analogues found in assays with strain TA 100 suggests that, like MX, they are direct acting mutagens operating primarily through base-pair substitution at the G-C pairs. Because certain compounds can be procarcinogens that are transformed into electrophiles (which then react with deoxyribonucleic acid, DNA) by the action of the cytochrome P-450 enzyme system within cells and tissues, it is necessary to test the compounds for mutagenicity in the presence of the exogenous xenobiotic metabolizing enzyme system, S9. Reduced-MX is a precursor to MX in the sense that it can be oxidized to MX, which is a more potent mutagen. This oxidation may possibly be carried out by the P-450 enzyme system, which would lead to a high mutagenic response when red-MX is tested in the presence of S9. However, this was not the case, as S9 was found to reduce the mutagenicity of red-MX. It would seem, therefore, that in vivo oxidation of red-MX is unlikely.

In strain TA 98, red-MX, ox-MX, and ox-EMX, even at the highest doses tested (1,000 ng, 2,000 ng, and 3,000 ng, respectively), did not elicit a mutagenic response. It would seem that these compounds do not operate as frameshift mutagens, at least not at these concentrations. The mutagenicity of red-MX in TA 102 was about the same as in TA 100 while ox-MX and ox-EMX were found to be nonmutagenic in TA 102 (at highest dose of 2,000 ng/plate and 3,000 ng/plate, respectively).

TABLE 3.1

Mutagenic Potencies of MX and the MX Analogues

Compound	Highest dose tested (ng/plate)	Mutagenic potency (net rev/nmol)					
		TA 100		TA 98		TA 102	
		-S9	+S9	-S9	+S9	-S9	+S9
red-MX (mol wt=200)	1,000	0.13±0.03	0.045±0.01	nm	nm	0.11±0.06	nm
ox-MX (mol wt=232)	2,000	0.36±0.04	nm	nm	nm	nm	nm
ox-EMX (mol wt=232)	3,000	0.03	nm	nm	nm	nm	nm
MX (mol wt=216)	16.4	22±2.1	nt	nt	nt	nt	nt

nm = not mutagenic (approximately equal to the spontaneous reversion rate)
nt = not tested for mutagenicity
mol wt = molecular weight

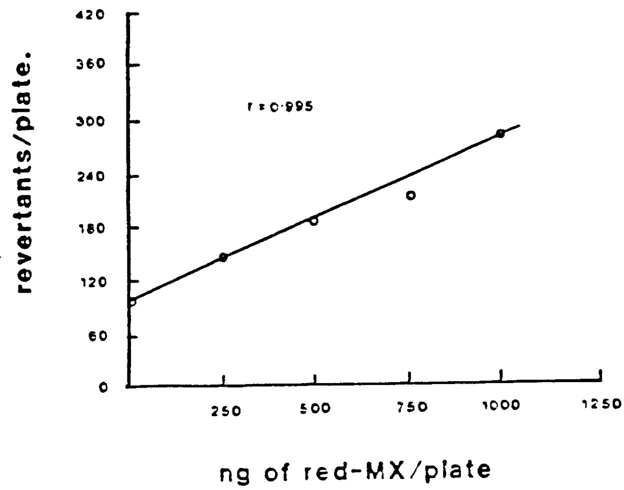


Figure 3.1. Mutagenic Response of ox-MX in Strain TA 100 (-S9)

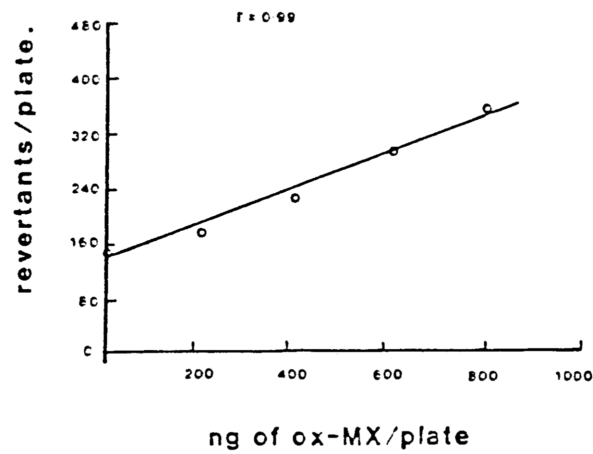


Figure 3.2. Mutagenic Response of red-MX in Strain TA 100 (-S9)

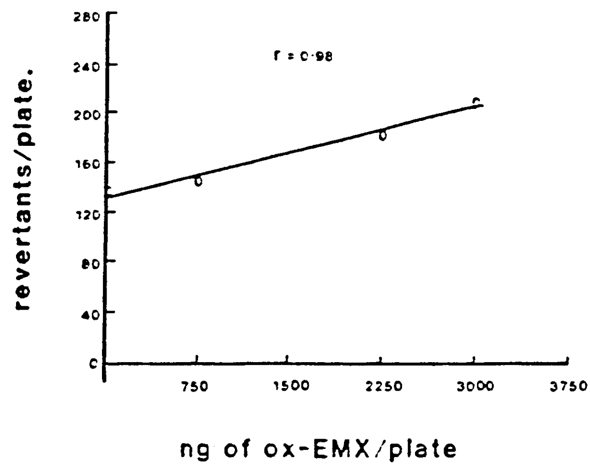


Figure 3.3. Mutagenic Response of ox-EMX in Strain TA 100 (-S9)

QUANTITATIVE ANALYSES OF THE MX ANALOGUES
IN EXTRACTS OF CHLORINATED WATER

The results of GC analyses of various amounts of ox-MX methylated with BF_3 in methanol showed good linearity (Table 3.2). Methylation with 2 percent H_2SO_4 did not seem to go to completion, and methylation using diazomethane did not work at all (not shown). The analyses of underivatized ox-MX showed poor linearity and reproducibility, particularly at low concentrations. Thus, extracts used for the determination of ox-MX and ox-EMX were derivatized with BF_3 in methanol prior to SIM analyses, while MX and EMX were determined in extracts methylated with 2 percent H_2SO_4 in methanol. Reduced-MX was determined in underivatized extracts. The mass spectra of methylated ox-MX and ox-EMX are presented in Figures 3.4 and 3.5.

SIM-mode GC/MS analyses of the extracts of chlorinated water showed all the MX and EMX analogues to be present (Tables 2.2 and 3.3). The concentration of ox-EMX was 20 to 40 times higher than the concentration of MX in corresponding samples. The other MX analogues were present at concentrations slightly higher than or equal to the MX concentration. Because of the relatively weak mutagenicity of the analogues, however, each compound contributed less than 1 percent to the overall mutagenicity, with the exception of ox-EMX in extract FA, which accounted for approximately 1.7 percent of the total activity.

TABLE 3.2

Results of Gas Chromatography Analyses of ox-MX

Derivatization procedure	Amount of ox-MX in sample (ng)	Analysis no. 1	2	3	4	Standard deviation (%)
Underivatized	260	227	238	323	265	±16
	130	197	127	152	139	±20
	26	11	23	17	24	±30
BF_3 in MeOH	13	14				
	26	26				
	39	38				
	52	50				

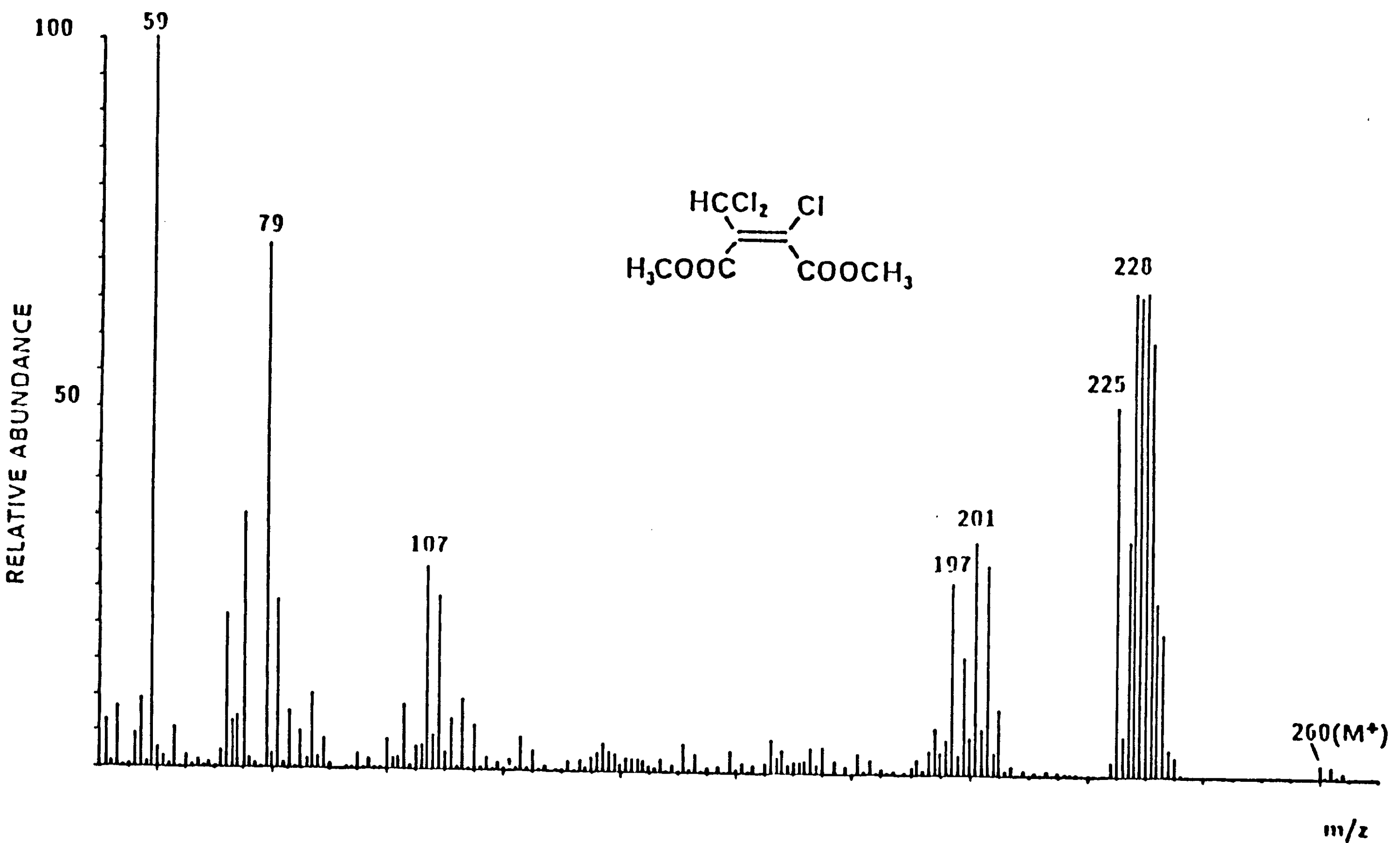


Figure 3.4. Mass Spectrum of Methylated ox-MX

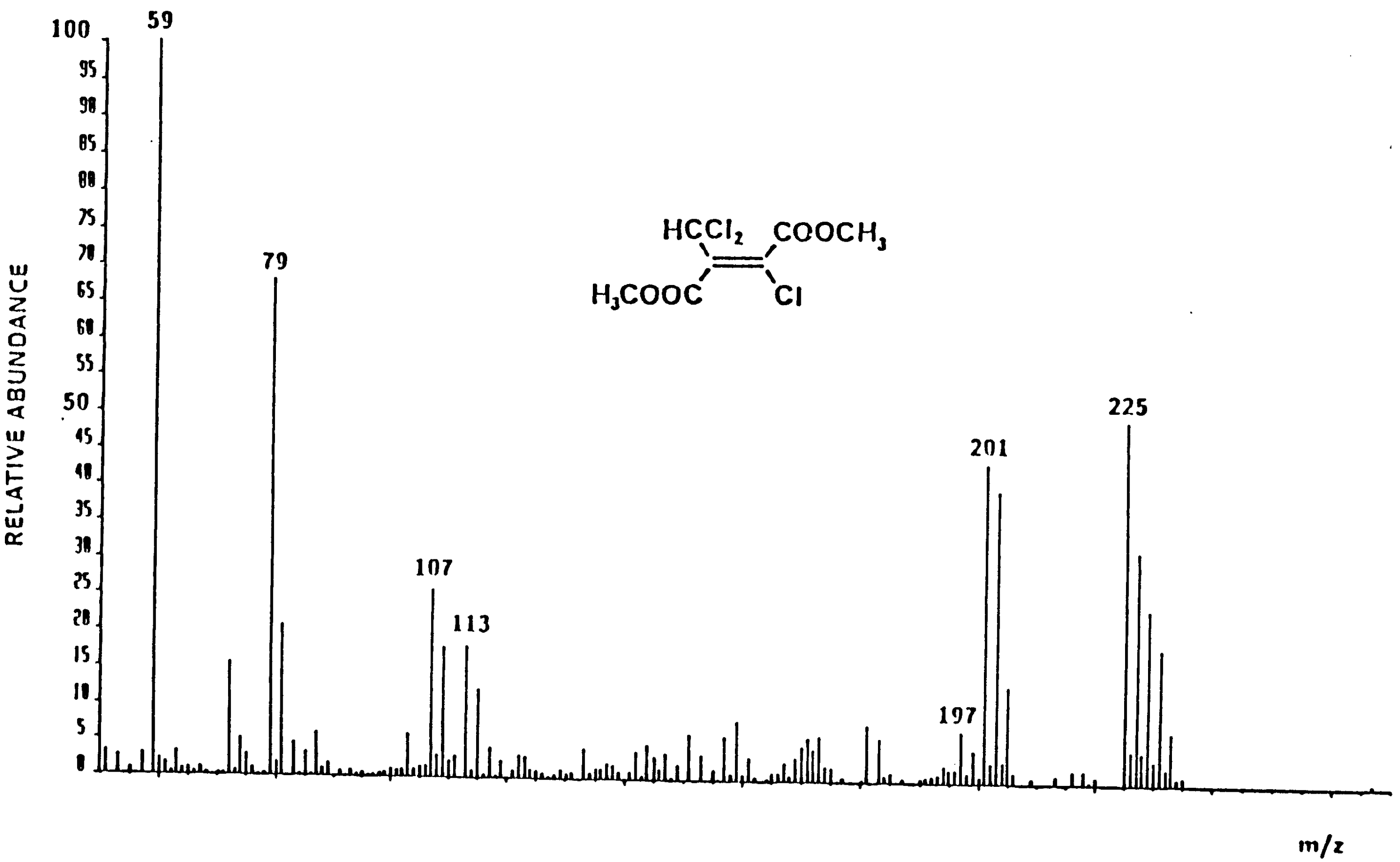


Figure 3.5 Mass Spectrum of Methylated ox-EMX

TABLE 3.3

Concentration and Mutagenicity Contribution of MX and MX Analogues in Extracts of Chlorinated Water

Sample	Mutagenicity (net rev/mL)	MX		EMX		red-MX		ox-MX		ox-EMX	
		Conc. (ng/L)	Mutagen. contr.* (%)	Conc. (ng/L)	Mutagen. contr.* (%)	Conc. (ng/L)	Mutagen. contr.+ (%)	Conc. (ng/L)	Mutagen. contr.+ (%)	Conc. (ng/L)	Mutagen. contr.+ (%)
FA	48	675	36	1,204	4	643	0.3	961	0.7	26,777	1.7
HW	21	260	32	526	4	370	0.4	306	0.5	5,081	0.7
DW	2.04	13	17	20	2	41	0.4	53	0.9	251	0.4

* Calculated on the basis of 5,600 and 320 net rev/nmol specific MX and EMX mutagenicity, respectively
+ Calculated on the basis of the mutagenicity reported in Table 3.1

SIM-mode GC/MS determinations of ox-MX were attempted on nonmethylated and methylated samples. Although GC analyses of pure ox-MX indicated that the analyses of unmethylated ox-MX do not give a strict linear correlation of amount of analyte to detector response (Table 3.2), the method was thought to approximate the concentration of ox-MX in the extracts. Analyses of unmethylated samples showed the concentration of ox-MX to be 6,830 ng/L, 1,160 ng/L, and 266 ng/L in extracts of FA, HW, and DW, respectively. These values were much higher than those found when the methylated samples were analyzed (Tables 3.3 and 3.4). In addition, the analyses of spiked underivatized DW extracts did not result in the expected increase in the concentration of ox-MX (Table 3.4).

Methylated pure ox-MX and the methylated spiked DW extracts, however, showed a linear increase in detector response with increases in the amount of ox-MX analyzed (Tables 3.2 and 3.4). Therefore, the concentration of ox-MX found in methylated extracts was assumed to represent the actual concentration of ox-MX.

The discrepancies between ox-MX concentrations found with underivatized extracts and those seen with derivatized extracts were most likely the result of interference from ox-EMX. (Oxidized-EMX was present in concentrations several times higher than the concentration of ox-MX, and ox-EMX might upon heating in the GC injector partly isomerize to ox-MX.) Indication of isomerization of ox-EMX upon heating is seen in the mass spectra recovered by probe inlet of ox-EMX (Figure 2.2). The fragment ions at m/z 214 and 216 are most likely due to the formation of the ox-MX anhydride in the heated probe. Analyses of methylated pure ox-MX and ox-EMX showed that isomerization does not occur after methylation.

Because the fragment ions of methylated ox-MX and ox-EMX that can be used for qualitative and quantitative analysis are the same, separation of the compounds on the GC column is critical. The only column found to give satisfactory, albeit incomplete, separation was a DB-5/60-m column (Figure 3.6). Owing to the high concentrations of ox-EMX in the extracts, some of the ion peaks overlap with the ion peaks of ox-MX (Table 2.2 and Figure 3.6); therefore, truly accurate analyses of these two compounds will require the use of a column that provides complete separation.

Similarly, the qualitative and quantitative determination of red-MX suffered from interferences from ions of a compound not completely separated from red-MX (Figure 3.7). The interference seemed to originate from the same compound that was produced by reactions of EMX with $NaBH_4$, which was earlier suggested to be an isomer of red-MX. This argument is based upon the similarity of retention times and the identical nature of the fragment ions of the two unknown compounds. Nevertheless, the quantitative results of the analyses of the DW extract spiked with red-MX showed that reasonable accuracy for the determination was possible (Table 3.4).

TABLE 3.4

Concentration of ox-MX and red-MX in Unspiked and Spiked Samples of Chlorinated Drinking Water

Compound	Spike level, ng/L --	50	100	200
ox-MX				
anhydride	266	--	403	429
methylated	53	--	191	244
red-MX				
(analysis 1)	41	103	--	--
(analysis 2)	33	92	164	--

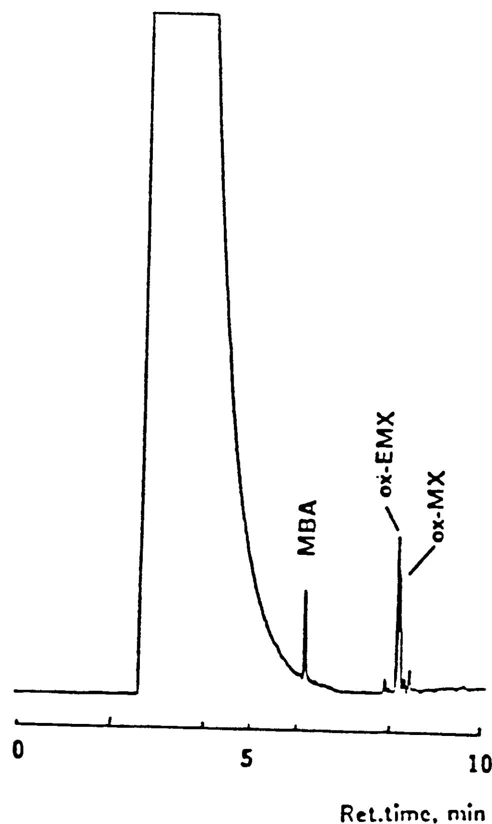


Figure 3.6. GC Separation of Methylated ox-EMX and Methylated ox-MX on a DB-5/60-m Fused Silica Capillary Column

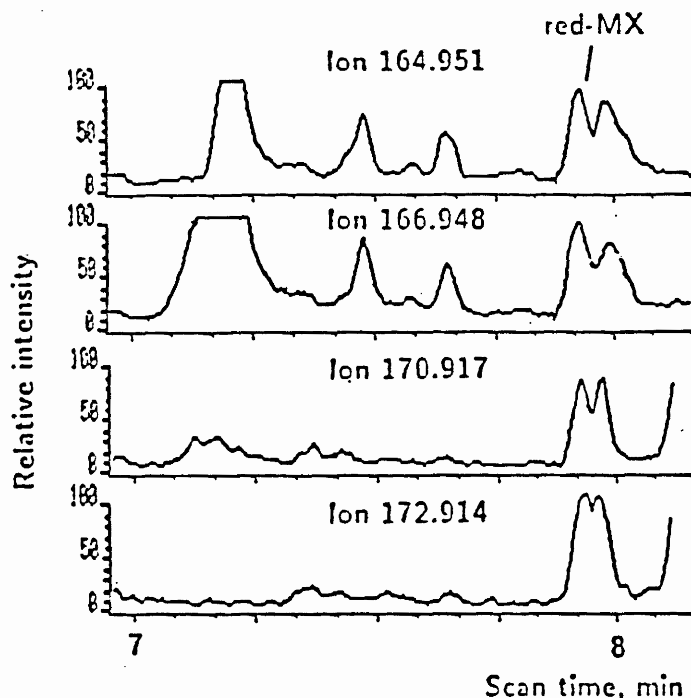


Figure 3.7. Examples of SIM Chromatograms of red-MX Ions Generated During Analyses of Underivatized Extract of Chlorinated Humic Water

FRACTIONATION OF EXTRACTS OF CHLORINATED FULVIC ACIDS

Prior to fractionation of the mutagens, experiments were conducted to determine the conditions for chlorination of aqueous fulvic acid solutions that would result in high mutagenic activity and, simultaneously, low amounts of MX. The results showed that the mutagenic activity and the concentration of MX and EMX are higher in samples chlorinated at pH 2 than in corresponding samples chlorinated at pH 7 (Table 3.5). The higher the chlorine dosage applied at pH 2, the higher the mutagenicity and the amount of MX and EMX produced. The highest absolute yield in mutagenicity not due to MX was found in sample H, the aqueous fulvic acid solution chlorinated at pH 2 to a Cl_2/TOC ratio of 2 (Figure 3.8). Extracts of this solution were thus considered suitable material for the study of non-MX mutagens. Because these extracts were found to be about 12 times more active in strain TA 100 than in strain TA 98, TA 100 was used to detect mutagenicity in material subjected to liquid liquid extractions and to chromatographic fractionation.

Liquid liquid extraction at various pH conditions of the chlorinated aqueous fulvic acid solution showed an almost constant increase in the extraction of mutagenicity with decreases in the pH of the water solution (Table 3.6). The results would suggest that the water contained a range of mutagenic

TABLE 3.5

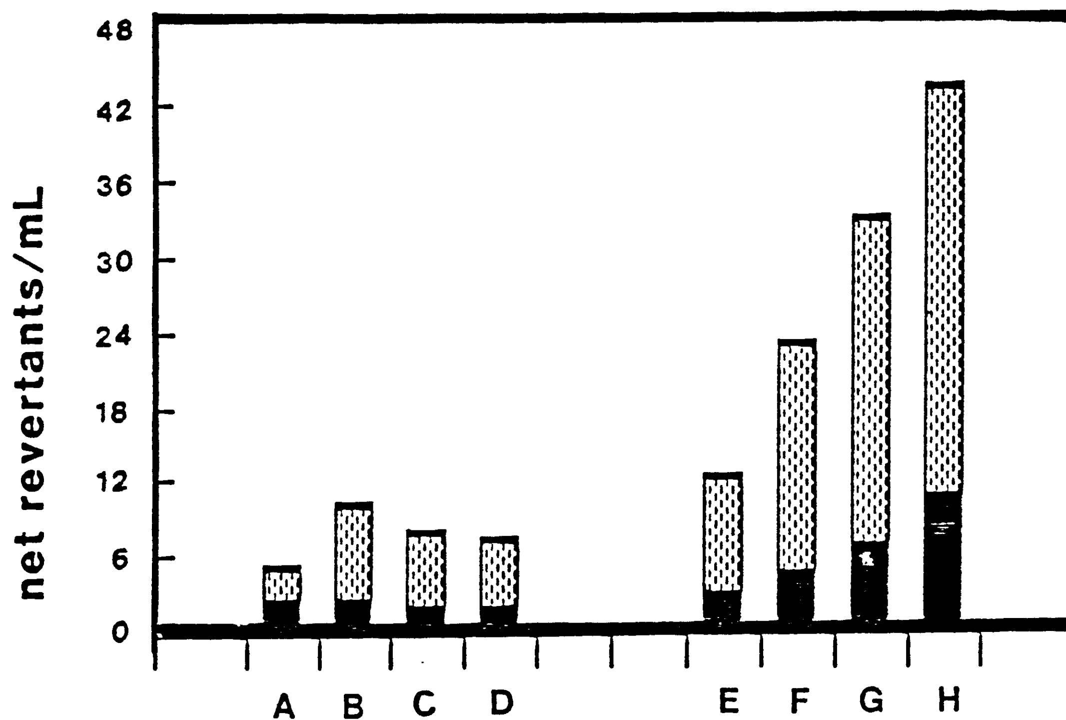
Mutagenic Activity and MX and EMX Concentrations in Aqueous Solutions of Chlorinated Fulvic Acids

Sample	pH*	Cl_2/TOC+	Mutagenicity (net rev/mL)	Concentration (ng/L)		Mutagenicity contribution				Total
						MX++		EMX++		
				MX	EMX	net rev/mL	%	net rev/mL	%	%
A	7.0	0.5	5.1	77	171	2.0	40	0.3	6	46
B	7.0	1.0	10.1	86	235	2.2	22	0.3	3	25
C	7.0	1.5	7.6	71	130	1.8	24	0.2	3	27
D	7.0	2.0	7.4	69	136	1.8	24	0.2	3	27
E	2.0	0.5	12.0	107	328	2.8	24	0.5	4	28
F	2.0	1.0	22.0	180	703	4.7	21	1.1	5	26
G	2.0	1.5	33.0	261	851	6.8	21	1.3	4	25
H	2.0	2.0	43.5	377	658	10.3	24	0.9	2	26

* Chlorination pH

\+ Total organic carbon (TOC) content of aqueous solution = 2.5 mg/mL; chlorination carried out at room temperature, in the dark

++ Calculated on the basis of 5,600 net rev/nmol and 320 net rev/nmol specific mutagenicity of MX and EMX, respectively



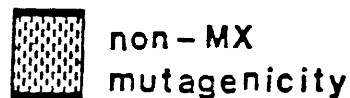


Figure 3.8. Contribution of MX to the Total Mutagenic Activity of Chlorinated Aqueous Fulvic Acid Solutions

compounds with various pKa values. However, the simultaneous increase in the yield of MX (pKa = 5.25) with decreases in pH indicates that the extractability of this mutagen is not based only on its acid properties. Perhaps mutagens of low molecular weight, such as MX, are bound to material of higher molecular weight (non- or partly degraded fulvic acids), and the extractability is dependent on the pKa of the higher molecular weight material.

Fractionation by RP-HPLC using the C_6 column showed that fractions H1, H2, and H3 contained mutagenic material while fraction H4 was nonmutagenic (Table 3.7 and Figures 3.9 and 3.10). MX was detected mainly in fractions H1 and H2; however, fraction H3 also contained considerable amounts of MX. The reason MX was found over a broad area of the chromatogram might be that the column was overloaded. Because MX accounted for only a small portion of the mutagenicity of the very active H3 fraction, the organic material in this fraction was further fractionated on the C_{18} column.

During stepwise gradient elution of the column, four fractions were collected (Figure 3.11), and the only fraction that contained mutagenic material was fraction F3. This fraction did not contain MX (pure MX was found to elute in the beginning of fraction F2). Nevertheless, the mixture of organic compounds in fraction F3 is still too complicated to allow for a positive identification of the compound(s) responsible for the mutagenicity of the fraction.

TABLE 3.6

Mutagenicity, MX and EMX Concentrations, and MX Mutagenicity Contribution in Extracts of Fulvic Acid Obtained at Various pH Conditions

pH	Mutagenicity (net rev/mL)	Concentration (ng/mL)		Mutagenicity contribution of MX	
		MX	EMX	net rev/mL	%
2.0	59	0.68	1.03	17	29
3.5	53	0.15	0.66	4	8
4.0	45	0.26	0.21	7	16
4.5	35	0.18	0.07	5	14
5.0	36	0.11	0.03	3	8
5.5	34	0.09	--	2	6
6.0	21	0.03	--	1	5

TABLE 3.7

Mutagenicity and MX Concentration in Fractions of Fulvic Acid

Fraction	Mutagenicity (net rev/mL)	MX conc. (ng/L)	Percent of mutagenicity due to MX*
H1	16	188	31
H2	25	205	21
H3	15	120	21
H4	+	--	--
H3/F1	+	#	#
H3/F2	+	#,**	#
H3/F3	31	#	#
H3/F4	+	#	#
H3/F1-F4++	33	#	#

* Based on a specific MX mutagenicity of 5,600 net rev/nmol

\+ Not mutagenic

\# Not analyzed

** Pure MX analyzed under similar chromatographic conditions was found to elute in the beginning of fraction H3/F2

++ Following work-up, the fractions were recombined and retested for mutagenicity

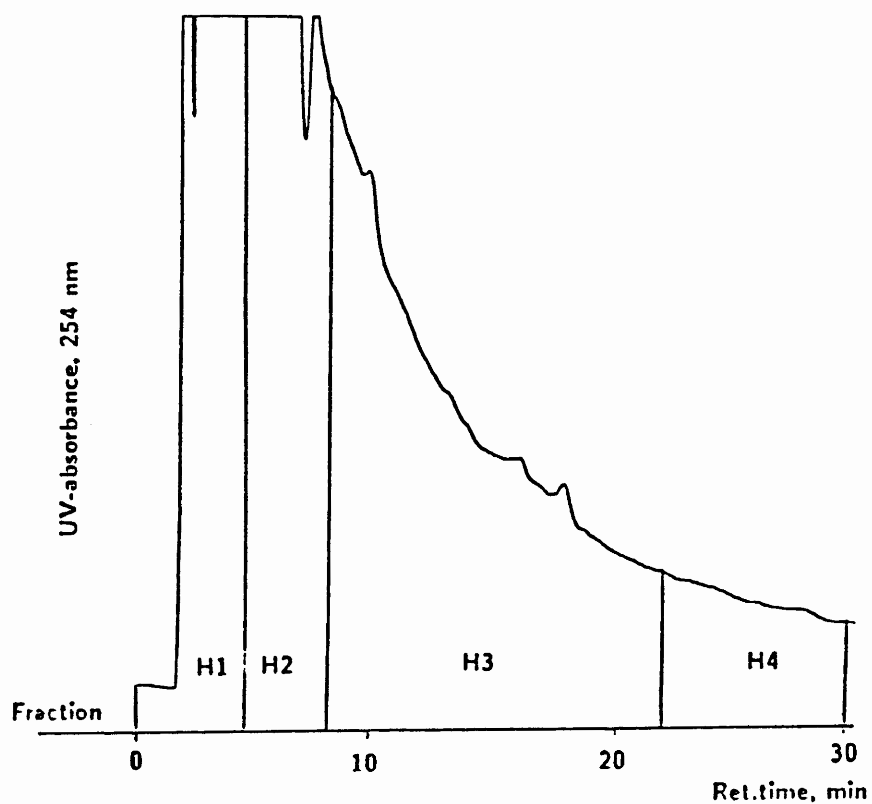


Figure 3.9. C_6 Column HPLC Fractionation of Extract of Chlorinated Aqueous Solution of Fulvic Acids. The eluent was 20 percent acetonitrile in 0.1 M phosphate buffer at pH 6.0. The flow rate was 1 mL/min.

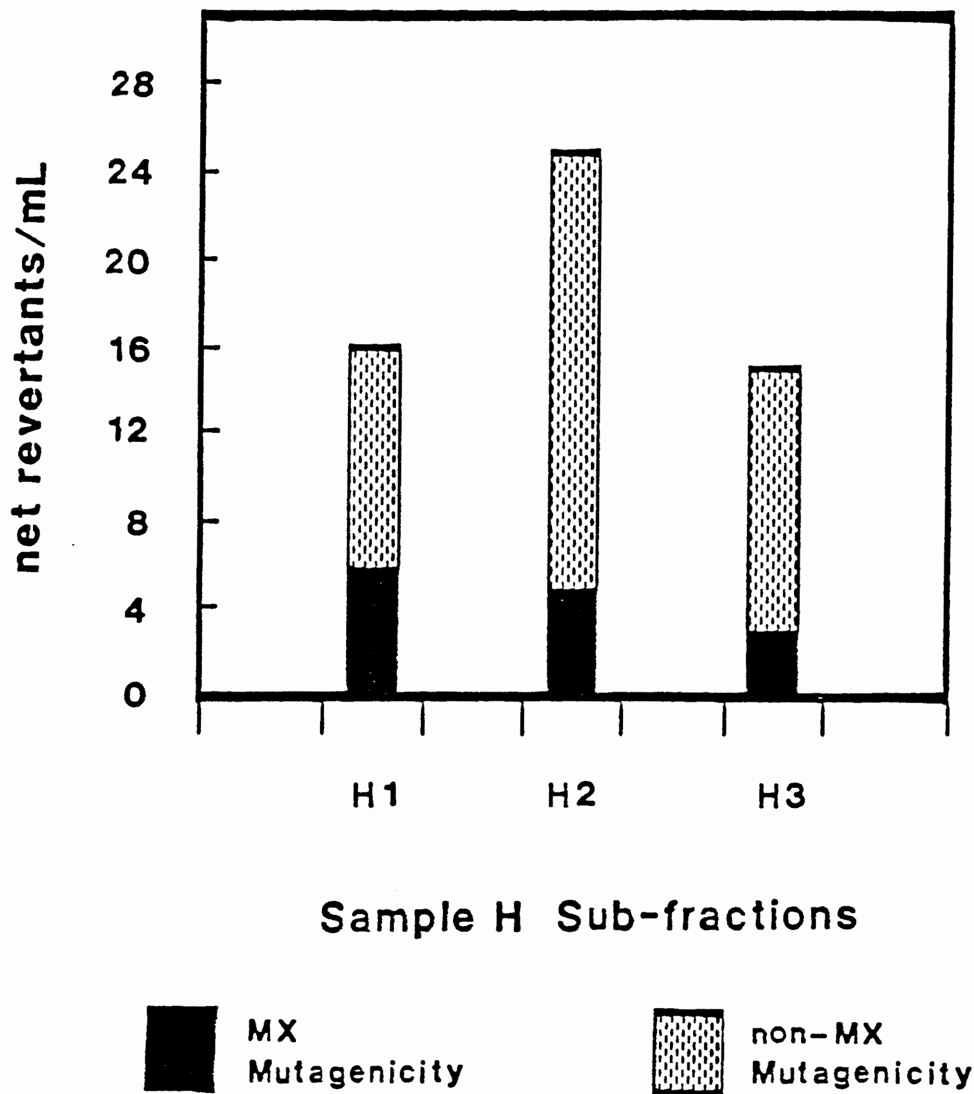


Figure 3.10. Contribution of MX to the Total Mutagenic Activity of Sample H Subfractions

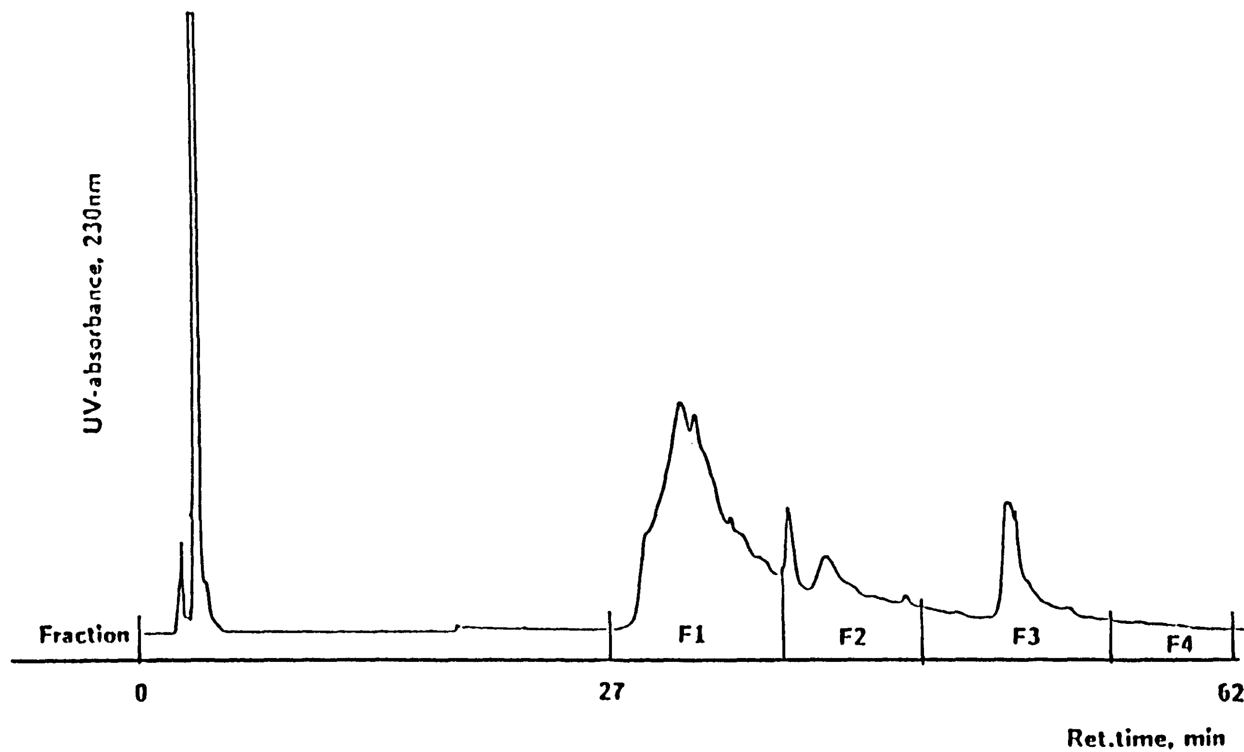


Figure 3.11. C_{18} Column Separation of the Organic Material in the C_6 Column Fraction H3. F1 to F4 are collected fractions. The eluent was acetonitrile (A) and 0.1 M phosphate buffer at pH 6.0 (B). Stepwise gradient: 0-10 min, 100% B; 11-21 min, 90% B; 22-32 min, 80% B; 33-43 min, 70% B; 44-54 min, 60% B; 55-64 min, 60-100% B. The flow rate was 1 mL/min.

CHAPTER 4

CONCLUSIONS

All the MX analogues studied in this work were found in the extracts of chlorinated water. Because the compounds were relatively weak mutagens, they neither individually nor collectively accounted for a significant amount of the mutagenicity of the extracts. MX remains the only major mutagen identified in chlorinated water. This work clearly demonstrated that the aldehyde group of MX is an important structural feature associated with the mutagenic potency of MX.

Although the present data indicate that red-EMX is not a potent mutagen, this finding has to be verified by the synthesis of the compound and by mutagenicity testing of pure red-EMX. Availability of the reduced and the oxidized forms of MX and EMX will enable kinetic studies to be undertaken of the formation and isomerization of the compounds in chlorinated water. Such kinetic studies might result in the identification of chlorination conditions that would minimize or hinder the formation of MX.

CHAPTER 5

RECOMMENDATIONS

In this study, the work on the identification of mutagens by an initial fractionation procedure was carried out in the extract of aqueous fulvic acid solutions chlorinated at pH 2 to a Cl_2/TOC ratio of 2 (i.e., sample H). This extract was found to yield the highest absolute mutagenicity due to mutagens other than MX. Repeated fractionation of the extract on C_6 and C_{18} RP-HPLC columns yielded a fraction (F3) that was highly mutagenic (31,000 net rev/L) and did not contain MX. The loss of mutagenicity of the parent FA extract during fractionation was remarkably low. Although the fractionation procedure resulted in a considerable purification of the mutagen(s), further fractionation should be carried out prior to GC/MS analyses of the constituents in the fraction.

The chromatographic characteristics of the material in fraction F3 indicate that the mutagenic compounds are less polar than MX and most likely contain acid functionalities. Therefore, the GC/MS analyses should be carried out on fractions derivatized by methylation. Complementary structural information should be obtained by recording both electron impact mass spectra and chemical ionization mass spectra. Additional information might be provided by the study of metastable peaks.

Of particular interest for further work is fraction H2 collected during the initial fractionation on the C_6 column. Although the fraction contains MX, the compound accounts for only part of the activity of the fraction. The isolation of the unknown mutagens could be carried out by a similar fractionation procedure as the one applied for fraction H3.

Finally, alternative methods of derivatization should be attempted for GC/MS studies. Careful consideration should be given to the possibility of derivative formation based upon the reactivity of the aldehyde groups, which appear to be an integral part of the mutagenicity of the MX family of molecules. Possibilities include formation of the oxime derivative with pentafluorobenzylhydroxylamine (PFBOA) and the hydrazone with 2,4-dinitrophenylhydrazine.

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ABBREVIATIONS AND ACRONYMS

ACN	acetonitrile
A-T	adenine-thymine
AWWA	American Water Works Association
AWWARF	American Water Works Association Research Foundation
^{o}C	degrees centigrade
Cl_2/C dosages	chlorine/carbon dosages
Cl_2/TOC weight ratio	chlorine/total organic carbon weight ratio
DBP	disinfection by-product
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DW	extract of drinking water
EMX	[geometric isomer of MX; Kronberg et al. 1988 gave formula as (E)-2-chloro-3-(dichloromethyl)-4-oxobutenoic acid (p. 42)]
FA	chlorinated fulvic acid sample
g	gram
G-C	guanine-cytosine
GC	gas chromatography
GC/MS	gas chromatography/mass spectrometry
g/L	grams per liter
^{1}H NMR	proton nuclear magnetic resonance spectroscopy
HPLC	high-pressure liquid chromatography
HW	extract of chlorinated humic water
Ka	acid ionization equilibrium constant
L	liter

m	meter
M	molar
M^+	molecular ion
MBA	mucobromic acid
mg/L	milligrams per liter
min	minute
mL	milliliter
mL/min	milliliters per minute
mm	millimeter
MS	mass spectrometer
mol wt	molecular weight
MX	3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone
m/z	mass-to-charge ratio
N	normal
ng	nanogram
nm	nanometer
nmol	nanomole
NMR	nuclear magnetic resonance
ox-EMX	oxidized-EMX--(E)-2-chloro-3-(dichloromethyl)-butenedioic acid
ox-MX	oxidized-MX--(Z)-2-chloro-3-(dichloromethyl)-butenedioic acid
PFBOA	pentafluorobenzylhydroxylamine
pH	negative logarithm of the effective hydrogen-ion concentration
pKa	$-\log_{1}{}^{0}K_a$
r	correlation factor
red-EMX	reduced-EMX = (E)-2-chloro-3-(dichloromethyl)-4-hydroxy-butenoic acid

red-MBA	reduced mucobromic acid = 2,3-dibromo-2(5H)-furanone
red-MX	reduced MX = 3-chloro-4-(dichloromethyl)-2(5H)-furanone
ret. time	retention time
rev/nmol	revertants per nanomole
RP-HPLC	reverse-phase high pressure liquid chromatography
S9	9,000 g supernatant fraction of tissue homogenate (used here to describe 9,000 supernatant fraction of livers of Aroclor 1254-treated male rats)
-S9	without S9
SIM-mode	selected-ion-monitoring mode
Std	Standard
TOC	total organic carbon
ug	microgram
uL	microliter
umol	micromole
USEPA	United States Environmental Protection Agency
UV	ultraviolet
v/v	volume per volume