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Identification and Quantification of the Mutagenic Compound 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (MX) in Chlorine-Treated Water

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The MX was first identified in kraft pulp mill effluents of a paper factory containing high concentration of non-volatile organic matter and high free chlorine concentration derived from the whitening process. These effluents had a strong mutagenic activity in the Ames test. The combined use of high performance liquid chromatography (HPLC), high resolution gas chromatography (GC) and mass spcctrometry (MS) techniques with the Ames test bioassay was able to isolate, characterize and identify the MX compound (Holmbom et al. 1981). Later, the MX was identified in drinking water as a disinfection by-product (Hemming et al. 1986; Meier 1988). Nowadays, the MX analysis is noteworthy because it could represent as much as the 60% total Ames test activity of chlorine treated water extracts (Kronberg and Vartiainen 1988a). This compound is the sole product in drinking water proved to be directly related to mutagenic activity in the Ames test. Recently, the reduced (red-MX), oxided (ox-MX), geometrical isomers (E-MX) and bromo-derivative forms of MX have been identified in water samples (Suzuki and Nakaniski 1995).

Ames test (Maron and Ames 1983) has been widely used for the genotoxicity evaluation of organic pollutants' water extracts. Ames and McCann (1981) estimated an 83 % of correlation between the ability of chemicals to induct mutations in bacterial assays and the induction of cancer in long-term animal tests. In comparison, chlorodibromomethane, the more active trihalomethane is 1.4 millions less Ames test active than the MX with only 0.004 net rev/nmol (Simmon et al. 1977), while the red-MX, ox-MX and E-MX forms are only 2% Ames active compared to MX response (Christman et al. 1990). Conversely, some MX bromo-derivatives have an Ames test response near to that of MX (Suzuki and Nakaniski 1995).

To prove the presence of MX in samples is very difficult because usually, the MX concentration is at trace levels (ng/L) and the compound is thermolabile. The analytical methodology for MX requires preconcentration of several liters of water, a clean up of the water extract, its derivatization and high resolution gas chromatography coupled to high resolution mass spectrometry (GC/HRMS) analysis to eliminate the interferences of other compounds. This paper describes the Ames test results obtained in Barcelona drinking water extracts and several intermediate stages of the potabilization process; the identification of MX by GC/HRMS and its contribution to the mutagenicity of the water extracts.

MATERIALS AND METHODS

Raw (Llobregat River), prechlorinatcd and ozonated water samples were collected in June. The carbon filtered and postchlorinated (drinking) samples were collected in October and May (“a” and “b”, respectively). They came from the Sant Joan Despí

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water treatment plant (Barcelona, NE Spain), carrying out a conventional treatment consisting of prechlorination, flocculation (settling), sand filtration, ozonization, granular activated carbon filtration and postchlorination.

Amberlite XAD-2 resin (Merck, Germany) was mixed and washed with methanol (Merck, HPLC grade) until it became clear according to the procedure described by Junk et al. (1974). 200 mL of clear XAD-2 resin was packed into a glass column (40 x 600 mm) and bed volumes were designed to accommodate 1.2 L of water. The XAD-2 column was washed by passing through 5 L of Milli-Q water (pH 2.0 with 0.1 N HCl -C. Erba, Italy). A total of 36 L of water (acidified to pH 2.0 with HCl) was intermittently passed through the XAD-2 column at a flow rate of 100 mL/min. After sampling, the XAD-2 column was washed with 2 L of pH 2.0 Milli-Q water and the residual water was blown off from the column with dry nitrogen. Then, the column was eluted with 800 mL of diethyl ether (C. Erba), the extract dried on anhydrous sodium sulfate (C. Erba) and reduced by evaporation to 1 mL. Bioassay aliquots were evaporated to dryness and redissolved in DMSO (Merck).

To derivatize the MX, the diethyl ether extracts were concentrated to 100 µL by a gentle stream of nitrogen and then, 1 mL of sulfuric acid (C. Erba) in methanol (2% v/v) was added. After 1 hr at 70°C, 2 mL of sodium bicarbonate (C. Erba) in water (2% w/v) were added. The methylated MX (MX-Me) was extracted with 2x1 mL of n-hexane (Merck), the extract was dried, reduced to a small volume and analyzed by GC/LRMS (low resolution) or GC/HRMS (high resolution).

A KoniK 3000C (S. Cugat, Spain) gas chromatograph (GC) interfaced to a TS-250 VG (Fisons, UK) mass spectrometer (MS) was used for the experiments in low resolution (500 resolution power). The experiments in high resolution (7500) were performed on a HP-5890 GC interfaced to an AutoSpec VG (Fisons, UK) MS. The mass spectrometers operated in the electron impact mode (70 eV), 200 µA trap current and 200°C source temperature. Full-scan mass spectra were obtained by scanning 60-250 amu with 2 sec/decade. For qualitative and quantitative purposes the MS operated in the SIR (selective ion recording) mode using PFK (perfluorokerosene) as a lock mass. Injections (2 µL) in the splitless mode (0.7 min) were made into a 30 m x 0.25 mm i.d., 0.25 µm film, DB-5 (J&W Scientific; Folsom, CA) fused-silica capillary column. The GC temperature program was: 60°C (1 min) to 280°C (5 min) at 4°C/min, having helium as carrier gas (12 psi). Temperatures of injector and interface were 250°C and 290°C, respectively. The quantification of MX in real samples was performed by comparison of the SIR-response in the real sample and the MX standard (Ultrafine Chemicals, UK).

The bioassay aliquots were redissolved in DMSO at 1:10 proportion (the extract equivalent to 1 L of sample in 10 µL of DMSO) and stored at 4°C until analysis. *Salmonella typhimurium* TA98 and TA100 strains were used for the Ames test as described by Maron and Ames (1983), with and without S9 microsomal activation (Organon Teknika Corp. USA). The experiments were performed in duplicate.

The Ames test results were expressed as Mutagenic Index (MI=[revertants of the sample (spontaneous+induced) / revertants in the negative control (spontaneous)] by liters equivalents of sample. Thus, potential variations in the spontaneous revertants rate among differents assays were eliminated and made the results more homogeneous.Therefore, the mutagenic zero (MI=l) is the spontaneous reversion. The criterion for mutagenicity detection in experimental samples was a concentration-dependent response exceeding the zero mutagenic by at least twofold. The criterion for toxicity was a negative dependent response between dose and MI, with and without cytotoxic effect.

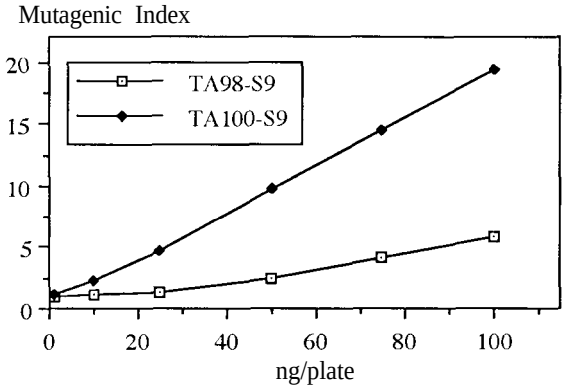

Figure 1. Mutagenic activity of the MX in the Ames test

RESULTS AND DISCUSSION

Figure 1 shows the Ames test results obtained for a pure standard of MX. Both strains were induced for mutagenicity, but the results for TA100-S9 experiments were superior than those for TA98-S9 experiments. A closed relationship between dose-mutagenicity (r^2= 0.9985) was observed for TA100-S9. We concluded that 1 nmol of MX (217 ng) was able to induce 8227 net revertants in TA100-S9 strain, that represents an increment of the spontaneous reversion rate of 42 fold. This value means that MX is one of the stronger mutagens assayed in the Ames test. Our results are in agreement with Kronberg et al. (1988b) who obtained 5600 net revertants for 1 nmol.

The TA100 is the best strain to analyze the MX because it produces Guanine-Cytosine pair substitutions. As MX is a direct action mutagen and the incorporation of the S9 fraction completely destroys its mutagenic activity (Meier 1988), the S9 has been seldom used in this work. Christman et al. (1990) showed that the structural arrangements of critical importance for conferring to MX its extreme mutagenicity are the *cis* arrangement of the $HCCl_2$ and Cl groups around the C-C double bond and the aldehyde groups that close the ring of the MX.

Table 1 depicts the Ames test results obtained for the different real samples studied. The observed general trend was a high toxicity that masked the mutagenic activity. The toxicity already appeared at a low dose (0.1 L/plate) in the initial stages of the treatment process while it was present at higher doses (0.5 L/plate) in drinking water. The Ames test of toxicity in Llobregat River water extracts has been already reported (Romero et al. 1991). In some cases, this toxicity was related to the surfactant concentrations in the water extracts (Romero et al. 1993). TA100 strain was more sensitive to toxicity that the TA98 strain, because both strains are not isogenic (Maron and Ames 1983).

The mutagenic activity was present practically below toxic doses in all samples. The TA98 strain showed more mutagenic activity in raw (2.25 to 0.125 L/plate) and filtered water (5.35 and 9.8 for 0.25 and 1.5 L/plate, samples a and b, respectively), while the TA 100 strain was more effective for drinking water extracts (6.78 and 5.72 for 0.25 and 0.15 L/plate, samples a and b, respectively). Conversely, the S9 fraction reduced the toxicity and mutagenicity in the studied raw, prechlorinated and ozonated water extracts.

Table 3 shows the net revertants/L values obtained for the studied water samples. However, it was not possible to calculate the net revertants/L by regression curves in samples with high toxicity.

Table 1. Ames test results of chlorinated water samples

SAMPLE	dose L/plate	TA98 (MI)		TA100(MI)	
		without S9	with S9	without S9	with S9
RAW WATER	0.125	2.25	1.0	0.87	1.16
	0.25	1.85 *	1.23	0.54 *	1.09
	0.5	0	1.8	0.01 *	1.32
PRECHLORINATED WATER	0.125	nt	nt	nt	1.16
	0.25	*	2.45	*	1.09
	0.5	0	3.43	0	1.32
OZONATED WATER	0.125	nt	nt	1.05	1.03
	0.25	0.98*	1.05	0.74*	1.2
	0.5	0	1.63	0.02*	1.12
CARBON-FILTERED WATER sample a	0.2	nt	nt	1.75	nt
	0.25	5.35	nt	0.55	nt
	0.5	3.26*	nt	0	nt
	1	0	nt	nt	nt
CARBON-FILTERED WATER sample b	0.25	2.98	nt	1.22	nt
	0.5	4.15	nt	1.47	nt
	1	7.4	nt	2.1	nt
	1.5	9.8	nt	1.36*	nt
	2	7.83*	nt	0.79*	nt
DRINKING WATER sample a	0.05	2.01	nt	2.22	nt
	0.1	2.57	nt	4.34	nt
	0.2	3.15	nt	5.2	nt
	0.25	3.46	nt	6.78	nt
	0.5	0*	nt	2.01*	nt
	1	0	nt	0	nt
DRINKING WATER sample b	0.05	2.39	nt	4.8	nt
	0.1	3.43	nt	5.57	nt
	0.15	4.55	nt	5.72	nt
	0.2	1.88	nt	0.44*	nt
	0.25	0.45*	nt	0.2*	nt
	1	0	nt	0	nt

MI: Mutagenic Index. nt: not tested. *: toxicity with cytotoxic effect.

Figure 2 shows the mass spectrum of MX-Me in GC/LRMS. The theoretical relative abundance for the cluster M-OCH_3]$^+$ in a compound with three chlorine atoms is 1:0.96:0.31 respectively for m/z 199:201:203 (identical distribution to the cluster at M-H]$^+$, figure 2). Nevertheless, the observed relative abundance for m/z 199:201:203 is 0.55:1:0.51, respectively. Charles et al. (1991, 1992) working in GC/HRMS explaincd these anomalous m/z abundances by a different fragmentation mechanism of MX-Me. The cluster at m/z 199-203 amu is formed from both M-OCH_3]$^+$ and M-CO]$^+$ losses of the MX-MC molecule. Table 2 compares the calculated exact mass for M-OCH_3]$^+$ and M-CO]$^+$ ions with the observed-measured mass for these ions in GC/HRMS. We worked in GC/HRMS at 7500 resolution power in the full scan mode. The relative abundance of the clusters M-OCH_3]$^+$ and M-CO]$^+$ in HRMS is approximately 1:0.96:0.31. The little differences between the theoretical and the measured ratios can be explained by taking into account the 12000 resolution power required to complete the resolution of the double cluster.

Therefore, the reuirements to search the MX and E-MX in HRMS and LMRS are different. Thus, the criteria that we followed to identify the MX in GC/MS in the SIR mode were: a) the retention times of suspected MX and the standard be identical. b) the relative abundance of the fragment ions M-OCH_3]$^+$ in the sample and the standard should not exceed a 10% variation (see Table 2).

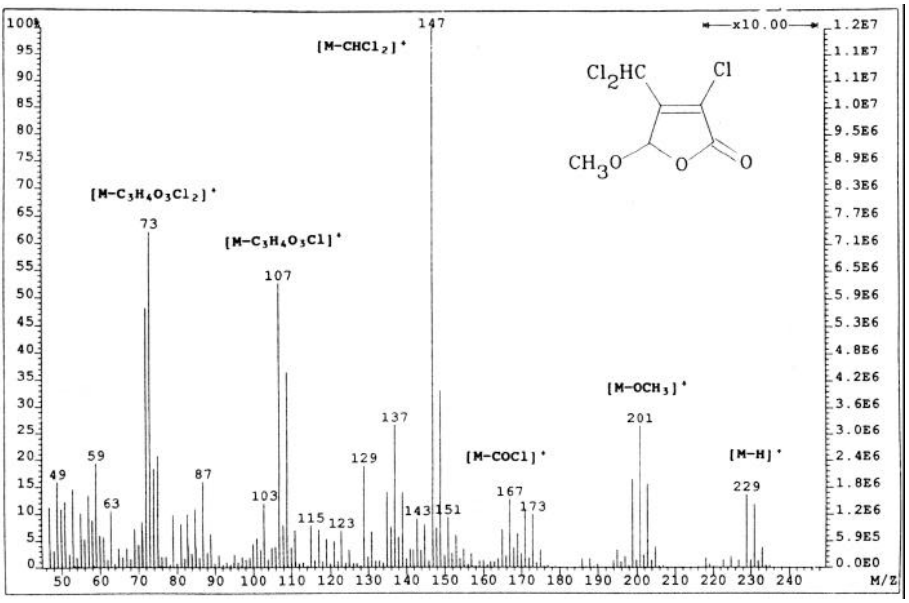

Figure 2. Mass spectrum of MX-Me in GC/LRMS

Table 2. m/z ions used in MX and E-MX search by LRMS and HRMS.

		Low Resolution (m/Δm = 500)		High Resolution (m/Δm = 7500)			
Compound	Fragment ion	m/z	Isotopic ratio[a]	m/z exact mass	m/z measured mass[d]	Theoretical ratio[b]	Real ratio[c]
MX-Me	M-OCH3	199	0.51	198.9121	198.9112	1.00	1.00
		201	1.00	200.9094	200.9062	0.96	0.93
		203	0.55	202.9061	202.9035	0.31	0.31
MX-Me	M-CO	-	-	200.9277	200.9262	1.00	1.00
		-	-	202.9247	202.9237	0.96	0.89
		-	-	204.9208	204.9207	0.31	0.32
E-MX-Me	M-OCH3	245	1.00	244.9540	-	1.00	1.00
		247	0.95	246.9510	-	0.95	0.98
PFK	LOCK	219	-	218.9861	-	-	-

a: Measured isotopic ratio of the MX spectrum in low resolution. b: Theoretical ratio for a compound with three chlorine atoms. c: Measured isotopic ratio of the MX spectrum in high resolution conditions. d: Mass measurements of the MX in high resolution conditions.

The search for MX-Me and EMX-Me in real samples by GC/LRMS was unsuccessful. Figure 3a-c shows the SIR of MX-Me in CG/LRMS of a drinking water extract. The methylated MX could not be identified because of the high background produced by interferences of other compounds that reduced the signal/noise ratio of the MX-Me.

Several authors have used HPLC to subfractionate the water extract to purify the MX in order to increment the signal obtained by low resolution. This procedure is long, expensive and complex. After Charles et al (1991, 1992) and Suzuki and Nakanishi (1990), the analysis of MX can be simplified by using high resolution mass spectrometry. In this case, no purification step is needed and the methylated water extract can be directly analyzed prior to derivatization by GC/HRMS.

Thus, MX extracts were analyzed by GC/HRMS at 7500 resolution power. Figure 3d-e depicts the SIR of a real drinking water sample which clearly shows the presence of MX according to the criteria previously defined. Figure 3f displays the

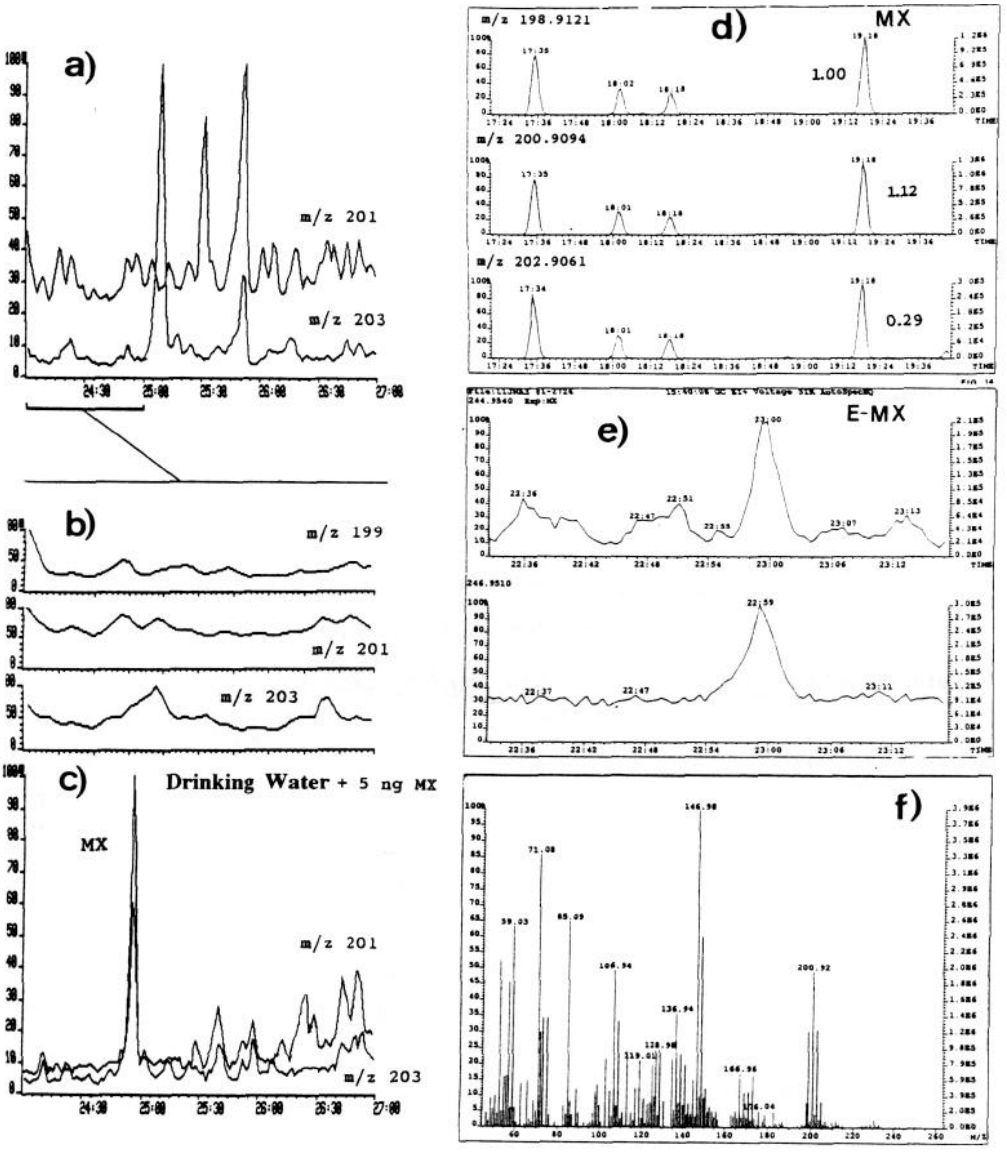

Figure 3. Search of MX-Me and E-MX-Me in drinking water samples by SIR GC/MS. a) SIR of MX-Me in GC/LRMS. b) enlarged region of MX. c) Analysis of MX-Me by SIR GC/LRMS of a drinking water sample spiked with 5 ng of MX-Me standard. d) SIR of MX-Me in GC/HRMS of a drinking water extract showing the presence of MX. e) SIR-GC/HRMS of E-MX, E-MX is also present. f) mass spectrum of MX-Me obtained by CG/HRMS full scan mode in a drinking water extract

MX spectrum in the full scan mode of a real drinking water extract, and thus the presence of MX in the studied samples was univocally demonstrated.

The E-MX-Me was also identified in the studied samples by HRMS (Figure 3e). Since its contribution to the mutagenic activity of the sample is low, no attempts were made to accurately quantify the E-MX. However, its level was estimated to be at 10-20% of the MX concentration.

The obtained MX concentrations (Table 3) varied between 0-30 ng/L, which is the range usually reported from various laboratories (Kronberg and Vartiainen 1988a; Kronberg et al. 1991; Kinae et al. 1992). MX had been only identified in chlorinated samples. The MX showed a behavior like other disinfection by-products at the potabilization plant: It is formed at the first stage of the treatment process (prechlorination), then decreases along the potabilization treatment, it is totally removed in the carbon filters and finally is formed again in the postchlorination step.

Table 3. MX concentrations and its contribution to the Ames activity in real samples

		Mutagenic activity (net revertants/L)				MX contribution
	concentration	TA98	TA100	TA98	TA100	to TA100
SAMPLES	MX (ng/L)	-S9	-S9	+S9	+S9	-S9 activity
Raw water	nd	T	T	79	140	-
Prechlorinated water	3	T	T	160	786	-
Ozonated water	0.7	T	T	55	0	-
Filtered water sample a	0.1	T	T	nt	nt	-
sample b	nd	260	150	nt	nt	**0**
Drinking water sample a	5.6	329	2552	nt	nt	**8.3%**
sample b	10	883	1897	nt	nt	**20%**

nt: not tested. T: toxicity.

The MX contribution to the Ames test activity in the drinking water samples ranged from 8-20% (Table 3) in agreement with the most usual values (20-30%) cited by several authors (Kronberg and Vartiainen 1988a; Kronberg et al. 1991); although even a 60% contribution had been reported (Kronberg and Vartiainen 1988a).

The small differences observed in the concentrations of MX in drinking water extracts could be attributed at first glance to seasonal variations as also observed for other disinfection by-products (i.e. THM, DHAN) in Barcelona's water treatment plant (Cancho et al. 1997), although the limited number of samples analyzed avoids any conclusion. On the other hand, Kinae et al. (1992) studied the seasonal variations of MX in ten cities of Japan showing similar values and trends as our samples. These seasonal variations could be related to physico-chemical properties of raw water, potabilization treatment processes and temperature. It is known that MX is very stable at low pH but at neutral pH and temperatures higher than 25°C it is transformed into E-MX (Backlund et al. 1990; Vartiainen et al. 1991).

The determination of brominated derivatives of MX has not been performed. Llobregat River has an average level of 0.5 mg/L of bromide when entering the water treatment plant. This high level arises from salt mine discharges in the upper course of the river. According to the results obtained for other disinfection by-products in Barcelona's drinking water with predominance of brominated and chlorobrominated species (Cancho et al. 1997), the presence of brominated derivatives of MX could be possible. The unavailability of standards did not allow us to identify its presence in our water extracts. Further research is needed to identify and to monitor the contribution of brominated derivatives of MX to the mutagenicity of Barcelona's drinking water.

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