Determination of genotoxic effects of methidathion alkaline hydrolysis in human lymphocytes using the micronucleus assay and square-wave voltammetry

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ABSTRACT

The interaction of pesticides with environmental factors, such as pH, may result in alterations of their physicochemical properties and should be taken into consideration in regard to their classification. This study investigates the genotoxicity of methidathion and its alkaline hydrolysis by-products in cultured human lymphocytes, using the square-wave voltammetry (square wave-adsorptive cathodic stripping voltammetry (SW-AdCSV) technique) and the cytokinesis block micronucleus assay (CBMN assay). According to the SW-AdCSV data the alkaline hydrolysis of methidathion results in two new molecules, one non-electro-active and a second electro-active which is more genotoxic than methidathion itself in cultured human lymphocytes, inducing higher micronuclei frequencies. The present study confirms the SW-AdCSV technique as a voltammetric method which can successfully simulates the electromodynamics of the cellular membrane.

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1. Introduction

The biological activity of a pesticide is influenced by its physical and chemical properties. Once a pesticide is introduced into the environment, its physicochemical parameters and processes, such as pH, redox potential, UV light, ionic strength, adsorption/desorption and transformation determine its fate [1–3]. The assessing of the impact of a particular pesticide and its products degradation on human health is difficult due to speciation of pesticides. The mechanism by which the pesticides exert their toxic effects on mammals has only been characterized for a few compound groups [1]. The genotoxic and mutagenic activities of certain agro-pharmaceuticals have been studied both with in vitro and in vivo systems, using indicators of genetic damage such as micronuclei (MN), single cell gel electrophoresis (SCGE), chromosomal aberrations (CA) and sister chromatid exchanges (SCE) [4–10]. The cytokinesis block micronucleus assay (CBMN assay) in human lymphocytes, developed by Fenech and Morley [11], uses cytochalasin-B, an inhibitor of actin polymerization, to prevent cytokinesis without blocking nuclear division [12–13]. As a result, binucleated (BN) cells are produced, which are scored for the presence of MN [12,14–15].

Methidathion \((C_6H_{11}N_2O_4PS_3)(\text{S-2,3-dihydro-5-methoxy-2-oxo-1,3,4-thiadiazol-3-ylmethyl} \quad \text{O,0-dimethylphosphorodithioate})\), Schematic 1, is a non-systemic organophosphorous pesticide intended to control insects and arachnoids in a wide range of cultivations. It was first registered in 1972 and is characterized as a Class I Toxic Compound (Highly Toxic) and as a Restricted Use Pesticide (RUP) by US Environmental Protection Agency [16]. Data from 1987 to 1997 indicate an average domestic use of approximately 241,000 lb, of active ingredient, per year [16–18]. Its LD50 ranges between 25 to 48 mg/kg for rats and 25 to 70 mg/kg for mice [19]. Methidathion and its derivatives are still in use, regardless their toxicity. This is due to their low persistence in the environment and their high effectiveness.

At alkaline pH, methidathion undergoes non-reversible hydrolysis [20]. In Schematic 2 a detailed study of the effect of pH on methidathion that has been made by Eto [21] is presented. The cleavage of the phosphoro-sulfur bond “P-S” results into two by-products. According to stripping analysis theory [22], the first product (Schematic 2) is a non-electroactive phosphono-compound, while the second product is a highly electro-active sulfhydryl-species (marked “H-SR”). Molecular structures with “H-SR” electroactive groups can react with sulfur-containing enzymes and coenzymes blocking their catalytic activity [23–24]. Enzyme inhibition may also occur by complex formation of the active “H-SR” group with metal ions of metal-containing enzymes [24–25]. In addition, the “H-SR” species can also act as a chelating agent...
for various metal ions \( [M^{n+}] \) such as \( \text{Fe}^{2+}, \text{Mn}^{2+}, \text{Cu}^{2+}, \text{Ni}^{2+}, \text{Hg}^{2+} \) and \( \text{Bi}^{3+} \) to form coordination complexes [25–28]. The toxicity is significantly enhanced if \( M^{n+}-\text{SR} \) complex can act as a fungicide, such as in the case of ziram: \( \text{Zn}^{2+}-(\text{SR})_2 \) [25–27].

Especially, the interaction of the \( S \)-containing molecules with \( \text{Hg}^{2+} \) ions has drawn the continuous attention of scientists over the past 50 years. The pioneering works of Kolthoff and Stricks [29] have contributed to the fundamental understanding of the physical chemistry of the interaction of cysteine/cystine with \( \text{Hg} \) [29]. The reaction of cystine at the \( \text{Hg} \)-electrode was comprehensively discussed by Heyrovský et al. [30]. Gregg and Tyler [31] paved the way for the polarography of dithiocarbamates compounds. Several studies have examined the electrochemical behavior of the cystine and cysteine on mercury electrodes and confirmed the considerable influence of \( \text{pH} \), supporting electrolyte as well as of the concentrations of cystine/cysteine on the double layer properties at the mercury/solution interface [28,32–34].

A common denominator in their action is the cell membrane activity. Their adsorption on the biological membranes can determine the surface activity of these molecules [24,35]. In a more general context, surface adsorption is widely recognized as a regulatory physicochemical mechanism for the interaction of pesticides with surfaces such as biological membranes [36], or the hanging mercury drop electrode surface (HMDE) [37].

Nowadays, among the various electrochemical techniques, the Electrochemical impedance spectroscopy (EIS) [38] and the square cathodic stripping voltammetry (SW-CSV) using a HMDE [22,39] can be used to determine the electroactive groups of molecular structures as well as to simulate electrodynamically the adsorptive ability of cellular membranes [2,40].

More precisely, the EIS is an in-situ non-invasive analytical technique for characterizing electrochemical systems [38,41]. This technique measures the impedance of the concerned electrochemical system over a range of frequencies. The EIS technique has been used for investigating membrane microstructure, membrane fouling and immediately changes that occurred by osmosis process on the zeolite membrane surface [41] and catalytic activity of cysteine and cystine on the electroreduction of \( \text{Bi}(\text{III}) \) ions [28].

Additionally, the HMDE has been employed for the analytical determination of dithiocarbamate pesticides, including ziram and thiram. They were addressing topics such as the polarographic determination of thiram [47], the analytical determination of ziram by anodic stripping voltammetry [48] and the analytical measurement of thiram by cathodic stripping voltammetry [49]. A detailed study of the thermodynamics of adsorption for two representative dimethyl-dithiocarbamate pesticides on the HMDE showed a cleavage of the disulfide \( S-S \) bond of the thiram near the HMDE at around \(-550\) mV and a strong adsorption of the dithiocarbamate products onto electrode surface [37,50].

According to the above, the methidathion upon its application in the field undergoes gradual alkaline hydrolysis, depending on soil’s \( \text{pH} \) (Schematic 2). So far, the effect of methidathion alkaline hydrolysis and its by-products have not yet been tested for their possible side effects.

In the present study, the SW-AdCSV voltammetric technique using a HMDE as simulator of cellular membrane and the CBMN assay were used in order to study the potential genotoxic effects of methidathion and its hydrolysis by-products in cultured human lymphocytes.

2. Materials and methods

2.1. Reagents and solution

All the experiments were performed with analytical grade chemicals. Methidathion (Riedel-de Haën 45572, purity > 99.7%, CAS-Number: 950-37-8) was used without further purification. Stock solution of methidathion (Water Solubility: 240 mg/l) was prepared with ultrapure Milli-Q water at a concentration of 50 \( \mu \text{g/ml} \) [18] at

Schematic 1. Molecular structure of methidathion.

Schematic 2. Reaction scheme on methidathion hydrolysis by Eto [21].
pH 7.4 and stored in the dark at 4 °C. To adjust the pH at pH 7.4 a NaOH/HNO₃ system was used. Standard solutions were prepared daily from stock solution by diluting to the appropriate concentration.

2.2. Voltammetric parameters

For the voltammetric measurements of methidathion, an electrochemical detector (Model TraceLab 50, Radiometer Analytical) was used to control the voltage of a three-electrode system as described earlier [37]. For all voltammetric measurements the ionic strength was adjusted at 1 mM with KNO₃ (Aldrich, >99.9%) at room temperature. Electrochemical reduction of methidathion is investigated at HDME using SW-AdCSV technique. The SW-AdCSV was used with the following parameters: Cell parameters—stirrer at 525 rpm, purge time 600 s, accumulation time (tacc) 300 s, waiting time 10 s, Hg-drop growth time 0.7 s, electrode area 3 mm² [2]. The electrode area was controlled by a pneumatic connection with nitrogen (99.999%) at 1.0 bar. Signal parameters — accumulation potential (Eacc) = 100 mV, E-initial = −100 mV, E-final = 800 mV, SW frequency 12.5 Hz, Scan increment 1 mV, SW amplitude = 50 mV. Current range — minimum 10 nA, maximum 10 mA.

In addition, the effect of Eacc on the peak current between the cellular membrane potential range (−100 mV up to +100 mV), was investigated. According to the data, the peak current varies <5% due to interactions between hetero-ring and the mercury electrode.

The pH measurements were carried out with a GLP21, CRISON pH meter.

2.3. CBMN assay in cultured human lymphocytes

Blood samples were obtained from two healthy non-smokers, without previous known contact with pesticides. Donors were aged between 20 and 25 years. Whole blood (0.5 ml) was added to 6.5 ml Ham's F-10 medium (Invitrogen), 1.5 ml fetal calf serum (Invitrogen), and 0.3 ml phytohemagglutinin (Invitrogen) to stimulate cell division. The appropriate concentrations were added 44 h post culture initiation. Cells were collected 6 μh after the addition of the chemicals and fixed with freshly made methanol/acetid acid (Riedel-de Haën/Merck) mixture (3:1 v/v) after mild hypotonic treatment and stained with Giemsa (Fluka) [8,51]. At least 1000 BN cells with preserved cytoplasm were scored per slide, for each donor and for each case, in order to calculate the frequency of MN. Standard criteria [15] were used for scoring MN. The cytokinesis block proliferation index (CBPI), given by the equation CBPI = [M1 + 2 M2 + 3 (M3 + M4)] / N, where M1, M2, M3 and M4 correspond to the numbers of cells with one, two, three, and four nuclei and N is the total number of cells [52], was calculated by counting at least 2000 cells in order to determine possible cytotoxic effects.

One millilitre of supernatant, derived from 72 h blood cultures in the presence of methidathion, was mixed with 9 ml of absolute ethanol [53]. The mixture was centrifuged at 1500 rpm for 10 min and 4 ml of the resulting supernatant was mixed with 10 ml KNO₃ buffer at final concentration 1 mM and transferred in the stripping voltammetry cell. Quantitative analysis was performed based on a calibration curve.

2.4. Statistical analysis

Statistical analysis of MN data was made by the G-test for independence on 2 × 2 tables. This test is based on the general assumption of the χ² distribution, but offers theoretical and computational advantages [54]. The G-test was evaluated using the data analysis statistical software Minitab.

3. Results

3.1. SW-AdCSV signal analysis

Under the conditions of our experiments i.e. a quasi-spherical drop exposed to a stirred solution at low concentration of methidathion, we consider that diffusion is the main mechanism that determines the approach of the analyte on the HDME surface [22,39,55–56]. So, electroactive species move towards the electrode surface by diffusion, while they are adsorbed via electrostatic interactions [22,39,55–56].

The SW-AdCSV signal for methidathion, contained a main peak with Epeak = −535 mV (vs Ag/AgCl 3 M KCl) at pH = 7.4, as seen in Fig. 1, in accordance to previously reported data [37,50]. The characteristics of this signal at pH 7.4 (i.e. Epeak, shape) are identical to those reported previously for sulfhydryl-species [37,50]. In the present work, the peak current, I(nA), measured from the signal baseline was taken as an analytical measure of methidathion adsorbed on the HDME. At acidic and neutral pH values 4.5 and 7.4 respectively, a low peak current is observed (Fig. 1, dotted and dashed lines respectively), while when dissolved at high alkaline, pH 11.5, and readjusted of the solution at pH-measurement, pH 7.4, the peak current of the signal is high (Fig. 1, solid line). As presented in Fig. 2A the signals’ peak current is strongly dependent on the pH-hydrolysis of the methidathion. For voltammetric measurements, Fig. 2A, it should be underlined that the pH-hydrolysis of methidathion-solutions were readjusted at pH 7.4. In aqueous solution, the alkaline hydrolysis of methidathion is strongly pH dependent [20], and was previously described by reaction (1) [21]. The protonation of methidathion has a pKa of 6.5 [57]. Accordingly, in aqueous solution and at pH over 8.5 >50% of methidathion is dissociated in one phosphono-compound-anion plus one sulfhydryl-species “H-SR”.

In order to test the effect of methidathion alkaline hydrolysis by-products in culture conditions neutral pH is required otherwise the cultured cells would be stressed. On the basis of this, SW-AdCSV experiments were carried out at pH 7.4. The electrode reaction at the Hg drop concerns only the electro-active sulfhydryl- group. According to electrochemical oxidation behavior studies of sulfhydryl-species [31, 47,58] the electrochemical reaction responsible for the SW-AdCSV response of “H-SR” onto HDME at pH 7.4 can be described by the electrochemical reaction (1).

\[
\text{Hg}^2+(-\text{SR})_2 + 2\text{H}^+ + 2e^- \rightarrow \text{Hg}^0 + 2\text{H}^- - \text{SR} \quad \text{E}_{\text{peak}} = -535 \text{ mV} \quad (1)
\]

This is a common mechanism for anionic sulfur species adsorbed at the Hg-drop surface, where the electroactive adsorbed species is a sparingly soluble mercuric complex formed between the anionic sulfur

**Fig. 1.** SW-AdCSV signals of 0.3 mg/l methidathion dissolved at pH: 11.5 (solid line), 7.4 (dash line), 4.5 (dot line). All measurements were carried out at pH 7.4.
lymphocytes treated with 3 μg/ml methidathion at pH 7.4. Induction of BNMN, MN and CBPI values in human lymphocytes treated with methidathion at different pH values for 10 min followed by adjustment to pH 7.4.

**Table 1**

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>BNMN MF (%) ± se</th>
<th>MN MF (%) ± se</th>
<th>CBPI MF ± se</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.0 ± 0.0</td>
<td>5.0 ± 0.0</td>
<td>2.03 ± 0.05</td>
</tr>
<tr>
<td>pH = 9.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5.0 ± 0.0</td>
<td>5.0 ± 0.0</td>
<td>2.16 ± 0.11</td>
</tr>
<tr>
<td>2</td>
<td>5.0 ± 0.0</td>
<td>5.0 ± 0.0</td>
<td>1.98 ± 0.95</td>
</tr>
<tr>
<td>3</td>
<td>6.0 ± 0.0</td>
<td>6.0 ± 0.0</td>
<td>2.12 ± 0.18</td>
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<tr>
<td>pH = 10</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>5.5 ± 0.5</td>
<td>5.5 ± 0.5</td>
<td>1.98 ± 0.03</td>
</tr>
<tr>
<td>2</td>
<td>6.0 ± 0.0</td>
<td>6.0 ± 0.0</td>
<td>2.02 ± 0.14</td>
</tr>
<tr>
<td>3</td>
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<td>9.0 ± 1.0</td>
<td>2.00 ± 0.07</td>
</tr>
<tr>
<td>pH = 10.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>5.5 ± 0.5</td>
<td>2.06 ± 0.05</td>
</tr>
<tr>
<td>2</td>
<td>6.0 ± 0.0</td>
<td>8.0 ± 1.0</td>
<td>2.12 ± 0.03</td>
</tr>
<tr>
<td>3</td>
<td>9.0 ± 1.0</td>
<td>9.0 ± 0.0</td>
<td>2.03 ± 0.12</td>
</tr>
<tr>
<td>pH = 11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>6.0 ± 0.0</td>
<td>6.0 ± 0.0</td>
<td>2.23 ± 0.08</td>
</tr>
<tr>
<td>2</td>
<td>8.0 ± 0.0</td>
<td>8.0 ± 0.0</td>
<td>2.16 ± 0.17</td>
</tr>
<tr>
<td>3</td>
<td>10.0 ± 0.0*</td>
<td>10.0 ± 0.0*</td>
<td>1.94 ± 0.02</td>
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<tr>
<td>pH = 11.5</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.0 ± 1.0</td>
<td>6.0 ± 1.0</td>
<td>2.05 ± 0.02</td>
</tr>
<tr>
<td>2</td>
<td>9.0 ± 0.0</td>
<td>10.0 ± 0.0</td>
<td>2.13 ± 0.09</td>
</tr>
<tr>
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<td>16.5 ± 0.5**</td>
<td>17.0 ± 0.0**</td>
<td>1.99 ± 0.09</td>
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<tr>
<td>pH = 12</td>
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<td></td>
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<tr>
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<td>6.5 ± 0.5</td>
<td>6.5 ± 0.5</td>
<td>2.20 ± 0.07</td>
</tr>
<tr>
<td>2</td>
<td>10.5 ± 0.5*</td>
<td>11.5 ± 0.5*</td>
<td>2.06 ± 0.06</td>
</tr>
<tr>
<td>3</td>
<td>16.0 ± 0.0**</td>
<td>17.5 ± 0.5**</td>
<td>2.09 ± 0.22</td>
</tr>
</tbody>
</table>

BNMN: micronucleated binucleated cells, MN: micronuclei, CBPI: cytokinesis block proliferation index, MMC: mitomycin-C, MF(%) ± se: mean frequencies (%±) ± standard error, 2000 binucleated cells scored per experimental point, *p < 0.0001 [G-test for BNMN and MN; χ² for CBPI].

3.2. CBMN assay

The genotoxic effect of methidathion was studied in three concentrations (1, 2 and 3 μg/ml) which represent the most commonly used ones [60–62]. Table 1 depicts the genotoxic effect of methidathion in the aforementioned concentrations. It is obvious that there is no statistically significant difference between the control and the treated cultures in the frequencies of either binucleated cells with micronuclei (BNMN), or micronuclei (MN). The positive control (MMC) revealed, as expected, statistically significant differences in both indices (BNMN and MN).

![Fig. 2](A) Electrochemical measurements: effect of pH-hydrolysis 0.3 mg/l methidathion on the peak current Id (mA) of SW-AdCSV signals (Exp. conditions: pH-measurement 7.4, Eps = −534 mV). (B) Measurements of micronuclei: MN (‰) values in human lymphocytes treated with 3 μg/ml alkaline methidathion solution at pH incubation 7.4 by using the parameters listed in Tables 1 and 2 as function pH-hydrolysis (where: * = p < 0.05, ** = p < 0.01, significant differences between control and treated cultures by G-test).

In our testing system, methidathion was not found to be genotoxic, as it did not induce increased frequencies of micronuclei at all the tested concentrations (Table 1). To our knowledge, there is no data on the genotoxic activity of methidathion with CBMN assay in human lymphocytes at such low concentrations. Our results are in line with previous reports showing that methidathion did not exert DNA oxidative damage in the liver of rats [61]. Moreover, methidathion did not exert sister chromatid exchanges with or without metabolic activating system in human lymphocytes at doses similar with our study, i.e. 10 μM which is approximately 3 μg/ml [62]. Additionally, in a recent study methidathion...
showed no genotoxicity in an in vivo system such as the Drosophila wing somatic mutation and recombination test [64]. The SW-AdCSV technique was used to investigate the physicochemical properties of methidathion. The purpose of this assay was not only to clarify the way methidathion acts, but also to detect the residues of this pesticide in human lymphocytes culture solutions. SW-AdCSV is a voltammetric technique that simulates electrodynamically the cellular membrane [2] and this assay is widely used for the investigation of physicochemical properties of a variety of compounds [22,45,65–76]. There have also been studies which used this method for the detection of compounds in human blood [22,74].

Methidathion is a pH sensitive compound that undergoes hydrolysis in alkaline environment [21]. The square wave voltammetry data for human lymphocyte cultures in the presence of methidathion’s alkaline–by-products revealed absence of by-products in culture medium. This indicates that >95% of methidathion was absorbed by the lymphocytes.

The major aim of the current study is to examine the genotoxic effects of the aforementioned concentrations of methidathion after hydrolysis by alkaline conditions (pH 9.5 up to 12). It should be underlined that the pH of the cultures was stable at 7.4 and pH alterations were made only in the stock solutions which after hydrolysis were readjusted at pH 7.4. In the CBMN assay, no increase in MN frequencies was observed up to pH = 10 with the hydrolyzed methidathion. For the higher dose tested (3 μg/ml) there is an increase in the frequency of MN at pH > 10 which reach a significant increase only in cases of pH ≥ 11. This indicates that the alkaline environment at high pH values can produce molecules that are much more genotoxic than the original one.

Vorkamp et al. investigated the fate of methidathion in biological waste during anaerobic digestion and showed that its hydrolysis accelerated by alkaline conditions (pH 10.5 and 12.8) and high temperatures 55 °C [63]. The findings of this study and the observed data from our experiments indicate that the abiotic hydrolysis of methidathion in alkaline environment, during anaerobic digestion of biological waste, can produce molecules that are much more genotoxic than the original one.

The alkaline hydrolysis of the phosphoro-sulfur bond “P-S” of methidathion into a non-electro-active phosphono-compound and to a highly electro-active sulfhydryl-species “H-SR” in relation to the observed SW-AdCSV and MN results suggests that the “H-SR” by-product is responsible for the observed increase of MN frequencies.

Surface adsorption is recognized as a regulatory mechanism for the interaction of pesticides with biological membranes [36]. For “H-S-” electroactive groups to react with sulfur-containing enzymes and coenzymes blocking their catalytic activity [24–25] it means that the “H-S-” containing molecules penetrate cellular membranes. In Fig. 2, the voltammograms from the samples derived from the culture media containing methidathion alkaline by-products indicate that the “H-SR” by-product possibly penetrates the lymphocyte membrane in order to act as an inducer of DNA damage.

Our findings lead to a possible assumption that several environmental factors and/or physicochemical parameters such as pH are able to alter the characteristics and the activity of pesticides and convert them from harmless and not genotoxic to potentially genotoxic. The above is corroborated by the studies of Stivaktakis et al. [2], according to which imidacloprid alters its genotoxic behavior towards human lymphocytes in the presence of nitrate and by Zachow and Uzumcu [77] which demonstrated that 2,2-bis-(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE), a metabolite of methoxychlor, inhibits steroidogenesis in rat ovarian granulosa cell in vitro.

5. Conclusions

The present data on the genotoxicity of methidathion confirm that alterations of the behavior of pesticides by environmental factors, such as pH, should be taken into consideration with regard to their classification. Briefly the current study can be summarized as follow:

- Alkaline hydrolysis of methidathion results in two new molecules: one non-electro-active and a second electro-active which is more genotoxic than the original molecule in cultured human lymphocytes.
- SW-AdCSV voltammetric technique confirmed that the electro-active by-product of methidathion’s alkaline hydrolysis is the responsible agent for the induction of micronuclei frequencies.

The present study proposed the SW-AdCSV as a voltammetric technique which successfully simulates electrodynamically the cellular membrane.

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References
