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High-performance liquid chromatography in stability studies of an organophosphorus insecticide in free form and after formulation as emulsifiable concentrate

Response surface design correlation of kinetic data and parameters

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Abstract

DCPE [α -(diethoxyphosphinoximino)dicyclopropylmethane] is an organophosphorus compound with good insecticidal activity. It is a yellow oily liquid, poorly soluble and unstable in aqueous media. The development of a useful, safe formulation, miscible with water, easy to use and retaining its activity was examined. First, the kinetics of the hydrolysis of DCPE were studied in various buffer solutions over the pH range 3–11 at 20–80°C by a reversed-phase HPLC method. Then the response surface methodology was used to investigate how the hydrolysis rate constant (response) was affected by temperature and pH (variables) over some specified region, and was described step by step. The degradation product of DCPE was isolated by extraction and identified by IR and ^1H NMR spectrometry. Also, the activation energy of DCPE hydrolysis was calculated from the dependence of the observed hydrolysis rates (k_{obs}) on temperature at constant pH. Finally, 30 emulsifiable concentrate formulations of DCPE were prepared by choosing different combinations of oil phases and emulsifiers. Five of the resulting emulsions retained their physical stability and were examined further for chemical stability of the active compound (DCPE) at different temperatures.

1. Introduction

α -(Diethoxyphosphinoximino)dicyclopropylmethane (DCPE) (I, Fig. 1), is a powerful insecticide that was synthesized in our laboratory [1]. It is a promising compound showing significant activity and structural originality owing to the presence and position of the two cyclo-

propane rings. It is a cholinesterase inhibitor and has high mammalian toxicity orally.

Organophosphorus compounds are widely used as insecticides and often acaricides [2]. Although they all have the basic property of inhibiting the action of cholinesterase at the ganglia, they enter the insect's body by different routes depending on the properties of the particular compound. Thus the group contains contact, stomach and systemic insecticides. After application, the organophosphorus compounds

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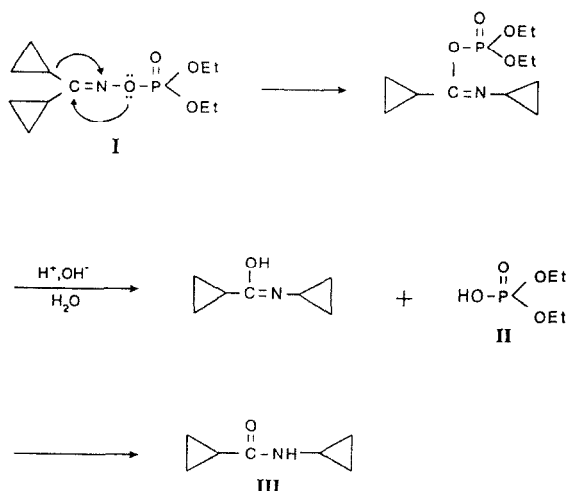


Fig. 1. Structure of I and its reactions.

are converted into their degradation products by the effect of the environment. Hydrolysis is the most common reaction that leads to detoxification. The products formed are usually less toxic than the initial pesticides. Between application of the active compounds and permitting access to the area (harvesting of the crop or other appropriate procedures), sufficient time must elapse for the active compound to be eliminated to a level considered to be safe. The shorter the time of detoxification, the safer the product is. DCPE in various emulsions has a detoxification period of 10–40 h, as shown by stability studies.

The decrease in the efficacy of pesticides depends on the environmental humidity and temperature, and therefore the degradation pathway and the decomposition kinetics are of major interest. In this study, a high-performance liquid-chromatographic (HPLC) method was developed for monitoring the degradation reactions. Further, the separation of DCPE from its degradation products and stability studies under different conditions were performed by the same method. The main degradation product was obtained following an experiment under stressed conditions and was isolated by extraction.

After the kinetic studies had been performed in buffer solutions under different conditions, in an attempt to stabilize DCPE, different emulsifiable concentrate (EC) formulations were pre-

pared. Most pesticides are formulated as ECs [3] and must retain their efficiency for at least 2 years under normal storage conditions. In all prepared EC formulations, DCPE remained unchanged for more than 1.5 years. By mixing the EC formulation with water an emulsion is prepared [oil in water (O/W) emulsion] and is applied in the form of a spray. An emulsion consists of two immiscible liquid phases, one of which is finely subdivided and uniformly dispersed in the other. Emulsions are thermodynamically unstable as a result of the excess free energy associated with the surface of the droplet. To minimize this effect a third component, the emulsifier, is added to the system to improve its stability. In this study, the effect of nine emulsifiers in 30 different EC formulations was tested.

Finally, the relationships between the degradation rate k of DCPE and the factors pH and T temperature were examined by using response surface methodology (RSM) techniques [4] on a personal computer (PC). According to this method we can build empirical models that use sequential experimentation techniques to survey a domain of interest and to focus on the most important variables and their effects. In other words, RSM is a collection of mathematical and statistical techniques that are useful for the modelling and analysis of problems in which a response of interest (here the rate constant k) is influenced by several variables (here temperature and pH). The objective is to find how the particular response is affected by the set of variables over a specified range.

2. Experimental

2.1. Materials

DCPE was synthesized and purified in our laboratory. Berol emulsifiers [5] were obtained from Berol Kemi (Stenungsund, Sweden). All other chemicals, including buffer components, were of analytical-reagent grade except methanol and acetonitrile, which were of HPLC grade.

Water was deionized and doubly distilled using a Millipore Milli-Q Plus water-purification system.

2.2. High-performance liquid chromatography

The HPLC assays for DCPE were performed on a Waters (Milford MA, USA) chromatographic system consisting of a pump (Model 590) and a multi-wavelength UV detector (Lamda-Max, Model 481). The separation of DCPE from its degradation product was performed on a 10- μm reversed-phase, $\mu\text{Bondapak C}_{18}$ column (300×3.9 mm I.D.) (Waters). A flow-rate of 1.5 ml min^{-1} was maintained and the effluent was monitored at 215 nm with a detector sensitivity of 0.05 AUFS. The sample volume was 20 μl (Rheodyne Model 717S injector fitted with a 20 - μl loop). In assay 1 the mobile phase was acetonitrile–water–methanol (40:50:10) and acetanilide ($\text{CH}_3\text{CONHC}_6\text{H}_5$) was used as an internal standard at a concentration of 4 mg ml^{-1} . In assay 2 the mobile phase was acetonitrile–water (55:45) and benzophenone (C_6H_5) $_2\text{CO}$ was used as an internal standard at a concentration of 0.33 mg ml^{-1} (because acetanilide is insoluble in EC formulations and therefore changing the mobile phase composition produces better separation). In both assays the mobile phases were vacuum filtered through a 0.45 - μm pore-size nylon membrane filter (Sartolon, Germany) prior to use. Quantification was performed by peak-height integration (Waters, Baseline-810 integration system for PC).

2.3. Kinetics

Citrate, phosphate and carbonate buffer solutions of various pH were used in all experiments. These buffer solutions were 0.1 M with respect to citrate, phosphate and carbonate, respectively, and were adjusted to a total ionic strength of 0.5 M with potassium chloride. The reaction was initiated by adding 13.1 mg of DCPE to 50 -ml volumetric flasks and diluting to volume with buffer solution to give an initial DCPE concentration of 1 mM (DCPE $M_r = 262$). A stock standard solution of acetanilide (4 mg ml^{-1}) was

prepared and stored in a refrigerator until further use as an internal standard. The reaction flasks were immediately placed in a water-bath at constant temperature (Tempette Thermo-regulator TE8D, Techne).

At appropriate time intervals, 450 - μl aliquots of the reaction mixture were removed from the flask and immediately added to a test-tube containing 50 μl of the internal standard stock standard solution. The samples were analysed for remaining DCPE by HPLC assay 1. The first-order rate constant for the disappearance of DCPE (k_{obs}) was determined from the slopes of linear plots. The logarithm of the percentage of remaining DCPE was plotted against time.

For the isolation of the degradation product of DCPE, 2 g (7.6 mM) of DCPE were dissolved in 20 ml of phosphate buffer solution (pH 7), the mixture was heated for 48 h on a water-bath at 80°C , then allowed to cool at room temperature and extracted repeatedly with diethyl ether. The combined ethereal extracts were washed with water and after drying over anhydrous Na_2SO_4 were filtered to leave an oil. The degradation product was obtained from this residue by crystallization with pentane. Recrystallization from diethyl ether–pentane gave the pure product in crystalline form. The structure of this product was investigated by ^1H NMR and IR spectrometry and is shown in Fig. 1 (amide III).

2.4. Preparation of emulsions

The typical process for the formulation of DCPE as an EC was as follows. First the oil phases (either an anhydrous non-polar solvent, such as xylene, or an oil, such as paraffin oil, soya oil, isopropyl myristate or isopropyl palmitate) were selected in which the active ingredient (DCPE) was dissolved. Then the emulsifiers were selected (Table 1). These emulsifiers are surfactants with high HLB (hydrophilic–lipophilic balance) values (>10), since O/W emulsions, such as DCPE emulsions, are favoured. The HLB value of the oil phase must be the same as that value of the emulsifiers. Also fundamental to the utility of the HLB concept is that the HLB values are algebraically additive. The emulsifiers

Table 1
Emulsifiers used for DCPE EC formulations and their HLB values

Emulsifier	HLB	Application
Berol 910	13.3	Non-ionic, can be used in formulations of insecticides
Berol 948	16.0	Non-ionic, can be used especially in formulations of organophosphates
Berol 930	14.7	Blend of anionic and non-ionic, can be used as "universal combination emulsifier" for many types of insecticides
Berol 938	13.4	As Berol 930
Berol 977	16.0	Non-ionic, can be recommended for use in formulations containing organophosphates
Berol 987	16.7	As Berol 977
Tween 20	16.7	Non-ionic, promotes O/W emulsions
Tween 80	15.0	As Tween 20
Span 80	4.3	Non-ionic, used only in combination with Tweens, for appropriate HLB values

were therefore used in combinations as better emulsions were usually obtained. Thirty EC formulations were prepared which consisted of the oil phase, the blend of emulsifiers and DCPE. The usual proportions before the emulsification were approximately 50% of DCPE, 45% of oil solvent and 5% of emulsifiers. Finally, the emulsification occurred on adding the continuous phase (water) gradually to the disperse phase. Generally, depending on the intended use, the water is added to the EC at rates varying from 1 part of concentrate to 4 or as much as 100 parts of water [6]. In this study the DCPE concentration in the final emulsions was approximately 5%. The emulsions first were examined for physical stability. The stable emulsions were further examined for the chemical stability of the active ingredient (DCPE) at various temperatures by RP-HPLC.

2.5. Response surface design

In the case of DCPE, the simple design in the 3^k system was used, which is the 3^2 design, with two factors, each of them at three levels. The polarity of the media was kept constant as we were interested only in the behaviour of DCPE in aqueous solutions. We refer to the three levels of the factors as low, intermediate and high. These levels will be designated by the digits 0 (low), 1 (intermediate) and 2 (high). For example, in a 3^2 design, 00 denotes the treatment

combination corresponding to factors A and B both at the low level. The treatment combination for this design is shown in Fig. 2. It is obvious that the system has $3^2 = 9$ treatment combinations and therefore there are eight degrees of freedom between these treatment combinations.

We used a statistical program [7] with strong design of experiments (DOE) capabilities to perform the calculations and to illustrate all the interactive graphics. The nine runs listed in Table 2 define a three-level response surface design in a two-factor design matrix. These nine runs were conducted in random order so as to nullify the effects of extraneous or nuisance variables. After the responses (k_{obs}) had been collected the system was ready for analysis.

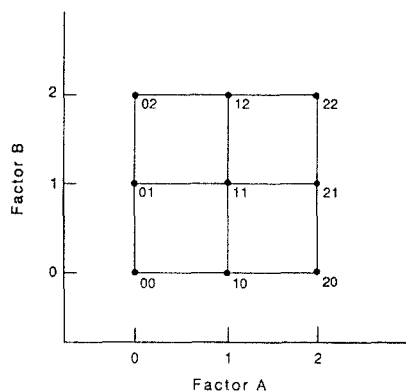


Fig. 2. Treatment combinations in a 3^2 design.

Table 2
Randomized combinations of two factors and the calculated responses

Run No.	pH	T	k
1	7	80	0.489
2	3	20	0.0075
3	11	20	0.009
4	11	80	0.451
5	7	20	0.008
6	11	50	0.131
7	7	50	0.121
8	3	80	0.511
9	3	50	0.101

2. Results and discussion

To clarify the degradation mechanism of DCPE in aqueous solutions, the degradation product was isolated by extraction. Then the degradation pathway was studied by monitoring the reaction buffer solutions using RP-HPLC.

3.1. Degradation analysis by RP-HPLC

DCPE was dissolved in various aqueous buffer solutions at various temperatures. The solutions were analysed by HPLC assay 1. Fig. 3 shows plots of the residual percentage amount of DCPE versus time in buffer solution of pH 7 at different temperatures. All the experimental

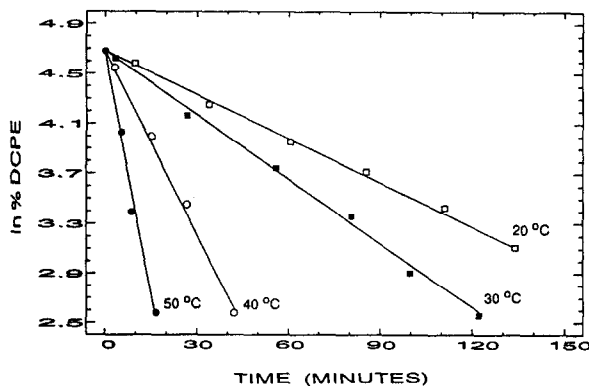


Fig. 3. Apparent first-order plots for the degradation of DCPE in buffer solution (pH=7, $\mu=0.5$ M) at various temperatures.

conditions and the rate constants for the degradation of DCPE are given in Table 2. In all instances the amide **III** was the only degradation product. This observation leads to the conclusion that the degradation pathway was due to a Beckman rearrangement [8] as shown in Fig. 1.

To calculate the activation energy for the degradation of DCPE in buffer solutions, the temperature dependence of the rate constants was obtained graphically according to the Arrhenius equation (Eq. 1). From the slope of the linear Arrhenius plot the activation energy was calculated to be 17.75 kcal mol⁻¹ ($r=0.99$).

$$\ln k = -\frac{E_a}{R} \cdot \frac{1}{T} + \ln A \quad (1)$$

3.2. Formulation of DCPE as EC

Anhydrous xylene (one of the commonest organic solvents in EC formulations) was used in this study. The emulsifiers used are specific for pesticides and have the trade-name Berol.

As there is no general rule for preparing a stable emulsion other than through experimentation, 30 different formulations were examined for physical and chemical stability. These formulations were all the possible combinations between the oil phases and the emulsifiers used in this study. Some of them were stored in an oven at 40°C and the chemical stability of DCPE was determined by HPLC assay 2. A sample of known concentration of each formulation was injected into the HPLC system and the DCPE content was calculated from a calibration graph. These experiments were repeated many times for all formulations in an oven and for a period of 18 months. The DCPE content of the formulations was found to be unchanged.

After the confirmation that DCPE remained unchanged in EC formulations, emulsification took place on adding the appropriate amount of water. In some instances spontaneous emulsions were formed. After shaking, the emulsions were left for 24 h. In some, sedimentation (the phenomenon of downward movement of dispersed droplets) appeared. The droplets were redispersed by shaking, as they retained their in-

dividuality. In others coalescence (the phenomenon of the ultimate separation of the two immiscible phases) occurred. Coalescence is irreversible and the emulsion could not be redispersed.

Finally, five emulsions retained their physical stability and these were selected to be examined further for the chemical stability of DCPE by HPLC assay 1 at 25 and 40°C. The compositions of these stable emulsions are given in Table 3. Fig. 4 shows the apparent first-order plots for the degradation of DCPE in stable emulsions at 25 and 40°C. It is evident from the plots that DCPE appeared to have the highest stability in emulsion No. 1. Rate constants and half-lives for the degradation of DCPE in stable emulsions are given in Table 4.

It is concluded from the study of physical and chemical stability that the best results were achieved by the use of non-ionic emulsifiers [9]. This may be due to the fact that non-ionics lack strong electrical charges and have high resistance to pH changes, electrolytes and polyvalent inorganic cations. Perhaps these emulsifiers readily form a film around each droplet of dispersed material. The main purpose of this film, which is usually a monolayer, is to form a barrier which prevents the coalescence of droplets that come into contact with one another. The film possesses some degree of surface elasticity and preserves its integrity, as results from previous physical stability studies.

In addition, the film and the helical conformation of the hydrophilic chains probably function by hindering the close approach of DCPE drop-

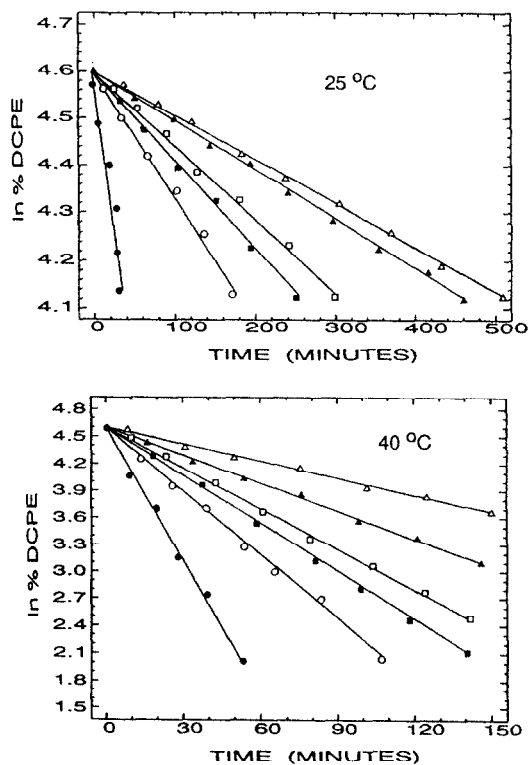


Fig. 4. Apparent first-order plots for the degradation of DCPE in stable emulsions at 25 and 40°C. ● = Aqueous solution; △ = formulation No. 1; ▲ = No. 2; □ = No. 3; ■ = No. 4; ○ = No. 5.

lets to water, thus decelerating degradation. Commonly, non-ionic emulsifiers, such as Berols and Tweens, consist of polyoxyethylene chains that extend outwards into the aqueous medium

Table 3

Compositions of the DCPE formulations that retain their physical and chemical stability

Formulation No.	Composition (g)
1	DCPE (2.1) + xylene (2.6) + Berol 977 (0.12) + Berol 987 (0.13)
2	DCPE (2.1) + xylene (2.5) + Berol 910 (0.13) + Berol 948 (0.13)
3	DCPE (2) + xylene (2.7) + Berol 930 (0.12) + Berol 938 (0.12)
4	DCPE (2) + soya oil (2.7) + Berol 930 (0.12) + Berol 938 (0.12)
5	DCPE (2) + isopropylmyristate (2.5) + Tween 80 (0.18) + Span 80 (0.07)

Table 4

Kinetic data for the chemical stability studies of DCPE in stable emulsions at 25 and 40°C

Formulation No.	k		$t_{1/2}$		r	
	25°C	40°C	25°C	40°C	25°C	40°C
Aqueous solution	$13 \cdot 10^{-3}$	$54 \cdot 10^{-3}$	51 min	12.85 min	0.993	0.995
1	$9.9 \cdot 10^{-4}$	$6.1 \cdot 10^{-3}$	11.7 h	113.6 min	0.983	0.991
2	$1.6 \cdot 10^{-3}$	$12.7 \cdot 10^{-3}$	7 h	54.6 min	0.993	0.988
3	$2 \cdot 10^{-3}$	$13.9 \cdot 10^{-3}$	5.8 h	49.9 min	0.988	0.985
4	$2.6 \cdot 10^{-3}$	$22 \cdot 10^{-3}$	4.45 h	31.5 min	0.985	0.986
5	$3.9 \cdot 10^{-3}$	$25.2 \cdot 10^{-3}$	2.96 h	27.5 min	0.994	0.991

and hydrocarbon chains that orientate in the non-polar environment.

3.3. Determination of temperature and pH effects on the degradation rate constant

Check of main effects and interactions

After completing the runs in the order listed in Table 2, the rate constants were calculated by HPLC assay 1. To determine which effects (linear or quadratic) of each factor or their interaction are significant, a two-way analysis of variance (ANOVA) was performed (Table 5). As $F_{0.05,1,3} = 10.13$ and $F_{AB} = MS_{AB} / MS_{\text{total error}} = 2.38$, we conclude that there is not a significant interaction between temperature and pH and the

only significant effect is temperature at a probability of $P = 0.05$ (5%).

Performance of a Pareto analysis

To assist in interpreting the results of this experiment, it is helpful to construct a standardized Pareto chart [10], which is similar to a Pareto chart, except that it shows the effect divided by its standard error. The chart includes a vertical line at the critical t -value for $\alpha = 0.05$ (Fig. 5). An effect that exceeds the vertical line may be considered significant. Table 6 shows the main effects of the factors temperature and pH (factors A and B), the quadratic effects of the factors (AA and BB) and the interaction between the two factors (AB). The last column in

Table 5

Analysis of variance for the examined design

Effect	Sum of squares	DF ^a	Mean square	F-ratio	P value
A (pH)	0.00013538	1	0.0001354	0.34	0.6064
B (T)	0.33915037	1	0.3391504	852.54	0.0001
AB	0.00094556	1	0.0009456	2.38	0.2208
AA	0.00003612	1	0.0000361	0.09	0.7858
BB	0.03289613	1	0.0328961	82.69	0.0028
Total error	0.00119344	3	0.0003978		
Total (corr.)	0.37435700	8			
R^2 0.996812			R^2 (adjusted for DF) 0.991499		

^a Degrees of freedom.

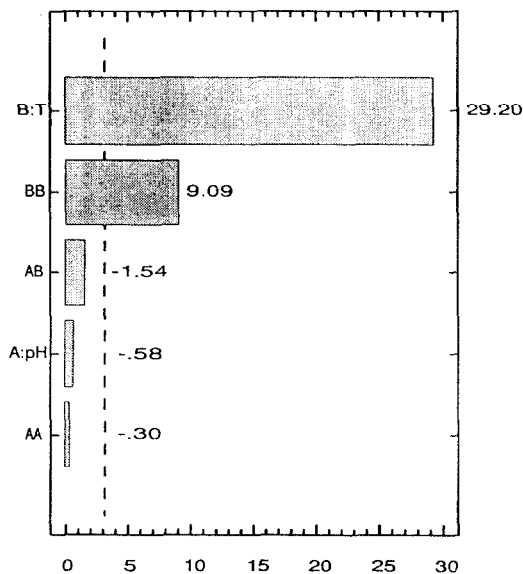


Fig. 5. Pareto chart for the factors effect on k_{obs} .

Table 6 displays the standard error estimated from the total error with three degrees of freedom. These results show that the linear effect of B is considerably larger than both the linear effect of A and the second-order effects (AB, AA, BB).

Generation of a predictive model

Using the significant main and interaction effects, a model that expresses rate constant as a function of temperature and pH was developed. This model can predict rate constants in the given region of the operating conditions. According to Table 6 and the Pareto chart (Fig. 5), we consider as significant the main and quadratic effects of temperature. The model was developed into PC software [7] that performs

Table 6
Estimated effects of the factors on k_{obs}

Average	0.1205 ± 0.0148663
A (pH)	$-9.5 \cdot 10^{-3} \pm 0.0162852$
B (T)	0.4755 ± 0.0162852
AB	-0.03075 ± 0.0199452
AA	$-8.5 \cdot 10^{-3} \pm 0.0282068$
BB	0.2565 ± 0.0282068

multiple regression analysis based on the following equation:

$$Y = C + B_1 \cdot \text{temperature} + B_2 \cdot \text{temperature}^2$$

where Y = predicted value of rate constant; C = intercept in regression equation; B_1 = coefficient for temperature; B_2 = coefficient for the quadratic effect of temperature.

This model does not include the other interaction terms because their effects are negligible and would not add to the model's predictive ability. Using the estimated constant and coefficient, the multiple regression analysis generates the prediction model:

$$Y = 0.078 - 0.006T + 0.000143T^2 (r = 0.998).$$

4. Conclusions

DCPE is a pesticide unstable in aqueous solutions. Its stability in aqueous solutions can be expressed with a predictive model by the use of a simple experimental design. Fortunately, its stability is increased and retains its activity after its formulation as an EC. The LD_{50} for DCPE has been determined in a previous study [11] and was found to be 10.86 mg kg^{-1} for male and 7.86 mg kg^{-1} for female Wistar rats after oral administration. Insecticidal studies [1] for contact toxicity have also been performed with four different insects: *Ceratitis capitata*, *Ectomyelois ceratonia*, *Musca domestica* and *Plodia interpunctella*. These studies support the conclusions that DCPE can probably be used either as a stomach poison or as a contact insecticide. The variation in the half-life of DCPE in emulsions gives the opportunity for the applicator to select the preferred formulation.

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