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A procedure for the assessment of the toxicity of intermediates and products formed during the accidental thermal decomposition of a chemical species

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ABSTRACT

The knowledge of the substances which form when a molecule undergoes chemical reactions under unusual conditions is required by European legislation to evaluate the risks associated with an industrial chemical process. A thermal decomposition is often the result of a loss of control of the process which leads to the formation of many substances in some cases not easily predictable. The evaluation of the change of an overall toxicity passing from the parent compound to the mixture of its thermal decomposition products has been already proposed as a practical approach to this problem when preliminary indications about the temperature range in which the molecule decomposes are available. A new procedure is proposed in this work for the obtainment of the mixtures of thermal decomposition products also when there is no previous information about the thermal behaviour of investigated molecules. A scanning calorimetric run that is aimed to identify the onset temperature of the decomposition process is coupled to an isoperibolic one in order to obtain and collect the products. An algal strain is adopted for toxicological assessments of chemical compounds and mixtures. An extension of toxicological investigations to human cells is also attempted.

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1. Introduction

Unpredictable dangerous substances may be formed and released to the environment during accidental events due to the loss of control of industrial chemical processes. The knowledge of these substances is required by the law in force in the European countries, Directive 2003/105/EC art.2 [1,2]. However, no theoretical tool is available to foresee the compounds which form during the thermal decomposition of a specific chemical molecule. In the same time, mainly because of the difficulties associated both (1) to the simulation of the operating conditions which take place during the loss of control of a chemical system and (2) to the analytical identification of the species which are present in very complex mixtures resulting from the thermal decomposition processes, experimental protocols are still lacking. For the first aspect a range of experimental techniques (thermogravimetry, scanning and adiabatic calorimetry, etc.) characterized by different temperature–time profile are indicated as suitable [3]. For the second aspect, in a previous paper [4] some of the authors proposed a procedure to deal with the problem of the formation of unwanted compounds during the accidental thermal decomposition of chemical species by measuring the associated change of an *overall toxicity*. The procedure was applied to two species, cumene hydroperoxide and ethyl parathion, for which information about the decomposition temperature ranges and the nature of the decomposition products was already known from a previous investigation by some of the authors. With this approach it was shown that during the thermal decomposition some compounds may form that are more toxic than the parent species. In this case it is necessary to fulfil the Seveso II directive [2] determining the nature and the properties of unwanted thermal decomposition products, in order to correctly estimate the consequences of explosive events and adequately plan the emergency response.

In the present work, a new approach is proposed to deal with organic substances when no information related to their decomposition temperature range is available. The procedure is based on two different calorimetric techniques and allows the decomposition of the compounds and the collection of their products. The evaluation of an overall toxicity of the collected samples is carried out by using an algal strain as in the previous paper [4]. Moreover, an attempt to determine the toxicity of thermal decomposition products of studied compounds on Human Umbilical Vein Endothelial

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Cells (HUVEC) is performed. This should provide a first assessment on the human health hazard associated with the chemical contamination caused by the thermal decomposition of a compound. The use of *in vitro* test for the risk assessment in man and other mammalian species in substitution of *in vivo* tests on animals is recommended by EU directives, as indicated in EC regulation n. 1907 (EC. 2006) concerning the Registration, Evaluation, Authorization and restriction of CHemicals (REACH) [5]. To evaluate the suitability of this new approach, the same chemical species as those used in the quoted paper are adopted in the present investigation, working *as if no* previous indications their decomposition temperature ranges were available. That is, the two chemical species (ethyl parathion and cumene hydroperoxide) are used in the present work completely neglecting the available information from previous investigations on their thermal decomposition behaviour. The approach followed in this work will be thus suitable in all situations when an evaluation is required of the toxicity of the decomposition products of chemical compounds with an unknown thermal behaviour.

2. Experimental

2.1. Thermal decomposition experiments

The organic species adopted in this investigation were firstly submitted to some scanning calorimetric runs at a fixed heating rate to identify the temperature range in which the compounds decompose. To this purpose a Radex oven [6] was used in scanning mode (1–2 K/min). Once the onset temperature was determined from the recorded thermogram, new runs at this temperature were carried out in the same oven in isoperibolic mode to collect at varying reaction time some samples to be submitted to ecotoxicological assessments.

A sample of 0.5 g (ethylparathion) or 0.75 g (cumene hydroperoxide) was firstly used in the scanning mode test and then four additional samples – each of the same mass – were submitted to isoperibolic tests and stopped at different reaction times to recover the mixtures of decomposition products.

2.2. Ecotoxicological assessments

2.2.1. Algae bioassay

Decomposition mixture from ethyl parathion and cumene hydroperoxide samples collected at varying reaction times during the isoperibolic calorimetric runs, were dissolved in acetone to have final solutions of 20 mg/ml. Each solution was stored in the dark at 277 K. The toxicity test was based on the measurement of the growth inhibition of the green unicellular alga *Pseudokirchneriella subcapitata*, strain UTEX 1648, according to EPA test 1000.3. Algal inocula corresponding to 10,000 cells/ml from laboratory cultures in mid exponential phase were grown in 100 ml Erlenmeyer flasks containing 50 ml of Bold Basal Medium (BBM) [7] and the tested compound at different concentrations. The flasks were incubated on a shaking apparatus, at 297 ± 1 K under continuous illumination at a light intensity of 90 $\mu E\,s^{-1}\,m^{-2}$. The tests were carried out in triplicate and in axenic conditions. A series of controls containing either BBM and the algal inocula, or BBM medium, the algal inocula and the same volume of acetone added to the test flasks were also prepared. The algal growth was followed for 96 h from the addition of the compounds, either counting the cell number with a Bürker blood counting chamber or measuring the absorbance increase at 550 nm with a Secoman colorimeter. The analysis of the data was performed in the following way. In all the tests, the concentration of each tested sample which differed significantly ($P < 0.05$) from that of control, was determined by hypothesis tests. Dunnett's test, after verifying the Shapiro–Wilk's test for normality and the Hartley's test for homogeneity of variance, was used. Calculations were performed using TOXSTAT 3.0 software [8]. The concentrations that cause 50% of effect (EC_{50}) on the assessment endpoint were determined by regression using a log-logistic model or linear interpolation of means [9].

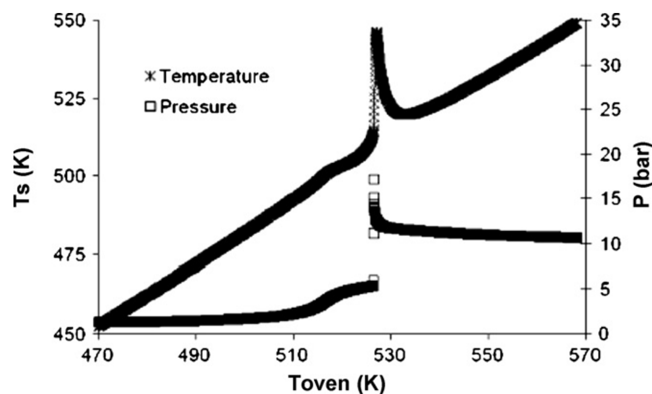

Fig. 1. Thermogram recorded under scanning conditions on an ethyl parathion sample.

2.2.2. Human Umbilical Vein Endothelial Cells bioassay

The decomposition mixtures from ethyl parathion and cumene hydroperoxide samples collected at varying reaction times during isoperibolic calorimetric runs were initially dissolved in 10 ml of DMSO. Human Umbilical Vein Endothelial Cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and grown in Endothelial Growth Medium (EGM-2) (Lonza Walkersville, MD, USA). Cell lines were maintained at 310 K in a humidified incubator containing 5% CO_2. The cells were plated on 96-well plates at a density of 2×10^3 cells per well in 100 μl of medium. Cytotoxicity was estimated by performing a reduction inhibition assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [10]. DMSO solutions containing decomposition mixtures were firstly diluted 1/10 and 1/100 in water, then 6.0 μl of each diluted solutions were added to each cell plate. After 48 h of incubation at 310 K, 10 μl of a stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in phosphate-buffered saline was added to the cells to a final concentration of 0.5 mg/ml in EGM-2 medium (final volume 100 μl). After 4 h of incubation, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution was removed and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide formazan salts were dissolved in 100 μl of 0.1N HCl in anhydrous isopropanol. Cell survival is expressed as the absorbance of blue formazan measured at 570 nm with an automatic plate reader (Victor3 Multilabel Counter, Perkin Elmer, Shelton, CT). Each experiment was performed in triplicate. A series of negative controls containing EGM-2 and 2×10^3 cells per well were prepared, as well.

3. Results and discussion

3.1. Ethyl parathion

In Fig. 1 the thermogram recorded in a scanning run by using the Radex oven at a constant heating rate of 1 K/min is shown. From this figure an “onset” temperature of about 503 K was determined. At this temperature some isoperibolic runs on new samples of ethyl parathion were subsequently performed. It is evident from the diagram (Fig. 2) that during these runs the energy generated from the thermal decomposition of the chemical substance caused a temperature increase (a maximum temperature gap of 7 K was recorded).

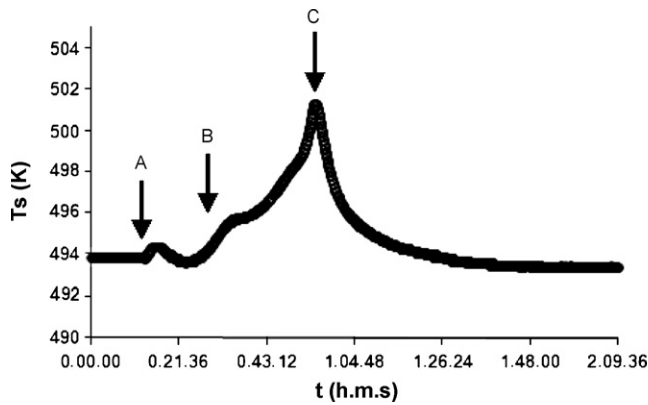

Fig. 2. Thermogram recorded under isoperibolic conditions on an ethyl parathion sample.

In the same figure, the times (A, B, C) at which single runs were stopped to recover the samples for ecotoxicological assessments are indicated.

The results obtained by testing on *P. subcapitata* the samples collected during the isoperibolic calorimetric runs are reported in Fig. 3.

P. subcapitata has an intermediate sensitivity to the sample collected at the beginning of the thermal treatment at 503 K with an EC_{50} value of 1.90 mg l^{-1}. The following two samples, corresponding to the middle and to the apex of the peak in Fig. 2, showed an EC_{50} of 0.40 and 3.9 mg l^{-1}, respectively. In a previous work [4] the toxicity of decomposition products from the thermal decomposition of ethyl parathion was assessed after two distinct heating treatments (448 and 458 K). The mixture collected after treating ethyl parathion at 458 K showed a higher toxicity to *P. subcapitata*, with an EC_{50} of 0.23 mg l^{-1} being that of untreated ethyl parathion equal to 1.0 mg l^{-1}. The experiments at 503 K carried out in the present study confirmed that the thermal treatment of ethyl parathion samples produces a mixture more toxic than the parent species. However, prolonging the treatment the toxicity decreases. Indeed, with the sample collected at the end of the calorimetric runs a reduction of inhibitory action on *P. subcapitata* growth is observed.

3.2. Cumene hydroperoxide

The same procedure was applied on some samples of cumene hydroperoxide. The thermogram obtained in a scanning run at a heating rate of 1 K/min is shown in Fig. 4. From this thermogram an onset temperature of about 393 K was derived. In Fig. 5 the thermogram recorded in an isoperibolic run at 393 K on a sample of cumene hydroperoxide is shown. The times (A, B, C, D) at which the single runs were stopped to recover the samples for ecotoxicological assessments are indicated by the arrows.

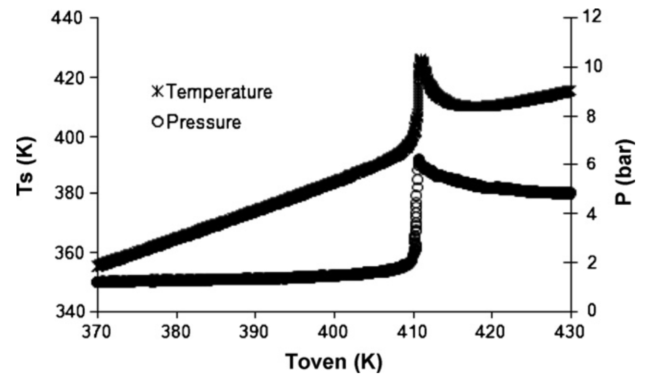

Fig. 4. Thermogram recorded under scanning conditions on a cumene hydroperoxide sample.

When they were tested on *P. subcapitata* the decomposition products originating from cumene hydroperoxide thermal decomposition at 393 K, presented EC_{50} values higher than that of the untreated compound (Fig. 6). Also in this case the obtained data are consistent with those found in the previous investigation on the same chemical compound [4].

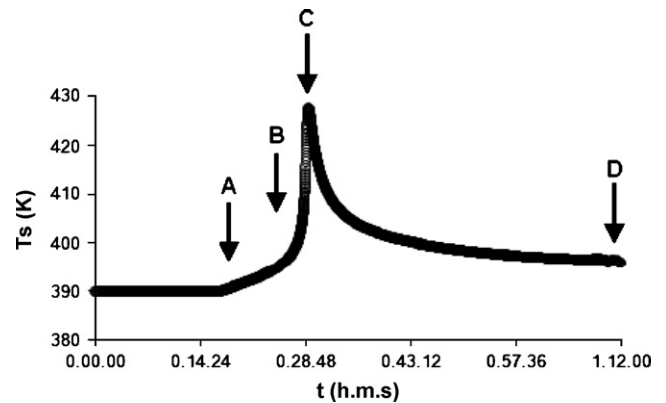

Fig. 5. Thermogram recorded under isoperibolic conditions on a cumene hydroperoxide sample.

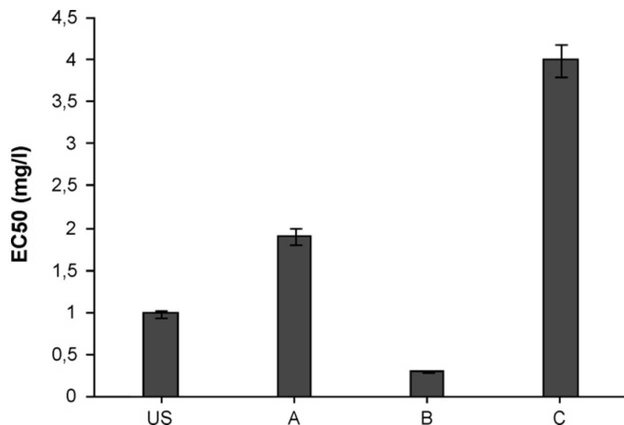

Fig. 3. Toxicity of ethyl parathion (US) and its thermal decomposition products (A, B, and C) towards *Pseudokirchneriella subcapitata* algal strain

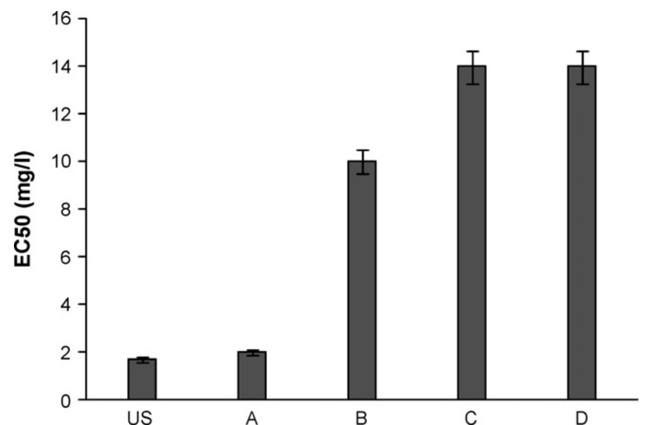

Fig. 6. Toxicity of cumene hydroperoxide (US) and its thermal decomposition products (A, B, C, and D) towards *P. subcapitata* algal strain.

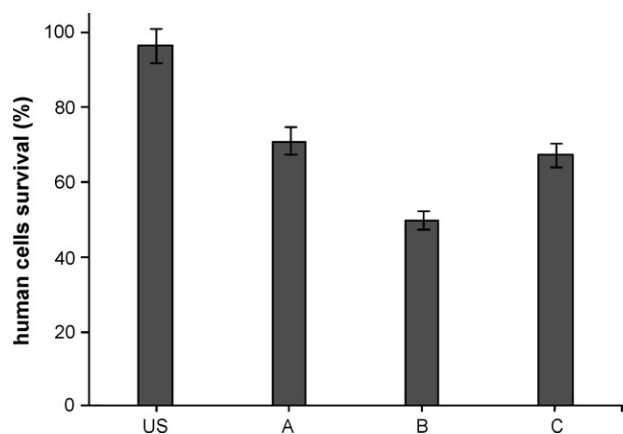

Fig. 7. HUVEC bioassay: cells survival after exposition to ethylparathion (US) and its thermal degradation products (A, B, and C).

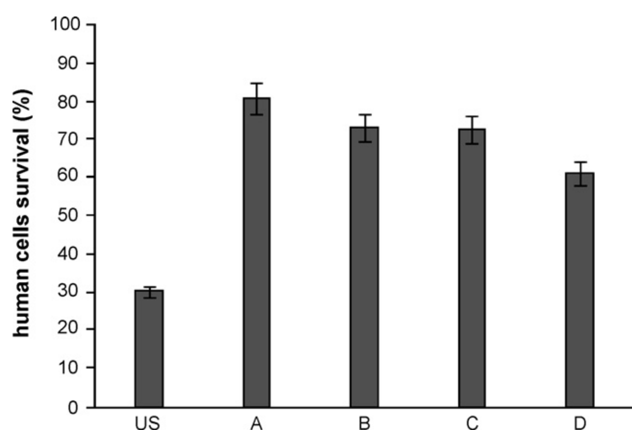

Fig. 8. HUVEC bioassay: cells survival after exposition to cumene hydroperoxide (US) and its thermal degradation products (A, B, C, and D).

3.3. Assessment on HUVEC

All the mixtures of decomposition products collected during isoperibolic runs on ethyl parathion and cumene hydroperoxide were also submitted to preliminary assessments on human cells according to the procedure described in Section 2.

The results of these tests (Fig. 7) show that ethyl parathion thermal degradation products are all more toxic than the parent compound and that in this case a reduction of the toxicity is observed towards the end of the run, as well.

Toxicity assay on the decomposition products of cumene hydroperoxide shows that these species are less toxic than the parent compound (Fig. 8). The results obtained on human cells (in the case of both ethyl parathion and cumene hydroperoxide) are thus consistent with those found on algae.

It is noteworthy to observe that the obtainment of data on human cells makes possible to foresee also the existence of some hazards for man due to the species formed during the thermal decomposition of a chemical substance. Moreover, this result is found at low cost, and avoiding the ethical concerns related to the animal testing [11,12].

4. Conclusions

In this study a new procedure to collect the intermediates and products coming from the thermal decomposition of organic substances – when no preliminary information of the temperature ranges of interest are available – was developed.

The results found on the thermal decomposition products of ethyl parathion and cumene hydroperoxide using the algal strain *P. subcapitata* are consistent with those obtained during a previous work in which preliminary indications on the decomposition temperature ranges were available. An extension of the investigations to the use of human cells (HUVEC) was done in the present work. A good correspondence between the responses of the two tests was observed although further experiments are necessary to confirm this trend. It is generally acknowledged that *in vitro* tests are less sensitive than *in vivo* cytotoxic assays, but in the case of both ethyl parathion and cumene hydroperoxide, human cell lines showed a sufficient sensitivity if compared with the alga *P. subcapitata*, and for the former the results indicate that cell lines were more sensitive to all degradation products than the algal cells. However, other tests on aquatic organisms (Crustaceans, Fish) belonging to different trophic levels and on different human cell cultures need to be considered for a full evaluation of the hazardousness of the decomposition mixtures, as requested by the current EU Guidelines.

Moreover, it is worthy to observe that since the real temperature–time profile followed by a chemical compound during an accidental thermal decomposition may result too complex to be simulated by a single calorimetric run, no conclusive remarks can be drawn taking into account the toxicity of only the decomposition products recovered in the present work. Further investigations are required using different experimental techniques (adiabatic calorimetry, thermogravimetry) to recover the mixtures of decomposition products and compare the results of the toxicity tests with those obtained in this work.

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