Effect of O_3/H_2O_2 molar concentration ratio at different pH values on cyclophosphamide degradation

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ABSTRACT

The effect of O_3/H_2O_2 liquid phase molar concentration ratio at different pH on cyclophosphamide degradation rate was evaluated. The combination of O_3/H_2O_2 leads to an increase of cyclophosphamide degradation rate, compared with single ozonation at the same pH and ozone dosages. It was found that cyclophosphamide degradation rate with O_3/H_2O_2 depends on pH for the molar concentration ratios under study, the fastest degradation being observed for molar concentration ratio near 3.4 at pH 7 (80% degradation in 3 minutes). Pseudo-first order reaction rate constants for cyclophosphamide degradation were determined under the experimental conditions of this study. Cyclophosphamide was not detected after 15 minutes of reaction, but only a partial mineralization was obtained, evidenced by a total organic carbon removal lower than 60%. By attenuated total reflection infrared spectroscopy and electrospray ionization tandem mass spectrometry, 4-ketocyclophosphamide was identified as one of the main reaction products at pH 7 and 3.4 molar concentration ratio.

Key words | advanced oxidation, cyclophosphamide, hydrogen peroxide, ozone

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NOMENCLATURE

C_i	cyclophosphamide concentration at each reaction time (M)
C_o	initial cyclophosphamide concentration (M)
k	pseudo-first order reaction rate constants (s^{-1})

INTRODUCTION

The processes based on oxidation of organic compounds by the highly reactive hydroxyl radicals are known as advanced oxidation processes (AOPs) (Glaze *et al.* 1987). These radicals can be formed in water by ozone and hydrogen peroxide or UV radiation, or by a combination of all three.

Many toxic compounds that are present in water and wastewaters can be removed by chemical oxidation. However, recent studies have shown the AOPs effectiveness in the degradation of cyanazine, different nitrophenols, chlorophenol, atrazine and organochloride compounds (Benítez *et al.* 1995; Ormad *et al.* 1997; Beltrán *et al.* 1998; Trapido *et al.* 2001). There is evidence that O_3/H_2O_2 processes accelerate the degradation of these compounds, which are very resistant to treatments with a single oxidizer. Satisfactory results have been obtained when wastewaters with high organic and microbiological contamination were treated by O_3/H_2O_2 for reuse (Langlais *et al.* 2001). The theoretical molar concentration ratio is 2, but in practice, sometimes the best results are reached at higher ratio values (Glaze *et al.* 1987; Beltrán 1997, 2004; Beltrán *et al.* 1998).

Methyl-tert-butyl ether has been detected in surface and underground waters in North America. Recently, methyl-tert-butyl ether degradation in contaminated waters by O_3/H_2O_2 treatment was evaluated. It was verified that oxidation rate and treatment efficiency initially increase with increasing hydrogen peroxide concentration, reach a maximum value, and then decrease with further increase of hydrogen peroxide concentration (Safarzadeh-Amiri 2001, 2002). Therefore, when O_3/H_2O_2 is applied to remove any pollutant, preliminary studies have to be performed to make this process cost-effective.

Cyclophosphamide, 2-[bis (2-chloroethyl) amino] tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide monohydrate (Figure 1), is a cytostatic fundamentally used for cancer treatment. The International Agency for Research on Cancer (Word Health Organization 1981) reported that it has mutagenic and carcinogenic effects. Wastewaters from cyclophosphamide production plants require safe treatment. Its removal is difficult and expensive, even at low concentrations. A high degree of degradation is demanded as the compound is highly refractory to conventional treatments, even with the use of ozone (Pérez 1999a, b). In this paper, the effect of O_3/H_2O_2 at different molar
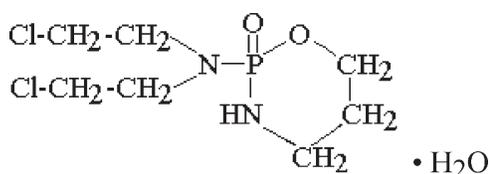

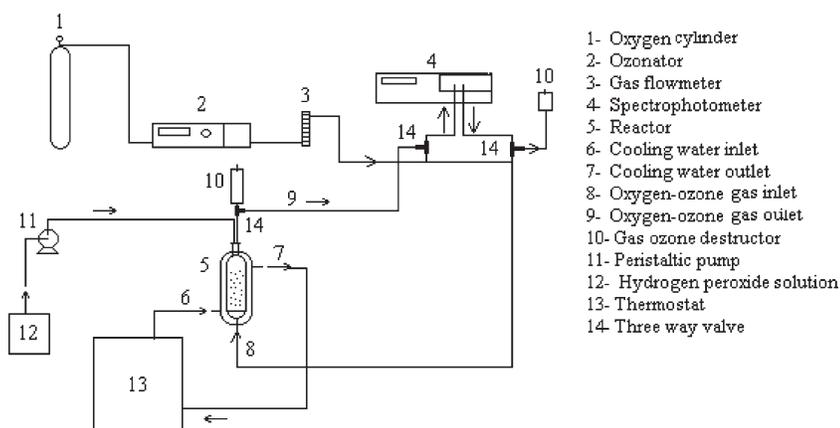Figure 1 | Cyclophosphamide molecular structure.

concentration ratios and pH values on the cyclophosphamide degradation rate was evaluated.

METHODS

Figure 2 shows the laboratory experimental installation. The reaction was carried out in a 100 ml reactor (5), provided with a porous glass diffuser, a gas inlet and a gas outlet. The reactor is coupled to a thermostat (13). The temperature was controlled at 298 ± 0.5 K. The oxygen–ozone mixture was obtained from an ozonizer (2) model AQOZO, CIOZONO (Cuba). Inlet and outlet gas ozone concentrations were determined at 256 nm in a spectrophotometer (4) model Ultrospec III, Pharmacia (UK). By properly switching the position of the three way valves (14) it was possible to lead inlet and outlet gas through the spectrophotometer to measure ozone concentrations. The inlet gas ozone concentration at the reactor was $45\,mg\,l^{-1}$ and the gas flow was $5\,l\,h^{-1}$, which remained constant in all the experiments.

The cyclophosphamide concentration was 1×10^{-3} M, considered to be the maximum expected concentration in production plant residual waters. Phosphate buffer solutions were prepared for pH 7 and 11, and borax/boric acid for pH 9. In all cases ionic strength was fixed at 0.15. Water previously ozonized and bidistilled was used. O_3/H_2O_2 feed molar concentration ratios to the liquid phase were 2.2; 2.55; 3.4; 4.4 and 6.8.

Henry's coefficient was previously determined at pH 7, considering the inlet ozone gas concentration and the dissolved ozone concentration, the latter obtained by using

Figure 2 | Laboratory experimental installation scheme.

the indigo method (Bader & Hoigné 1981). Taking into account the selected inlet gas ozone concentration, the gas flow and Henry's coefficient, the ozone moles transferred in the time unit were calculated.

Taking into account this value, as well as the desired feed O_3/H_2O_2 molar concentration ratios, 6.03×10^{-3}; 3.91×10^{-3}; 5.2×10^{-3}; 3.02×10^{-3} and 1.95×10^{-3} M hydrogen peroxide solutions were prepared. Ozone and hydrogen peroxide were fed simultaneously. Hydrogen peroxide solutions were added to the reactor at 1 ml min^{-1} with a previously calibrated peristaltic pump (11) during the reaction time, being dispersed and mixed into the reactor by the bubbling gas flow.

Samples were taken at 3-minute intervals up to 30 minutes of reaction time. The cyclophosphamide concentration was determined by high performance liquid chromatographic technique (HPLC) with a UV detector model 2501, KNAUER (Germany) at 210 nm and with a RP-18 column. The analyses were carried out under isocratic conditions. The mobile phase was a water/acetonitrile solution 60:40 v/v at 1 ml min^{-1} (detection limit 1×10^{-6} M). Total organic carbon (TOC) values were determined in a 5050 SHIMADZU TOC Analyzer (Japan) in samples taken at the beginning, during and at the end of the reaction. TOC values were obtained in hydrochloric acid acidified samples after a sparging time to remove inorganic carbon content. Standard solutions of potassium hydrogen phthalate were used for calibration curves.

The experimental conditions to obtain the reaction products of cyclophosphamide degradation with O_3/H_2O_2 were: initial cyclophosphamide concentration of 10^{-1} M, O_3/H_2O_2 molar concentration ratio 3.4, pH 7, and reaction time 60 minutes. The reaction products were separated, extracted and concentrated. The separation was performed by preparative HPLC using a 2248 LKB chromatograph (Sweden). The mobile phase was water/acetonitrile (75:25 v/v) at 15 ml min^{-1}. A reversed phase preparative column (RP18) was employed and 1 ml sample was injected. Spectrophotometric detection was carried out at 206 nm. Fractions corresponding to each chromatographic peak were collected at the detector outlet. Each collected fraction was submitted to three chloroform liquid–liquid extractions and afterwards samples were dried with bubbling nitrogen gas and kept at 263 K until analysis.

For product identification, in the cases where it was possible, spectra were obtained by infrared spectrophotometer with coupled ATR model FT/IR-460, JASCO (USA) and electrospray mass spectrometer (ESI-MS) Q-TOF-2, Micromass (UK) operating in the positive ion mode. Capillary voltage and cone voltage were 2.7 kV and 35 V, respectively. The cone temperature was 353 K. The MS data were processed by MassLinx software version 3.5.

RESULTS AND DISCUSSION

Figures 3, 4 and 5 show the experimental results obtained in the cyclophosphamide degradation with ozone only and using three O_3/H_2O_2 molar concentration ratios at different pH. The hydrogen peroxide addition increased the compound degradation rate, compared with ozone treatment, although the same initial cyclophosphamide concentration (1×10^{-3} M), pH and ozone dose values were applied.

In these figures, curves corresponding to O_3/H_2O_2 treatments were plotted until cyclophosphamide concentration could be detected. For example, with the molar concentration ratio 3.4 at pH 7 at 3 min of reaction time, about 23% of cyclophosphamide remains unreacted (Figure 3), but after 6 min of reaction time this compound could not be detected. Taking into account the sensitivity of ultraviolet detection for this compound at the working wavelength, with a detection limit of 1×10^{-6} M, it can be considered that a considerable amount of compound degradation was obtained within 15 minutes of reaction time under all the experimental conditions employed. When applying ozone only, 0.959×10^{-3} M (96%) remained without degrading at pH 7 after six minutes reaction time.

This is due to the great selectivity of molecular ozone and/or because the specific reaction rate constant is not high. Then the oxidation is slow and, as a result, the compound is partially degraded. When hydrogen peroxide is added in the presence of ozone, the cyclophosphamide oxidation by a free radical pathway is favoured. In particular, the very reactive OH radicals formed in the ozone decomposition cycle, which react in a non-selective way and have high reaction rate constants (in the order of 10^9 M^{-1} s^{-1}), are responsible for cyclophosphamide degradation.
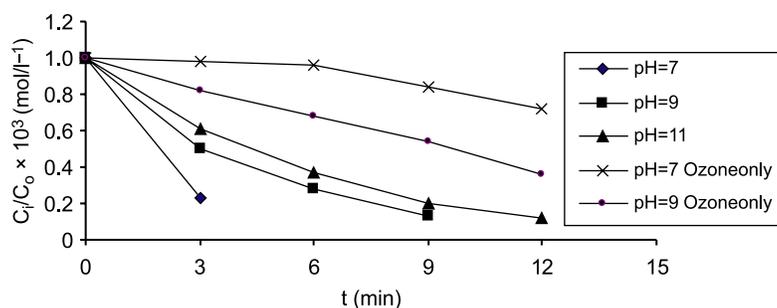
Figure 3 │ Effect of H_2O_2 addition and pH on cyclophosphamide degradation rate. O_3/H_2O_2 molar concentration ratio 3.4.
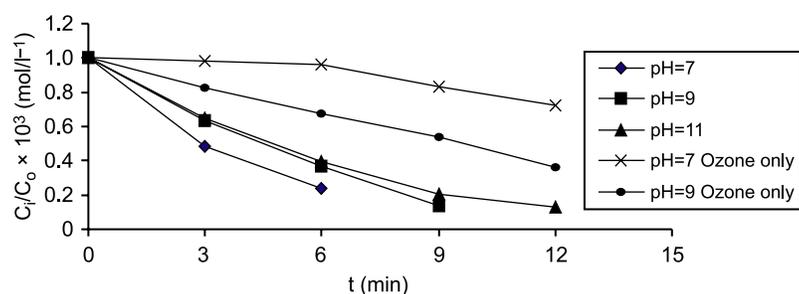

Figure 4 │ Effect of H_2O_2 addition and pH on cyclophosphamide degradation rate. O_3/H_2O_2 molar concentration ratio 2.55.
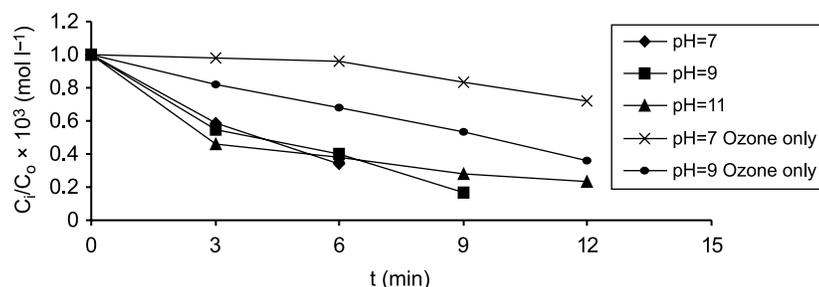

Figure 5 │ Effect of H_2O_2 addition and pH on cyclophosphamide degradation rate. O_3/H_2O_2 molar concentration ratio 6.8.

It is worth mentioning that, at pH 7 without hydrogen peroxide, partial ozone degradation into hydroxyl radicals could be expected. These radicals could be also responsible to some extent for the cyclophosphamide degradation observed under these conditions.

The cyclophosphamide degradation rate when hydrogen peroxide is added to the ozonization is dependent on pH value. This is shown in Figures 3, 4 and 5. When the treatment is carried out with molar concentration ratio 3.4, the greatest pH effect on degradation rate was obtained. Different degradation rates are observed when pH changes. On the other hand, for the molar concentration ratio 2.55, a slight positive effect is appreciated at pH 7 compared with

higher pH values. However, no significant pH effect was obtained with 6.8 molar concentration ratio.

At pH 7, where the best degradation rates were obtained, additional values of molar concentration ratios 2.2 and 4.4 were included in the study. Then, the presence of an optimum at molar concentration ratio around 3.4 at pH 7 was clearly observed (Figure 6). So, the excess of hydrogen peroxide concentration (when molar concentration ratio is smaller than 3.4) has no positive effect on the acceleration of compound oxidation, probably because OH radicals react with both hydrogen peroxide and $H_2O^{\cdot}$ (hydroperoxide) radicals. These radicals are formed during the first steps of the reaction. Therefore, competitive
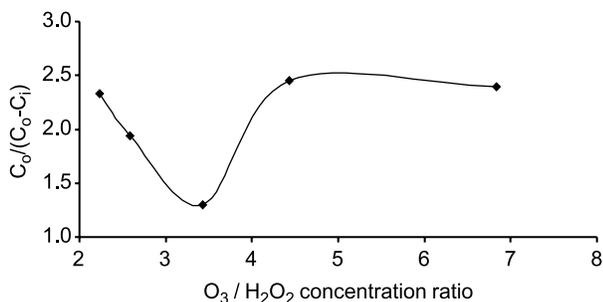
Figure 6 | Effect of the O_3/H_2O_2 molar concentration ratio value on the cyclophosphamide degradation rate at pH 7 and 3 min of reaction.

reactions occur, which can decrease the availability of radicals for the cyclophosphamide oxidation.

On the other hand, no influence of the hydrogen peroxide was observed at concentrations lower than the value corresponding to the molar concentration ratio 3.4. The hydrogen peroxide concentration can be insufficient to generate enough OH radicals to increase the cyclophosphamide degradation rate. Considering this behaviour, before the treatment, it is important to evaluate the effect of O_3/H_2O_2 molar concentration ratio to achieve the highest process effectiveness.

Other authors have reported a maximum degradation rate of several compounds around a determined O_3/H_2O_2 ratio. For example, the application of a hydrogen peroxide concentration of about 10^{-3} M for atrazine and 10^{-2} M for its main by-products (deethylatrazine and deisopropylatrazine) at pH 7 led to the highest oxidation rates during O_3/H_2O_2 oxidation (Beltrán 1997). Glaze & Kang (1989) also observed an optimum value of H_2O_2/O_3 (w/w) ratio during advanced oxidation of tetrachloroethylene and explained it as an ozone mass limited process. Safarzadeh-Amiri (2002) observed that, by O_3/H_2O_2 treatment, the methyl-tert-butyl ether degradation efficiency in contaminated waters reached a maximum value at peroxide concentrations near $225\,mg\,l^{-1}$. Results of the O_3/H_2O_2 process in discoloration of dyehouse effluents revealed a maximum rate at pH 7.5 and applied H_2O_2 dose of 0.5×10^{-3} M (Arslan *et al.* 2000). The reaction rate of O_3/H_2O_2 degradation of nitrophenols evaluated by Trapido *et al.* (2001) presented an optimum about 2×10^{-3} M.

Results in the present paper for the different experimental conditions indicate that cyclophosphamide was largely degraded between 6 and 15 minutes reaction time. However, the percentage TOC removal at 30 min of reaction time was only between 38.6 and 58.6%. The smallest degradation time and the highest percentage of TOC removal were reached at the O_3/H_2O_2 molar concentration ratio 3.4 and pH 7. This means that, although cyclophosphamide could not be detected, reaction products were only partially degraded to CO_2, and complete mineralization was not attained.

Table 1 | Pseudo-first order reaction rate constants for cyclophosphamide degradation under different experimental conditions

O_3/H_2O_2 molar concentration ratio	pH	Cyclophosphamide half lifetime (s)	$(k \pm SD) \times 10^3$ (s^{-1}) (n = 5)
3.4	7	86	8.1 ± 0.1
	9	185	3.8 ± 0.3
	11	408	1.7 ± 0.2
2.55	7	174	4.0 ± 0.2
	9	206	3.4 ± 0.3
	11	244	2.8 ± 0.2
6.8	7	232	3.0 ± 0.2
	9	211	3.3 ± 0.1
	11	314	2.2 ± 0.2

Determination of kinetic parameters

The reaction can be described by a first order kinetics with respect to cyclophosphamide for all the experimental conditions. The empirical pseudo-first order reaction rate constants (k) for cyclophosphamide degradation were calculated on the basis of experimental data. The highest k and the smallest half lifetime values of the compound were reached for O_3/H_2O_2 molar concentration ratio 3.4 and pH 7 (Table 1).
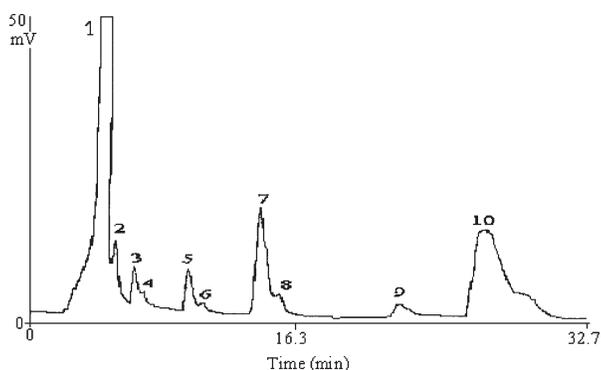

Figure 7 │ Reaction product chromatogram.

Many authors have proposed reaction mechanisms and have determined kinetic constants for the O_3/H_2O_2 system. Staehelin and Hoigné (1982) studied O_3/H_2O_2 reaction at different pH and H_2O_2 concentrations in both pure and natural waters. Glaze & Kang (1989) proposed a kinetic model that describes the oxidation of micropollutants in water. Beltrán *et al.* (1998) studied the mechanism and kinetics of atrazine oxidation for this system, and recently Beltrán (2004) carried out an extensive analysis of this reaction system. Here, the kinetics of this process is considered as a heterogeneous gas–liquid reaction. These studies showed that kinetics of real O_3/H_2O_2/pollutant systems is rather complex and only behaves as a first order kinetics under simplified conditions.

Reaction products

Figure 7 shows the chromatogram of a sample of reaction mixture using O_3/H_2O_2 molar concentration ratio 3.4 at pH 7. Peak 1 fundamentally consists of phosphate buffer pH 7, which is the sample solvent. Here some reaction products could also elute. Peak 10 corresponds to non-reacted

Result of Peak Picking

No.	Position	Intensity
1:	3119.30	86.2594
2:	3073.01	83.3407
3:	2926.45	84.0056
4:	2847.38	88.5168
5:	2792.42	89.4266
6:	1691.27	45.6632
7:	1457.92	81.0331
8:	1434.78	83.9506
9:	1411.64	74.6737
10:	1377.89	66.9073
11:	1341.25	52.3321
12:	1292.07	54.0613
13:	1259.29	68.6221
14:	1247.72	56.5187
15:	1228.43	38.4042
16:	1206.26	53.4243
17:	1147.44	54.4203
18:	1105.01	57.1468
19:	1080.91	64.8010
20:	1027.87	51.3592
21:	1011.48	49.9994
22:	985.45	51.3756
23:	920.84	41.0107
24:	901.56	53.3996
25:	869.74	56.2337
26:	810.92	56.7030
27:	790.67	47.6447
28:	754.99	59.7326
29:	705.82	40.3540
30:	647.00	42.1401
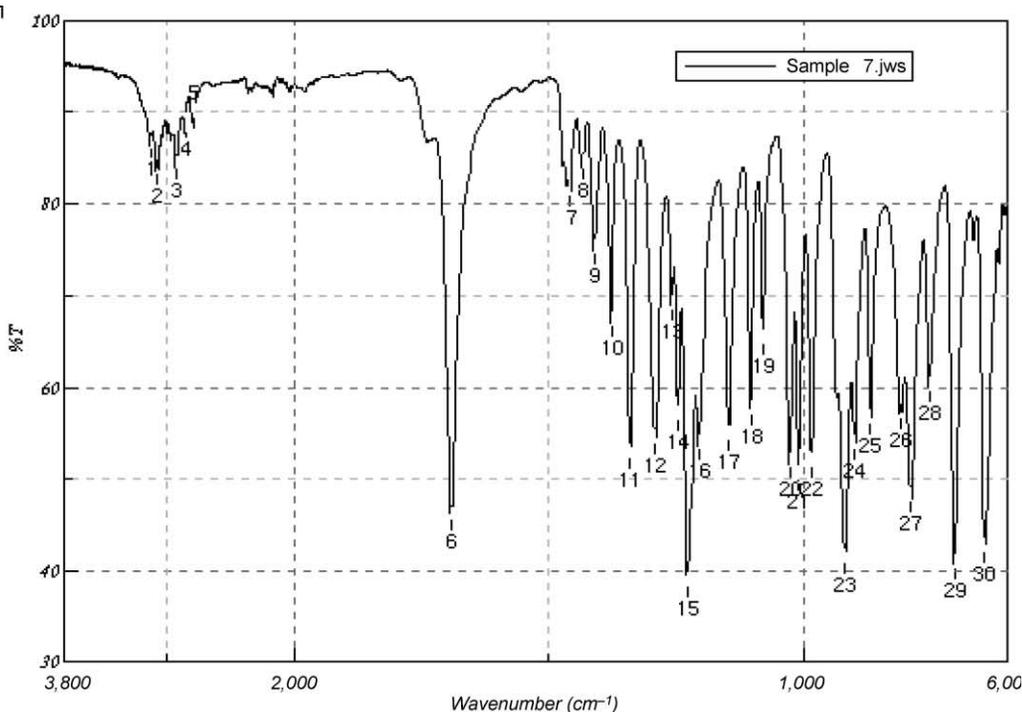
Figure 8 │ Sample 7 infrared spectrum.
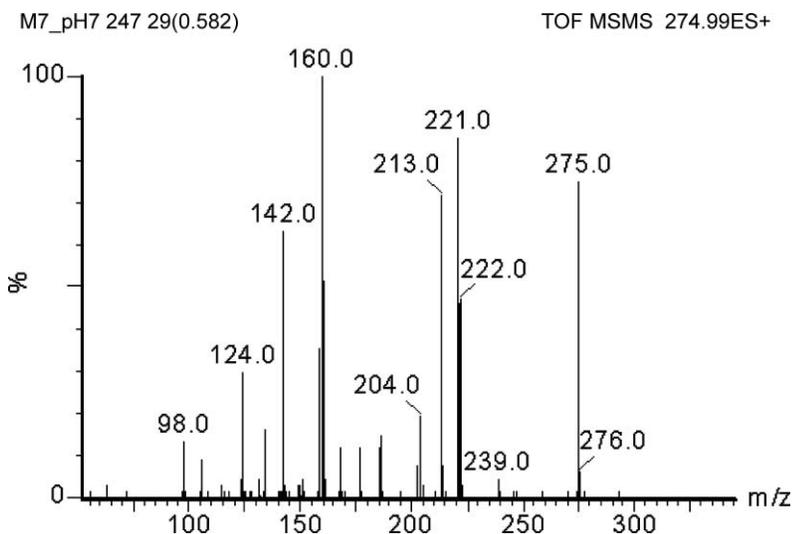
M7_pH7 18(0.369) Cm(17:38)
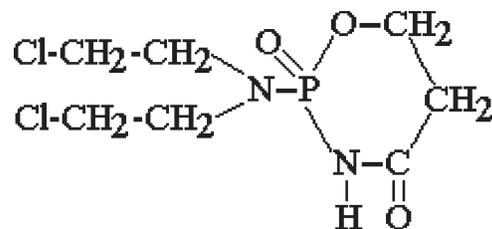

Figure 9 │ Sample 7 ESI mass spectrum.

cyclophosphamide. Only the product isolated in peak 7 could be identified by infrared spectroscopy and mass spectrometry techniques. Under the experimental conditions employed the rest of the reaction products remained unidentified because of their low concentrations in the reaction mixture.

Figure 8 shows the infrared spectrum of sample 7 (peak 7). Characteristic absorption bands are observed at 3,119, 3,070, 1,691, 1,228, 790, 754, 705 and 647 cm^{-1}.

In the ESI mass spectra of this sample (Figure 9) two groups of signals are observed: m/e 275 is the molecular ion

Figure 11 │ 4-ketocyclophosphamide molecular structure.

$(M+)$ and m/e 297 $(M + Na)+$. The accompanying characteristic cluster pattern corresponds to the chlorine isotopic distribution.

Taking into account the molecular mass ion obtained in 275, the infrared spectrum and the tandem mass spectrum (Figure 10) for m/z 275 ion, 4-ketocyclophosphamide was identified (Figure 11). This compound has been identified before by Peter *et al.* (1976) as one or the main reaction product in the synthesis of 4-hydroxycyclophosphamide by cyclophosphamide ozonization at 273 K in acetone/water mixture.

CONCLUSIONS

- The combined O_3/H_2O_2 treatment is effective in degrading cyclophosphamide.
- The highest cyclophosphamide degradation rate was reached at 3.4 O_3/H_2O_2 molar concentration ratio and pH 7.

M7_pH7 247 29(0.582)   TOF MSMS 274.99ES+

Figure 10 │ Tandem mass spectrum of the m/z 275 ion.

- Considerable cyclophosphamide degradation was obtained under the employed experimental conditions. However, only a partial mineralization was achieved.
- Under the experimental conditions of the study, 4-ketocyclophosphamide was identified as one of the main reaction products.

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