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ANALYSIS OF ORGANOPHOSPHORUS INSECTICIDES IN BIOLOGICAL SAMPLES BY SELECTIVE ION MONITORING GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

Gas chromatography with chemical ionization or electron impact mass spectrometry and selected ion monitoring provided a simple and sensitive method for measuring organophosphorus insecticides. Chemical ionization produced higher-mass ions which might increase the selectivity and sensitivity of the assay. The recovery of organophosphates from saline and urine was greater than 75%. The recovery of these compounds from plasma was less than the saline because of the binding of insecticides to plasma protein. Insecticides with lower LD_{50} values showed lower recovery from plasma than organophosphates with higher LD_{50} values.

INTRODUCTION

Organophosphorus insecticides are widely used to control household, agricultural and forestry pests. The application of insecticides for controlling insect pests has recently shown a tendency towards combination of two or more insecticides for diverse reasons, such as for increasing their effectiveness, for controlling more than one pest at a time, and for increasing the storage life of insecticides. The insecticidal property and the mammalian toxicity of organophosphorus insecticides are believed to be because of their inhibition of acetylcholinesterase[1]. Other esterases present in blood and tissue of mammals are also inhibited by the organophosphates[2,3]. The reaction of organophosphates with esterases (other than acetylcholinesterase) may serve to reduce the amount of free organophosphates available for inactivation of acetylcholinesterase[4]. Along with esterases, mammalian tissue also contains certain phosphorylphosphatase activity which hydrolyses and inactivates organophosphorus compounds[5–7]. The amount of free organophosphates in tissues is further reduced by these enzymes. As a result, the amount of organophosphates excreted in the urine will be considerably less than the amount of exposure. These factors may also affect the amount of active residue if organisms are exposed to multiple organophosphorus insecticides. Therefore, a simple and sensitive method for the quantification of or-

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ganophosphorus insecticides is required to monitor animals exposed to several organophosphorus insecticides. Several gas chromatographic (GC) methods have been developed for the analysis of insecticides[8–12]. The GC methods are time consuming, since they always require clean-up of crude extract by either silica gel or charcoal columns. The purpose of this investigation was to develop a highly sensitive, simple and rapid assay for determining the levels of organophosphorus insecticides in aqueous and biological samples by using a chemical ionization (CI) and electron impact (EI) gas chromatography–mass spectrometry (GC–MS). Unlike GC methods, this method does not require the cleanup of crude extract.

EXPERIMENTAL

Materials

The GC–MS system used was a Hewlett-Packard Model 5987 with electron impact (EI) and chemical ionization (CI), and an integral gas chromatograph. The column used in the analysis of organophosphorus insecticides was a DB-5 fused-silica column (25 m) purchased from PolyScience. Other chemicals were purchased from Sigma.

Extraction procedure

Plasma. Plasma samples containing various amounts of organophosphate mixture (10–200 ng/ml) were mixed with ethyl acetate (5.0 ml/ml plasma) and rotoracked (50 rpm) for 15 min. The samples were centrifuged at 1500 *g* for 5 min at 4°C. The organic layer was separated into another tube. The aqueous layer was extracted twice with ethyl acetate and the organic layer was pooled with the previous ethyl acetate extract. The organic layer was dried at 50°C under nitrogen and the dried residue was redissolved in 100 μl of ethyl acetate. A 1.0-μl volume of extract was injected directly into the GC–MS system. The recovery from plasma samples was determined by comparing the amount of organophosphates added and the amount recovered. The extraction efficiency of insecticides was determined by a double extraction method described by Singh *et al.*[4]. Plasma samples (containing 100 ng/ml of organophosphate mixture) were extracted with ethyl acetate as described above. The aqueous and organic layers were separated. The organic layer was dried and subjected to GC–MS analysis. The aqueous layer was extracted again with ethyl acetate. The amount of organophosphates present in the second ethyl acetate extract was determined. It was consistently found that the second extraction contained an additional 20% of the free extractable organophosphates. These observations indicate that *ca.* 75–80% of the free extractable organophosphates were extracted by the first ethyl acetate extraction.

Urine. Urine samples were mixed with various amounts of organophosphate mixture (10–40 ng/ml). The pH of each sample was adjusted to 7.4 with phosphate buffer (1.0 *M*). Urine samples were centrifuged at 1000 *g* for 30 min. Clear samples were transferred into another tube, mixed with ethyl acetate (10 ml/ml urine), and rotoracked for 15 min. Samples were centrifuged for 5 min, the organic layer was transfered into another test tube, and dried at 50°C under nitrogen. The dried residue was redissolved in 100 μl of ethyl acetate and 1.0 μl of extract was injected directly into the GC–MS system. Recovery was determined by comparing the amount of organophosphates added and the amount recovered after extraction. Extraction ef-

ficiency was determined by the double extraction method described for plasma.

Saline. The procedure for the extraction of organophosphorus insecticides from saline or other aqueous solutions was similar to that for urine.

GC–MS conditions

The following GC conditions were used: inlet temperature, 200°C; initial oven temperature, 70°C; oven temperature program, isothermal at 70°C for 1.0 min, then increasing at 10°C/min to 280°C; run time, 30 min. For electron impact ionization the mass spectrometer's source pressure was $4.5 \cdot 10^{-6}$ torr, the source temperature was 200°C, and the electron energy was 70 eV. For chemical ionization using methane as the reagent gas, the source pressure was $2.0 \cdot 10^{-4}$ torr, the source temperature was 150°C, and the electron energy was 200 eV. Injections into the GC inlet were performed using Hamilton microliter syringes. The concentrations employed in generating the mass spectrum of each organophosphate were from 10 to 40 ng/μl.

Quantitative analysis

To establish a standard curve, known amounts of organophosphorus insecticides, ranging from 10 to 50 nmol/ml, were added to 1 ml of ethyl acetate, and 1.0 μl of the various concentrations were injected into the GC–MS system. The integrated areas of the peak were directly proportional to the amount of organophosphates injected upto a concentration of 250 nmol/ml. A standard curve was drawn by plotting to concentration of standards at the x axis, and the integrated peak area at the y axis. The concentration of organophosphates in various samples was determined by using a linear regression program which compared the integrated area of the sample with that of the standard curve.

RESULTS AND DISCUSSION

Thirteen insecticides, ranging in LD_{50} 2–1300 mg/kg, were selected for this study. The three groups represented in these insecticides are: (i) phosphorodithioates (Phorate, Cygon, Guthion, Di-syston, Malathion, Ethion, and Zolone); (ii) phosphorothionates (Diazinon, Ronnel and Co-ral); and (iii) phosphates (DVVP, Phosdrin, and Naled) (Fig. 1).

EI ionization of organophosphorus insecticides

The ions formed from these insecticides by EI ionization are shown in Fig.

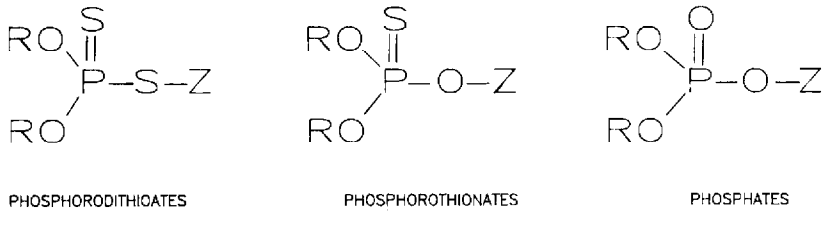
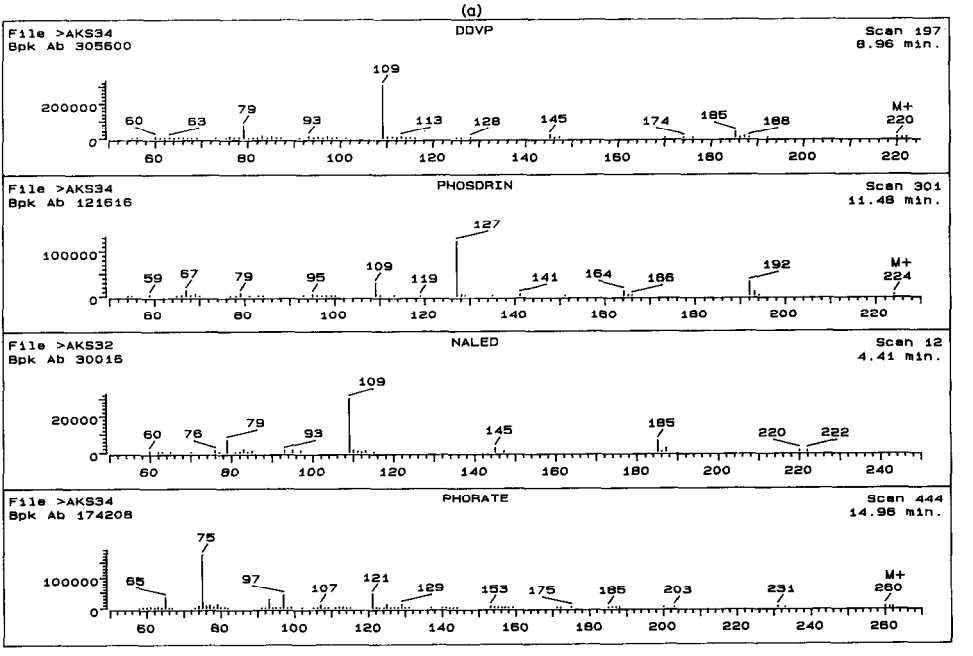


Fig. 1. Basic structures of the three groups of organophosphorus insecticides used in this study.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200000
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0
60 80 100 120 140 160 180 200 220
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Bpk Ab 121616
PHOSDRIN
Scan 301
11.48 min.
127
100000
59 67 79 95 109 119 141 164 186 192 M+ 224
0
60 80 100 120 140 160 180 200 220
File >AKS32
Bpk Ab 30016
NALED
Scan 12
4.41 min.
109
20000
60 76 79 93 145 185 220 222
0
60 80 100 120 140 160 180 200 220 240
File >AKS34
Bpk Ab 174208
PHORATE
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75
100000
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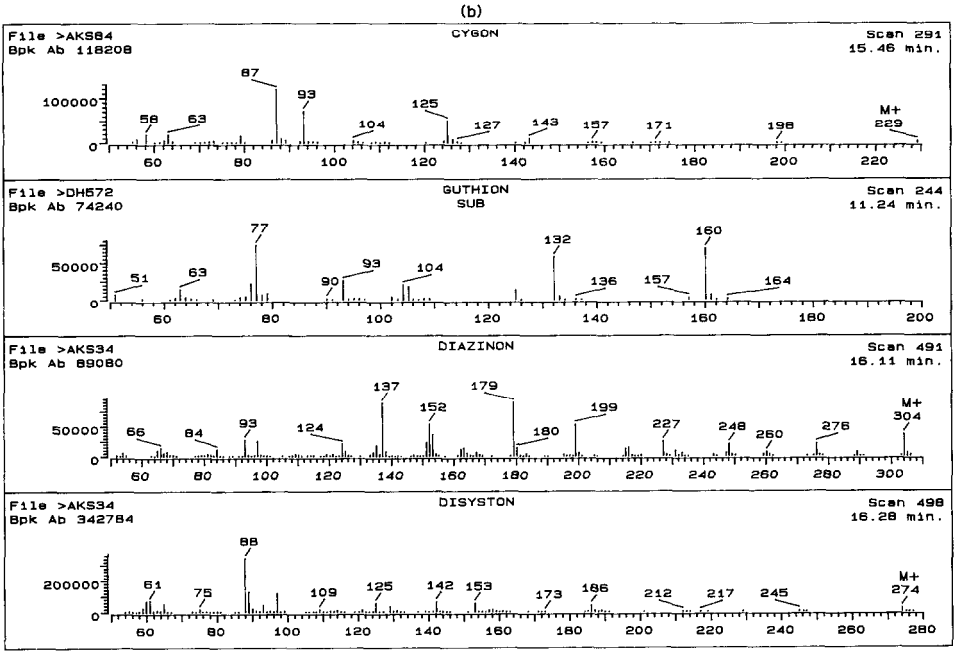
0
60 80 100 120 140 160 180 200 220 240 260


(b)
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CYGON
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87
100000
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0
60 80 100 120 140 160 180 200 220
File >DH572
Bpk Ab 74240
GUTHION
SUB
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11.24 min.
77
50000
51 63 90 93 104 132 136 157 160 164
0
60 80 100 120 140 160 180 200
File >AKS34
Bpk Ab 89080
DIAZINON
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137
179
50000
66 84 93 124 152 180 199 227 248 260 276 M+ 304
0
60 80 100 120 140 160 180 200 220 240 260 280 300
File >AKS34
Bpk Ab 342784
DISYSTON
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200000
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0
60 80 100 120 140 160 180 200 220 240 260 280

Fig. 2.

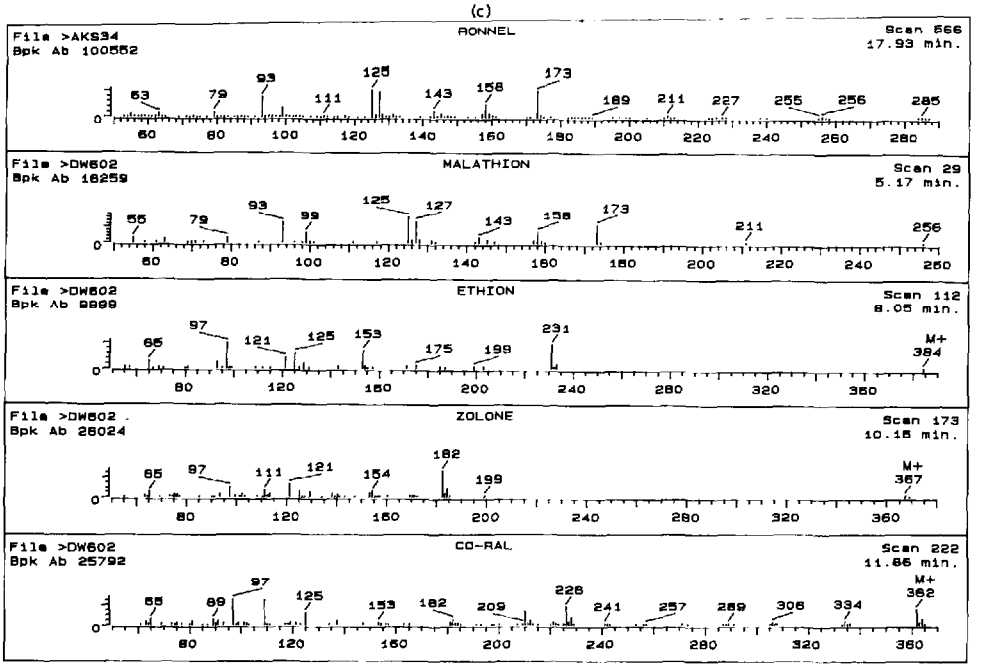


Fig. 2. EI mass fragmentation patterns of (a) DVVP, Phosdrin, Naled and Phorate; (b) Cygon, Guthion, Diazinon and Di-syston; (c) Ronell, Malathion, Ethion, Zolone and Co-ral.

2a–c. These ions are produced by many processes, such as rearrangement, alkyl and hydrogen migration, and alpha- and beta-cleavage[13].

The ion *m/z* 77 for Guthion represents a phenyl ion formed through the fragmentation of the alkyl tail and subsequent hydrogen migration. The ion of *m/z* 87 for Cygon is thought to be N-methylthioacetonitrile $[(SCH_2CNCH_3)^+]$ formed through both alpha-cleavage and subsequent dehydration. In Di-syston, rearrangement of the alkyl tail yields the tetrahydrofuran ion of *m/z* 88 $[(C_4H_8S)^+]$. The ion of *m/z* 97 is characteristic of both phosphorodithioates and phosphorothionates formed through the Quayle rearrangement $\{[(HO)_2PS]^+\}$[14]. An ion common to the phosphates is *m/z* 109 $\{[(CH_3O)_2PO]^+\}$. Ion *m/z* 125 is the thio analogue of *m/z* 109$\{[(CH_3O)_2PS]^+\}$ and is found in the spectra of Cygon, Ronnel, Malathion, and Co-ral. Double hydrogen migration is postulated for the *m/z* 127 ion found in Phosdrin $\{[(CH_3O)_2(OH)_2]^+\}$ while the *m/z* 137 ion of Diazinon and Co-ral is formed through simple cleavage $\{[(C_2H_5O)_2PO]^+\}$[13]. Characteristic of both phosphorodithioates and phosphorothionates with ethoxy groups is ion *m/z* 153 $\{[(C_2H_5O)_2PS]^+\}$ formed through simple cleavage and found in the spectra of Phorate, Diazinon, Disyston, Ethion, Zolone, and Co-ral[15,16]. The base peak in the spectra of Guthion is *m/z* 160 which represents the alkyl group $[C_8H_6N_3O]^+$ as does the base peak of Malathion $\{[C_8H_{13}O_4]^+$, *m/z* 173$\}$, both the result of beta-cleavage. Ion *m/z* 179 for Diazinon presents a more complex situation where an ethyl migration to the alkyl group following beta-cleavage is proposed $\{[C_{10}H_{15}ON_2]^+\}$. Naled and DDVP both exhibit ion *m/z* 185 from the same partial fragmentation of the alkyl tail

$\{[(CH_3O)_2POOCHCCl]^+\}$. Phorate and Ethion are similar in sharing a *m/z* 231 ion of identical structure through the same process $\{[(C_2H_5O)_2PSSCH_2S]^+\}$. Phorate's molecular ion *m/z* 260, Ronnel's M-Cl ion *m/z* 285, and Co-ral's molecular ion *m/z* 362 were all present in sufficient abundance.

Chemical ionization of organophosphorus insecticides

Fig. 3a–c show the CI mass spectra of 13 organophosphorus insecticides. The chemical ionization process is a softer ionization which produces higher-mass ions than EI ionization[17–20]. Holmstead and Casida[21] obtained significantly greater abundances of [M + 1] and [M − 1] ions in CI as compared to EI ionization. We observed that the [M + 1] ion was the most intense ion in the spectra of DVVP, Phorate, Cygon, Diazinon, and Co-ral. The abundances of [M + 1] ions were 50–75% in the spectra of Phosdrin, Di-syston, Malathion and Ethion. The spectra of these insecticides also indicated that the ion which was present in highest abundance was M − 31 (loss of $-OCH_3$ group) for Phosdrin, M − 61 for Di-syston, and M − 185 [loss of $-SP(S)(OC_2H_5)_2$] for Ethion. The abundances of the [M + 1] ion for Naled, Guthion, and Zolone was less than 10%. The *m/z* 160 ion was present in highest abundance in the spectra of Guthion, this represents the alkyl tail of the molecule. Ions at *m/z* 221 and 223 for Naled were formed by the loss of Br, and the ion at *m/z* 184 for Zolone was formed by the loss of $^+CH_3–S–C_2H_5$ from the molecule. Diazinon and Ronell produced the [M + 29] ion which represent the addition of C_2H_5 ion to the insecticide molecule.

Selection of ions for the analysis of organophosphorus insecticides

The EI and CI ions which are present in high abundance for the thirteen insecticides are listed in Table I. In the case of EI, the ions selected for monitoring were characteristic of the particular family of the insecticide, unique to the insecticide,

TABLE I

IONS WHICH ARE PRESENT IN HIGH ABUNDANCE FOR EACH INSECTICIDE

M = molecular ions.

	m/z (relative abundance)	
	EI	*CH_4CI*
DVVP	109(100), 185(11)	221(100) M + 1, 223(64)
Phosdrin	127(100), 109(24)	192(100), 225(75) M + 1
Naled	109(100), 185(18)	221(100), 223(65)
Phorate	75(100), 97(28), 153(5)	199(28), 261(100) M + 1
Cygon	87(100), 125(55)	199(54), 230(100) M + 1
Guthion	77(100), 160(94)	160(100), 318(4) M + 1
Diazinon	179(100), 137(85), 153(35)	305(100) M + 1, 333(16) M + 29
Di-syston	88(100), 97(36), 153(20)	213(100), 275(51 M + 1
Ronnel	285(5), 125(97), 97(18)	323(100) M + 1, 349(21) M + 29
Malathion	173(70), 125(100)	129(100), 331(50) M + 1
Ethion	97(100), 231(93), 153(69)	199(100), 385(75) M + 1
Zolone	97(39), 153(15)	184(100)
Co-ral	97(100), 362(60), 125(46)	363(100) M + 1

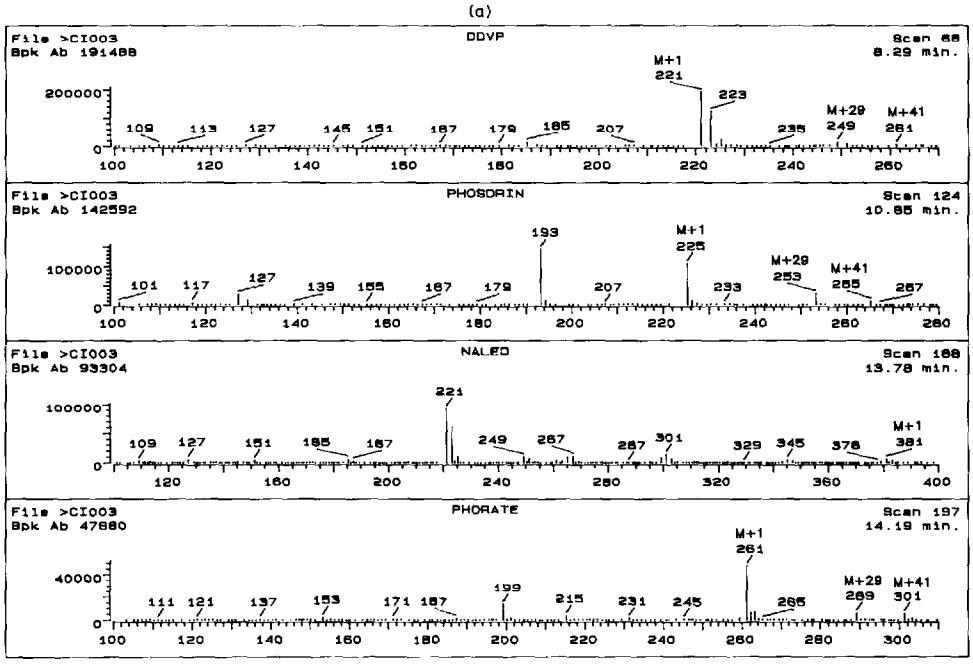
TABLE II

PROGRAMMING OF THE GC–MS SYSTEM TO MONITOR SELECTED IONS FOR THE ANALYSIS OF ORGANOPHOSPHORUS INSECTICIDES

SIM Program	*Group No.*	*Group start time (min)*	*Group run time (min)*	*No. of ions in each group*		*Ions monitored (m/z)*	
				EI	*CI*	*EI*	*CI*
1	1	7	20	17	17	109, 185, 127, 185, 75, 77, 87, 88, 97, 125, 137, 160,153, 173, 231, 153, 362	221, 223, 192, 225, 160, 199, 230, 261, 213, 275, 305, 323, 349, 129, 331, 184, 363
2	1	7	3	2	2	109, 185	221, 223
	2	10	3	2	2	127, 109	191, 225
	3	13	2	2	2	109, 185	221, 223
	4	15	2	9	9	75, 77, 87, 88, 97, 153, 137, 160, 125	160, 199, 230, 261, 305, 318, 333, 213, 275
	5	17	2	4	4	125, 97, 173, 125	323, 349, 129, 331
	6	19	3	3	3	97, 231, 153	199, 331
	7	22	5	5	2	97, 153, 125, 362	184, 363

or present in the highest abundances. The CI ions were selected only on the basis of their abundances. Other low-abundance ions could be included in ion monitoring. However, in our experience, increasing the number of low-abundance ions monitored decreased the sensitivity of the assay. Two different SIM programs were used to monitor the organophosphorus insecticides (Table II). In program 1, all 17 ions were monitored in one group. Whereas in program 2, ions were divided into seven groups (Table II). Each group had specified group-start and group-run times, and ions monitored (Table II). Although program 1 was simple to use, results of this study indicated that program 2 was several times more sensitive than program 1 since the number of ions monitored for each insecticide could be increased in the program 2. The urine, saline and plasma data reported in this study were obtained by using SIM program 1.

Our capillary column provided excellent separation of all 13 pesticides in saline, urine and plasma samples. Fig. 4 and 5 show the separation of 13 pesticides in CI and EI modes respectively. Fig. 6 shows the separation of organophosphorus insecticides added to plasma and analyzed in EI mode. Sass and Fisher[22] have reported that methane CI was at least 10 times more sensitive than EI. This study demonstrated that the CI method was more sensitive for Naled, Cygon, Guthion, Zolone and Diazinon; the EI method was relatively sensitive for Di-syston, Ronell, Malathion, Ethion and Co-ral; and the EI and CI methods were equally sensitive for DVVP, Phosdrin and Phorate. Since CI produced higher-mass ions than EI, and since significant abundances of M + 1, M − 1, M − 31, and M − 29 ions were produced by CI; it is proposed that the CI method might be more specific for these insecticides than the EI method.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221
223
M+29
249
M+41
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PHOSDRIN
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193
M+1
225
M+29
253
M+41
285
287
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NALED
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221
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M+29
289
M+41
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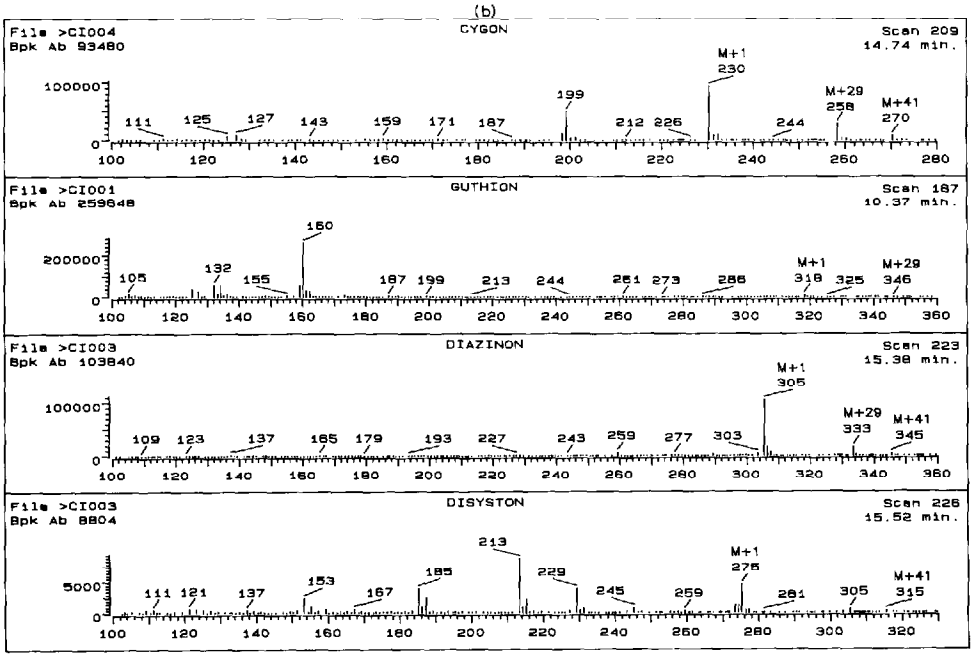
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M+29
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M+41
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GUTHION
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160
M+1
318
M+29
346
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Bpk Ab 103840
DIAZINON
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M+1
305
M+29
333
M+41
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213
M+1
275
M+41
315

Fig. 3.

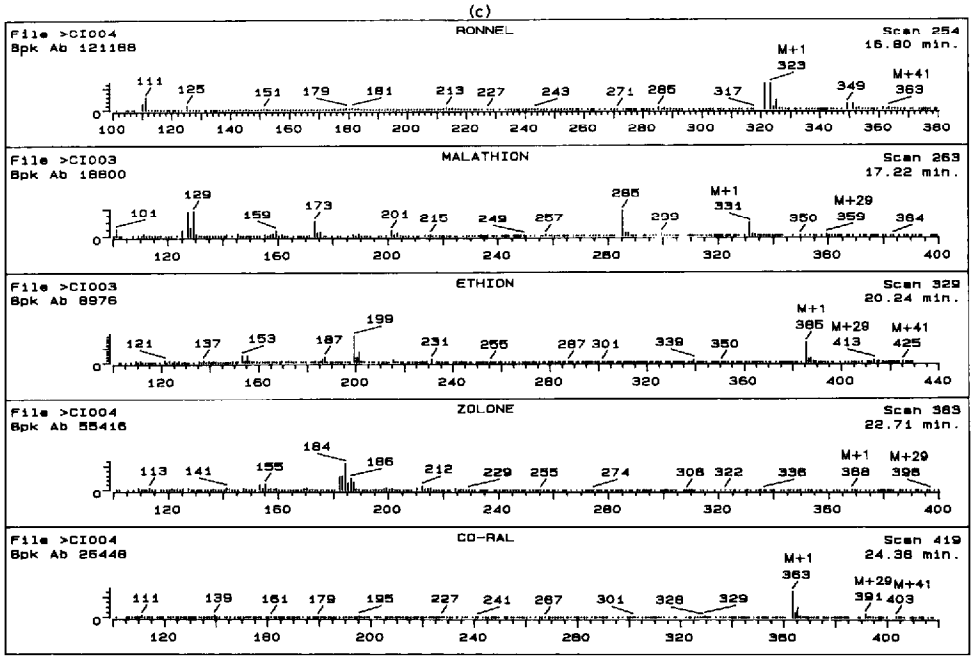

Fig. 3. CI spectra of (a) DVVP, Phosdrin, Naled and Phorate; (b) Cygon, Guthion, Diazinon and Disyston; (c) Ronell, Malathion, Ethion, Zolone and Co-ral.

Extraction efficiency and recovery of insecticides from saline, urine and plasma

The recovery of organophosphorus insecticides from urine, saline and plasma is shown in Fig. 7a and b. A linear relationship was observed between the amount of insecticides added and the amount recovered from both urine and saline. The extraction efficiency from urine or saline was 75–80% for each insecticide.

Although a similar procedure was utilized for the extraction of insecticides added to plasma or saline, the overall recovery was less in plasma than saline (Figs. 7a and b, and 8). Since a proper internal standard is not known for organophosphorus insecticides, the extraction efficiency of free, extractable insecticides from plasma was determined by using a double extraction method described by Singh *et al.*[4]. It was observed that approximately 80% of the free extractable insecticides present in the plasma was extracted by using the extraction procedure described above. Therefore, low recovery of insecticides from plasma might be the result of binding of insecticides to plasma esterases and other proteins. Sterri *et al.*[2], Fonnum and Sterri[3] and Singh *et al.*[4] have reported that mammalian blood contains several enzymes which bind with free organophosphorus compounds and reduce the level. Several other extraction procedures, such as deproteinization of plasma with perchloric acid before extraction, extraction of plasma samples in the presence of salt, and extraction of plasma samples by acetonitrile, were tried but did not improve the recovery of organophosphorus insecticides from plasma (data not shown). Perchloric acid which is known to hydrolyze organophosphorus compounds[4], caused a time-dependent decay in the concentration of organophosphorus insecticides.

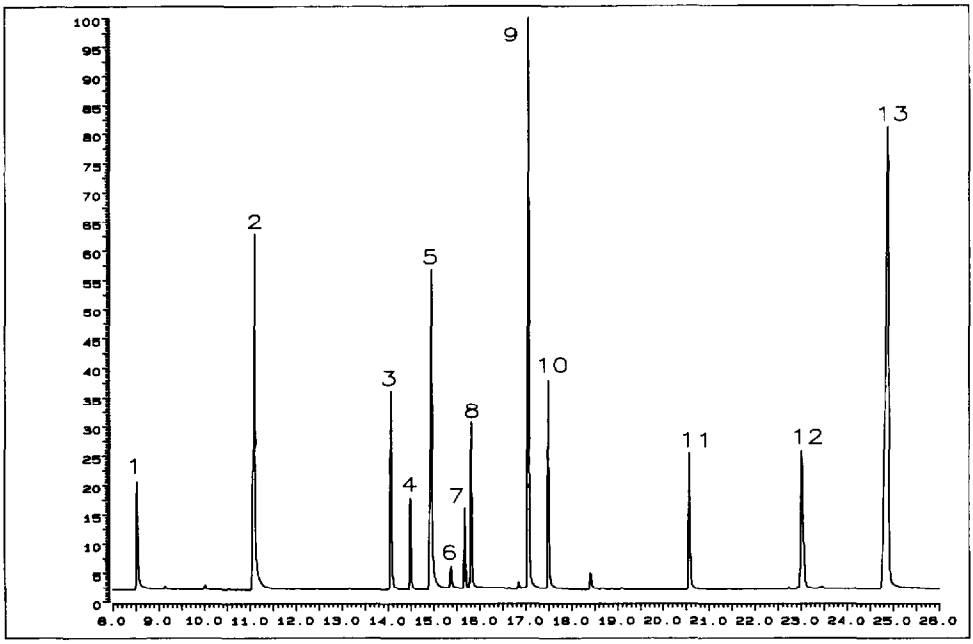

Fig. 4. Chromatographic separation of a mixture containing 0.1 ng/μl of 13 organophosphorus insecticides by EI-SIM, program 1. Ions monitored are listed in Table II. Peaks: 1 = DVVP; 2 = Phosdrin; 3 = Naled; 4 = Phorate; 5 = Cygon; 6 = Guthion; 7 = Diazinon; 8 = Di-syston; 9 = Ronnel; 10 = Malathion; 11 = Ethion; 12 = Zolone; 13 = Co-ral.

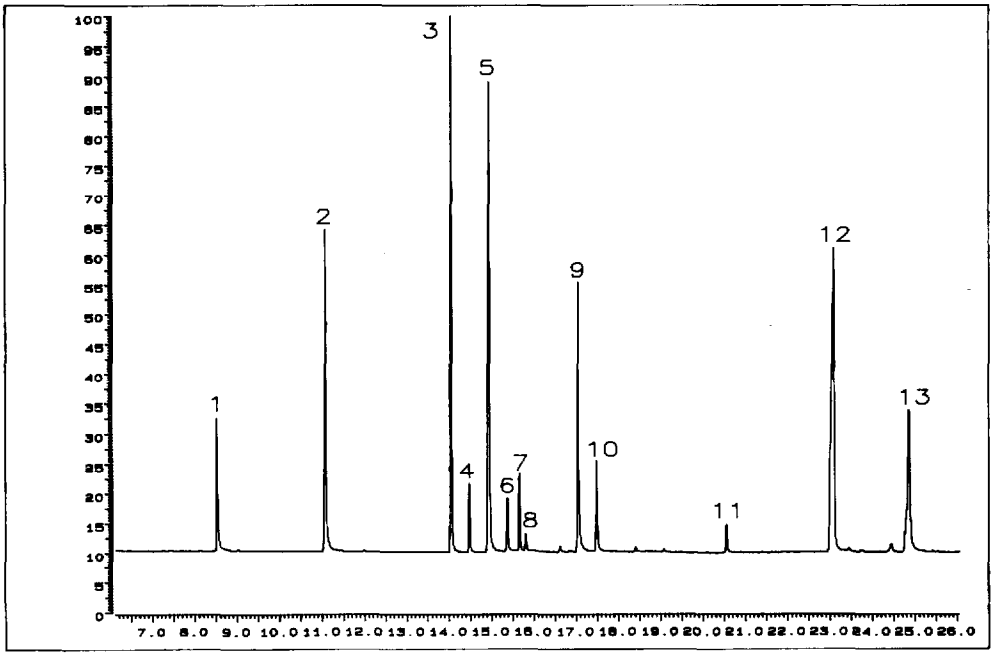

Fig. 5. Chromatographic separation of a mixture containing 0.1 ng/μl of 13 organophosphorus insecticides by CI-SIM, program 1. Ions monitored are listed in Table II. Peaks as in Fig. 4.

The results of this study indicate an interesting relationship between the LD_{50} value of an insecticide and its recovery from plasma. The recovery of Phorate, Phosedrin, Di-syston and Guthion (LD_{50} 2.5, 6.7 and 11 mg/kg respectively) was less than 10%; the recovery of Co-ral, Ethion, DVVP, and Zolone (LD_{50} 40, 65, 80 and 100 mg/kg respectively) was 15–30%; and the recovery of Cygon, Diazinon, Malathion and Ronel (LD_{50} 250, 500, 1000 and 1225 mg/kg respectively) was > 40% when 50 ng of each insecticide was added to 1 ml of plasma (Fig. 8). Increasing the dose resulted in a significant increase in extractable insecticides in the plasma. At a dose of 200 ng insecticides/ml plasma, 50–70% of the insecticides were recovered. However, the insecticides which had lower LD_{50} values showed lower recovery (Fig. 8). These observations suggest that at a lower dose most of the organophosphates added to plasma were present in an enzyme- (or protein-) bound form. Compounds which had a lower LD_{50} value exhibited a relatively higher reactivity to plasma proteins than the compounds which had relatively high LD_{50} values. The results find support with our previous observation[4] that the recovery of Sarin (an organophosphorous compound with LD_{50} 0.25 mg/kg) from plasma was less than the recovery observed in this study for Phorate (LD_{50} 2.6 mg/kg). As the dose was increased, the amount of free extractable insecticides increased in the plasma, which might be the result of the saturation of organophosphate-binding proteins.

From the results of this study it is concluded that (i) both EI and CI-selected ion monitoring provided a simple and sensitive method for measuring organophosphorus insecticides, (ii) CI produced higher-mass ions which might increase the se-

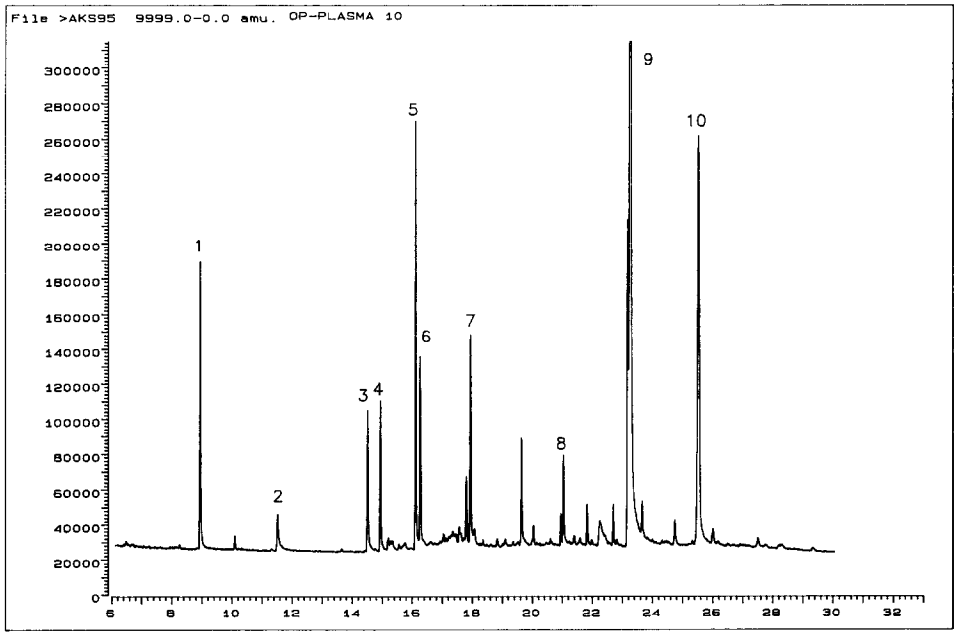

Fig. 6. Chromatographic separation of a mixture of organophosphorus insecticides added to plasma. The insecticides were extracted by a method described earlier. One μl of ethyl acetate extract was injected into the GC–MS system. Peaks: 1 = DDVP; 2 = Phosdrin; 3 = Naled; 4 = Phorate; 5 = Diazinon; 6 = Di-syston; 7 = Malathion; 8 = Ethion; 9 = Zolone; 10 = Co-ral.

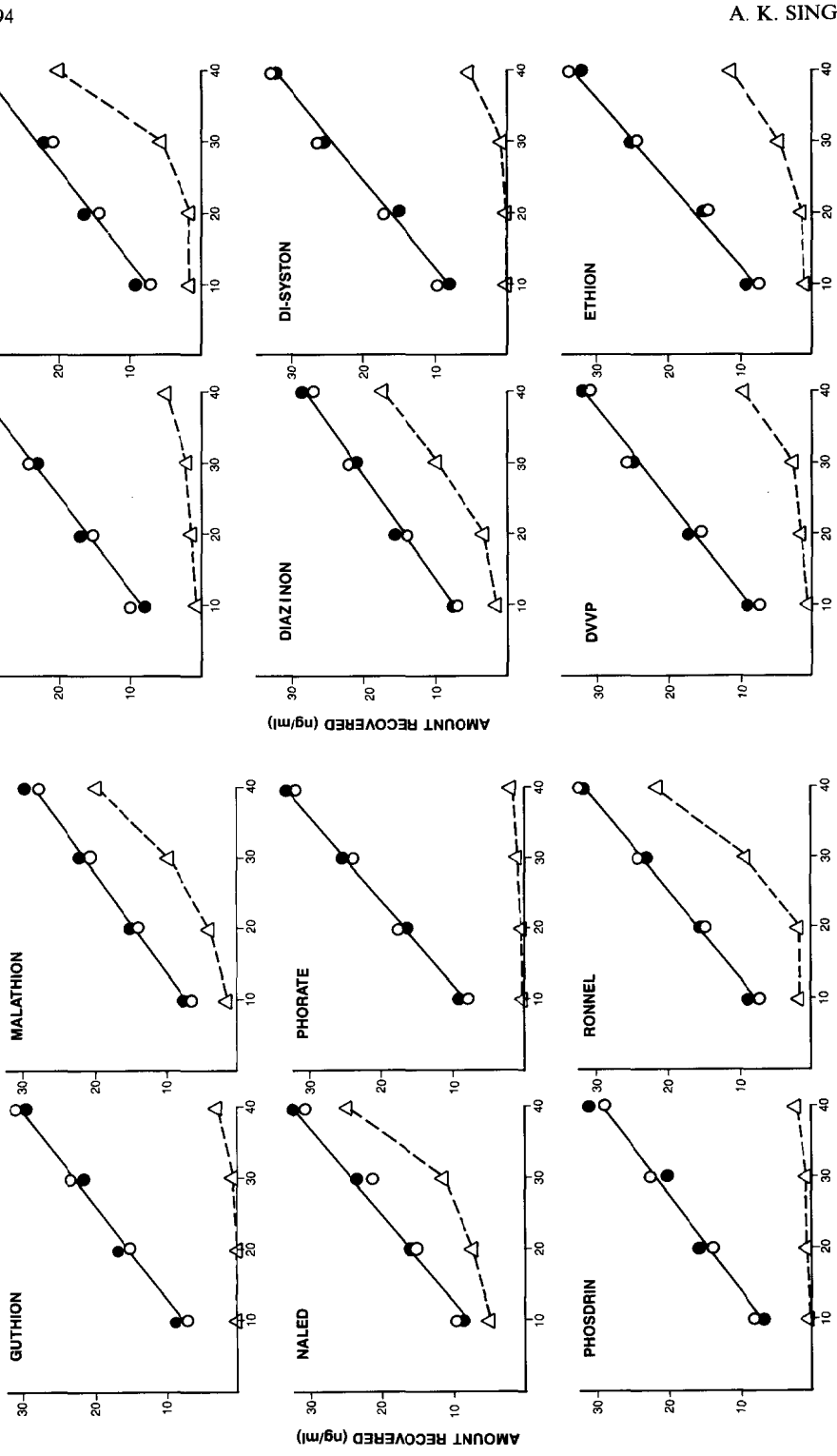

Fig. 7. Recovery of free organophosphorus insecticides from saline, urine and plasma at 10, 20, 30 and 40 ng/ml dose. Values are mean ± S.D. ○ = saline, ● = urine and △ = plasma.

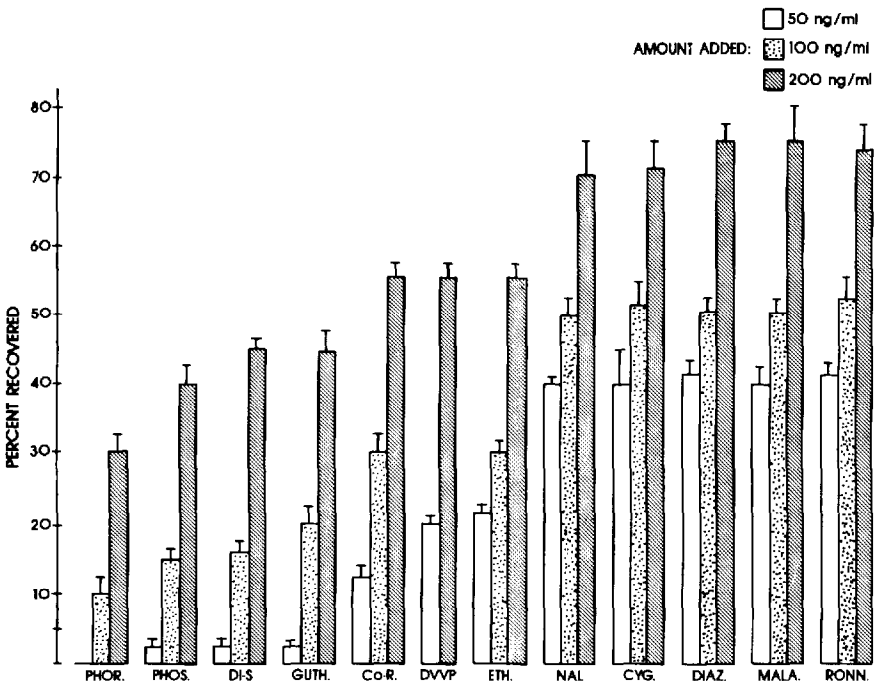

Fig. 8. Recovery of free organophosphorus insecticides from plasma when 50, 100 and 200 ng were added into 1.0 ml of plasma. Values are mean ± S.D.

lectivity and sensitivity (for certain insecticides) of the assay, (iii) the recovery of organophosphates from plasma was less than saline or urine because of the binding to plasma enzymes and proteins, and (iv) the organophosphates with lower LD_{50} values exhibited lower recovery from plasma than the organophosphates with higher LD_{50} values.

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