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Mechanisms of Composition Change and Toxic Potentiation of Chloramidophos Emulsifiable Concentrate during Storage

SHANSHAN ZHOU,[†,‡] DATONG ZHANG,[†] HUAYUN YANG,[†] YING ZHANG,[‡] AND WEIPING LIU[†,*]

Research Center of Green Chirality, College of Biological and Environmental Engineering, Zhejiang University of Technology, Hangzhou 310032, People's Republic of China; and Institute of Environmental Science, College of Environmental and Resource Sciences, Zhejiang University, Hangzhou 310027, People's Republic of China

Storage instability is one of the serious problems that greatly restrict pesticide use. Routine checks on the composition and toxicity of 30% emulsifiable concentrates (EC) of chloramidophos (CP) during storage indicated that 78.6% of the active ingredient had decreased, whereas the anti-acetylcholinesterase (AChE) activity of the formulation was potentiated by 3.5 times. To understand the mechanism for these changes, detailed knowledge of the products present and their effects on anti-AChE potential deserves attention. It was likely that the basis for these changes was methanol, the cosolvent of CP EC, because when the purified CP was stored in methanol at 50 °C for 2 weeks, CP drop and toxic potentiation similar to those observed in CP EC also appeared. The major products of the CP−methanol reaction mixture were isolated and identified by HPLC and GC-MS, respectively, and their inhibitory potentials against AChE and effectiveness as potentiators were assessed. Following redetermination of the main product (*O*,*S*-dimethyl-[(2,2,2)-trichloro-1-methoxyethyl]phosphoramidothioate (MCP)) and high anti-AChE material (methamidophos), which were preconfirmed in the reaction mixture in CP EC, it was successfully demonstrated that the majority of CP in the formulation had been transformed to a new stable compound, MCP. Meanwhile, formation of another product, methamidophos, resulted in toxic potentiation.

KEYWORDS: Chloramidophos; pesticide formulation; storage stability; toxic potentiation

INTRODUCTION

Methamidophos (Me, *O*,*S*-dimethylphosphoramidothioate; **Figure 1**), which was first registered in 1972, is a broad-spectrum acaricide−insecticide used to control pests on vegetables and various crops such as cotton, tobacco, and potatoes (*1*, *2*). It has even been one of the top 10 organophosphorus pesticides (OPs) sold worldwide (*3*) and is greatly responsible for humans surviving hunger and poverty. Me has also been the most used pesticide in China, accounting for >15% of China's 1.2 million tons of annual pesticide application (*4*, *5*). However, as a highly hazardous pesticide (*2*, *6*), Me has caused numerous poisoning cases (*2*, *7*, *8*), and the authorities of China have stated that all of the production, sale, and use of Me must be completely banned before December 31, 2008 (*9*). As a result, to fill this great gap in agrochemicals, it has been desirable to introduce some safe and cost-effective substitutes.

A new organophosphorus insecticide, chloramidophos (CP, *O*,*S*-dimethyl (2,2,2-trichloro-1-hydroxyethyl)phosphoramidothioate; also known as chloramine phosphorus; **Figure 1**) was provisionally registered as an effective alternative to Me in 2005 and has been widely applied in some provinces in China. Our

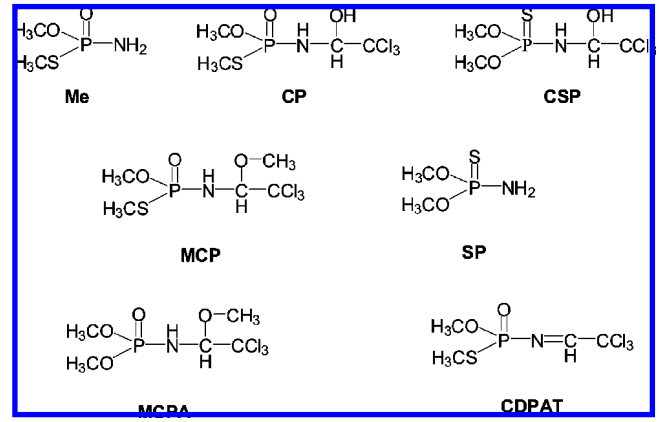

Figure 1. Compound structures of Me, CP, CSP, MCP, SP, MCPA, and CDPAT.

* Author to whom correspondence should be addressed (telephone +86-571-8832-0666; fax +86-571-8832-0884; e-mail wliu@zjut.edu.cn).

[†] Research Center of Green Chirality, Zhejiang University of Technology.

[‡] Institute of Environmental Science, Zhejiang University.

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previous study using a thermal assay found that the stability of CP in the solid phase was poor but that it could be improved when it was inclusion-complexed with β-cyclodextrin (*10*). The present study instead focused on the storage stability of the exclusively commercial formulation of CP, that is, 30% emulsifiable concentrates (EC). Because the formulated pesticide is often stored for long periods, a variety of toxic agents that are not present originally may form. Moreover, in some cases, the toxicological properties of the formulations will be modified by the newly formed compounds, and therefore samples must be re-evaluated for purity depending on the storage conditions. One of the most famous examples is the potentiating activity of the *S*-methyl isomer of malathion on the mammalian toxicity of its tropically stored water-dispersible powder formulation (*11*). If the formulations of OPs contain a hydroxylic organic solvent, like the 30% EC of CP which includes methanol as the cosolvent, the storage stability of the formulation should be carefully examined for possible changes in chemical composition and biological action, because hydroxylic organic solvents may catalyze the hydrolysis or directly replace amide or ester groupings within the OPs (*12*, *13*). In this study, we discovered that the amount of the active ingredient in CP EC had greatly decreased during its half-year of storage at room temperature. Contrary to expectation, the acetylcholinesterase (AChE) inhibitory potential of this formulated CP increased after storage. With the purpose of finding the particular mechanisms of disappearance of CP in the stored CP EC, the products produced were identified. Moreover, their inhibitory potentials against AChE and effectiveness as potentiators were determined to assess the relationship of CP drop and toxic potentiation. These corresponding results may offer useful evidence to determine whether CP EC can continuously be used in agriculture.

MATERIALS AND METHODS

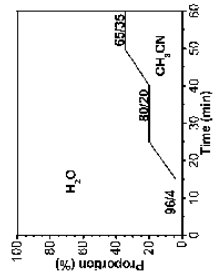
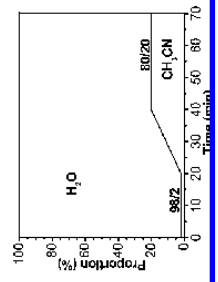
Chemicals. Racemic Me with a purity of 99.0% was purchased from Kefa New Technology Development Co. (Shenyang, China). The analytical standard (>98%), technical grade (about 90%), and 30% EC (trade name, Rosi Ling; certificate LS20051354) of CP as well as the analytical standard (about 97.3%) of *O*,*O*-dimethyl-(2,2,2-trichloro-1-hydroxyethyl)phosphoramidothioate (CSP; **Figure 1**) were kindly provided by Wuhan Zhongxin Chemical Engineering Co. Ltd. (Wuhan, China). Acetylthiocholine iodide (ATCh-I), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and AChE from *E. electricus* (EE-AChE, type V-S) were purchased from Sigma Chemical Co. (St. Louis, MO).

O,*S*-Dimethyl-[(2,2,2)-trichloro-1-methoxyethyl]phosphoramidothioate (MCP; **Figure 1**) was synthesized on the basis of our previous patent (*14*). Briefly, the technical CP (10.0 g) was stored in 20 mL of methanol at 50 ± 1 °C in the dark for 7 days and then evaporated under vacuum, reprecipitated from ethano,l and filtered. The crude product was further purified by silica gel chromatography, yielding the desired MCP as a white powder. The purity (>98%) of the compound was determined by HPLC: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.37 (3H, d, J = 15.2 Hz, $-SCH_3$), 3.69 (3H, s, $C-OCH_3$), 3.84 (3H, d, J = 12.8 Hz, $P-OCH_3$), 3.95 (1H, t, J = 21.2 Hz, NH), 4.93 (1H, dd, J = 4.0, 18.4 Hz, $-CH-$); ^{13}C NMR (100 MHz, $CDCl_3$) δ 12.42 ($-SCH_3$), 53.63 ($P-OCH_3$), 58.47 ($-OCH_3$), 91.73 ($-CCl_3$), 100.33 ($-CH-$); MS [electrospray ionization (ESI)], *m*/*z* calcd, 302.3 ([M] + H^+); found, 302.3. Elemental analysis (%): calcd C, 19.85; H, 3.66; N, 4.63; found C, 19.73; H, 3.56; N, 4.47.

Other chemicals and solvents were of analytical or HPLC grade.

General Information. HPLC analyses were carried out on a Jasco LC-2000 series HPLC system (Jasco, Tokyo, Japan) equipped with a variable-wavelength UV-2075 detector. GC-MS was conducted on a TraceGC 2000 equipped with a TraceMS mass selective detector (Finnigan, MA). ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Varian Mercury Plus 400 (400 and 100 MHz, respectively)

Table 1. HPLC Conditions for Isolation and Quantification of CP and Its Products

	column	Elution gradient chart	Flow rate (mL/min)	Detection wavelength (nm)	Oven temperature (°C)	Inject volumn (μL)
Isolation	Own-made C_{18} [a]		1.5	200	30	50
Quantification	Shimadzu Shim-pack VP-ODS C_{18} [b]		1	200	30	20

[a] 250 mm × 8.0 mm, Merck Purospher RP-18, 5 μM. [b] 150 mm × 4.6 mm, 5 μM.

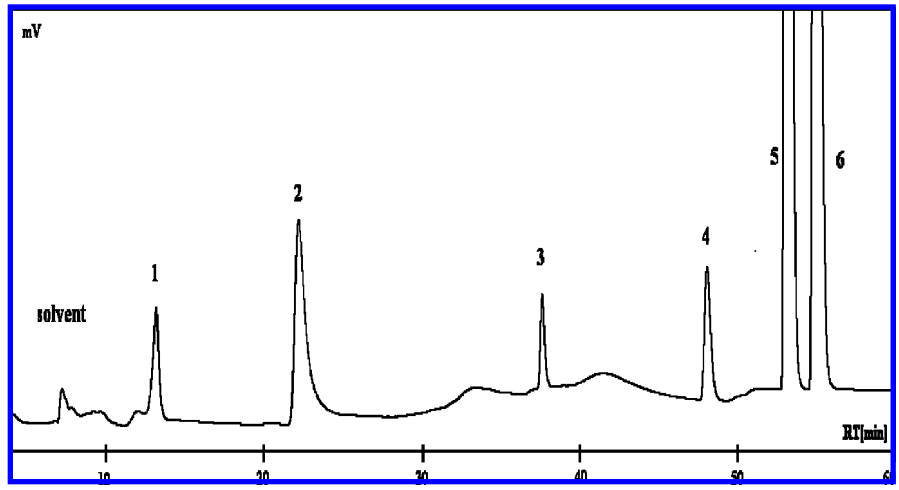


Figure 2. Representative HPLC chromatogram of the stored CP−methanol solution. Peaks: 1, Me; 2, CSP; 3, CP; 4, MCPA; 5 and 6, MCP. The operating conditions are shown in **Table 1** (isolation).

NMR spectrometer (Palo Alto, CA) at room temperature. Direct injection mass spectra were measured with a Finnigan Trace DSQ mass spectrometer (ESI) (Silicon Valley, CA). Elemental analysis was performed using a Thermo Finnigan Flash EA 1112 Elemental Analyzer (Milan, Italy). AChE activity was determined by a Bio-Rad model 680 microplate reader (Bio-Rad Laboratories).

Storage of Technical and Commercial Products of CP at Room Temperature. Samples (5.0 g) of freshly prepared CP (in both technical and formulated states) were sealed in glass ampules and placed at room temperature for half a year (February−July; daily mean temperature ranging from 0 to 30 °C; average temperature about 20 °C). At the end of storage, aliquots were analyzed for the CP by HPLC and tested for acute cholinergic toxicity against EE-AChE. Corresponding data of recently manufactured technical (purity about 90%) and 30% EC of CP were also measured for comparison.

Thermal Storage of the Analytical Standard of CP in Methanol. A solution of 750 mg of CP (>98%) in 1.5 mL of anhydrous methanol was closed in a vial and warmed at 50 ± 1 °C for 2 weeks. At 0, 7, and 14 days, aliquots were taken out for determination of their anti-AChE potentials against EE-AChE and identification of the main products. The extent of the anti-AChE activity of the decided products was also evaluated.

Isolation and Characterization of the Main Products in the CP−Methanol Reaction Mixture (RM). Aliquots from the 14-day RT samples were injected into a semipreparative C_{18} column to isolate the main products. The detailed parameters for separation of the main products are shown in **Table 1**. The isolated products were manually collected at the column outlet and immediately extracted with dichloromethane/acetone = 1:1. They were evaporated under vacuum and redissolved in ethyl acetate. All of the separated products were then identified by GC-MS.

GC-MS Analysis. A HP-5 MS fused silica capillary column (30 m × 0.25 mm, film thickness = 0.25 μm, cross-linked 5% diphenyl and 95% dimethylpolysiloxane, Agilent, Wilmington, DE) was used with nitrogen as the carrier gas (1.0 mL/min). The injection port temperature was 180 °C, the interface was 280 °C, and the oven temperature was increased by 10 °C/min from 80 to 250 °C. Electron impact ionization was performed at 70 eV. The MS scan range was set from 25 to 500 Da.

Quantification of the Products and CP. Quantities of the decided products and CP were measured using HPLC by comparing the area of the peaks with that of the standards. **Table 1** provides the operating conditions. Samples for the HPLC assay were dissolved with CH_3CN with a concentration of 5 g L^{-1}. Except for those reagents of analytical grade, the other samples were filtered through a 0.45 μm pore membrane filter.

AChE Inhibition Assay in Vitro. Anti-AChE potentials of the samples were determined by calculating their respective concentrations that inhibited half of the AChE (from *E. electricus*) activity (IC_{50}).

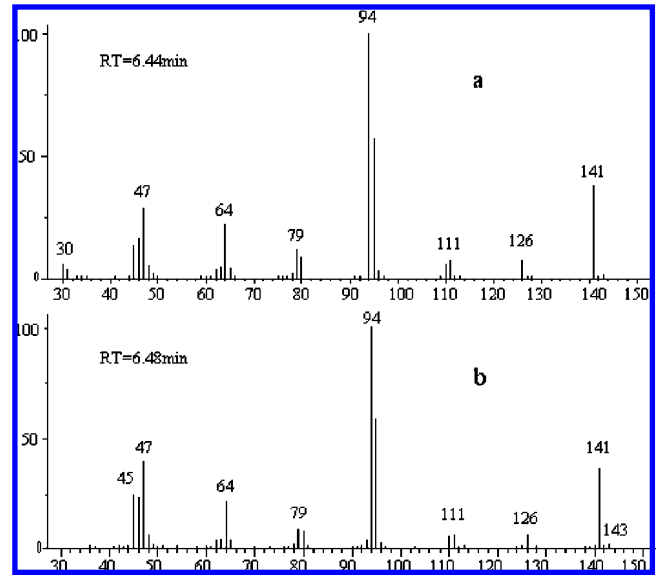


Figure 3. Mass spectra of compound **1** (**a**) and analytical standard of Me (**b**). RT represents the retention time of the sample from the GC spectra.

Table 2. Data Relating to Active Ingredient Content and Anti-AChE Activity of Technical and Formulated CP before and after Storage[a]

	30% EC[b]		technical product[b]	
	active ingredient, %	AChE IC_{50}, mg L^{-1} [c]	active ingredient, %	AChE IC_{50}, mg L^{-1}[c]
before[c]	29.60	31.09 ± 1.22	88.82	16.81 ± 0.66
after[d]	6.32	8.82 ± 0.32	81.29	20.00 ± 0.70

[a] All values are means ± SDs of the mean (n = 4). [b] Both the formulated and technical CP were resolved in acetone at proper concentration. [c] Freshly prepared. [d] Stored for half a year at room temperature.

Working solutions of the enzymes were made in 0.1 M potassium phosphate buffer (pH 8.0), in which the hydrolysis rates of ATCh-I were approximately 0.05−0.10 absorbance unit/min. In a typical experiment, each of five test tubes containing 180 μL of diluted enzyme solution was treated with 20 μL solutions of inhibitors at various concentrations that inhibited enzymatic activity by 10−90%. At the same time, control samples were also prepared using 20 μL of PBS (pH 8.0) in place of the inhibitor solution. The final concentrations of solvents (acetone or ethanol) in both the AChE−inhibitor solution and AChE−control solution were fixed at 0.5% (v/v). Each solvent had been confirmed not to react with the inhibitors during the experiment

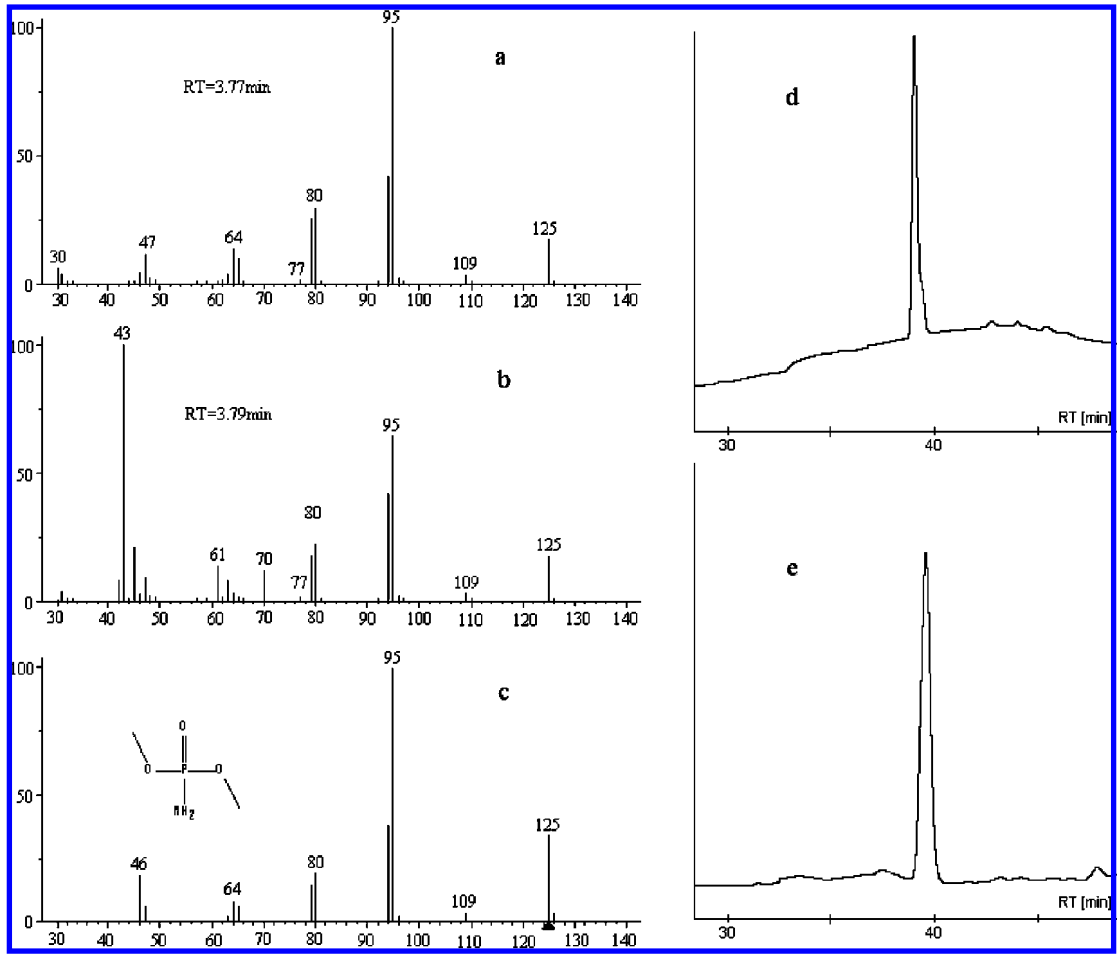


Figure 4. Mass spectra of compound **2** (**a**), CSP (**b**), and SP (**c**) and HPLC of compound **2** (**d**) and CSP (**e**). The HPLC conditions are shown in **Table 1** (quantification).

period, and their effects on AChE inhibition were negligible. The mixture was incubated at 37 °C for 30 min, and then 20 μL of the AChE−inhibitor solution (or AChE−control solution) was taken to measure the residual activity of AChE.

AChE activity was spectrophotometrically determined at 37 °C according to a modified Ellman method (*15*). All of the above tests and measurements were performed in four replicates. IC_{50} was calculated by the logit transition model (*16*).

Statistical Analysis. All data are expressed as mean ± SD. Student's *t* test at a significance level of 0.05 was used to compare the differences between groups.

RESULTS AND DISCUSSION

Effect of Storage on Technical and Formulated CP. This study arose from the changes observed in both composition and AChE inhibitory potential (**Table 2**) of CP EC during its half-year storage at room temperature. **Table 1** provides data which show that the anti-AChE activity of CP EC was potentiated by a factor of about 3.5 times after storage, whereas 78.6% of the active ingredient CP synchronously disappeared. In contrast to the formulated CP, AChE inhibition of the technical CP that was stored under the same condition was decreased only a little compared to that of a fresh solution. This result is likely because of a minor degradation of CP during the period of storage, as determined by HPLC (**Table 2**). As far as it is possible to discriminate between the technical and formulated versions of CP, it is reasonable to presume that some new compounds with high anti-AChE potential have formed in the stored CP EC, and the formulating agents are likely responsible for the instability of CP when it is formulated. The formulating agents of CP EC are very complicated, including the solvent benzene, a cosolvent methanol, a skin penetration promoter azone, and many polymer emulsifiers. In addition to the complexity of the formulated substances, materials for the synthesis of the technical CP also involve a lot of impurities (such as the technical Me with the purity of only 73%). Therefore, isolation of the compounds that were produced by the decomposition of CP in the formulation is very difficult. As an alternative method, we initially supposed that some certain formulation constituents are the main factors inducing the instability of CP. Then, following storage of highly purified CP with the proposed formulating ingredient, an effort was made to purify the main products as well as the potentiating materials out of the RM. Mechanisms responsible for the changes in CP EC cannot be concluded until the identity of the products of the main reaction or high-anti-AChE compounds have been confirmed in the RM of CP EC.

Chloramidophos is a hemiaminal and thus reactive in acidic alcohols, such as methanol (*17*). Therefore, we suppose that these reactions may play an important role in the decomposition and anti-AChE activity change of CP in EC, and thus we analyzed the spontaneous reactions of CP in methanol in this study. First, we mixed the analytical standard of CP with methanol and then incubated the solution at 50 ± 1 °C. The major products in the heated CP−methanol solution were collected and characterized by semipreparative HPLC and GC-MS, respectively. Then, the amount of the decided products and their anti-AChE contributions to that of the RM were evaluated for determining the mechanisms from both composition and

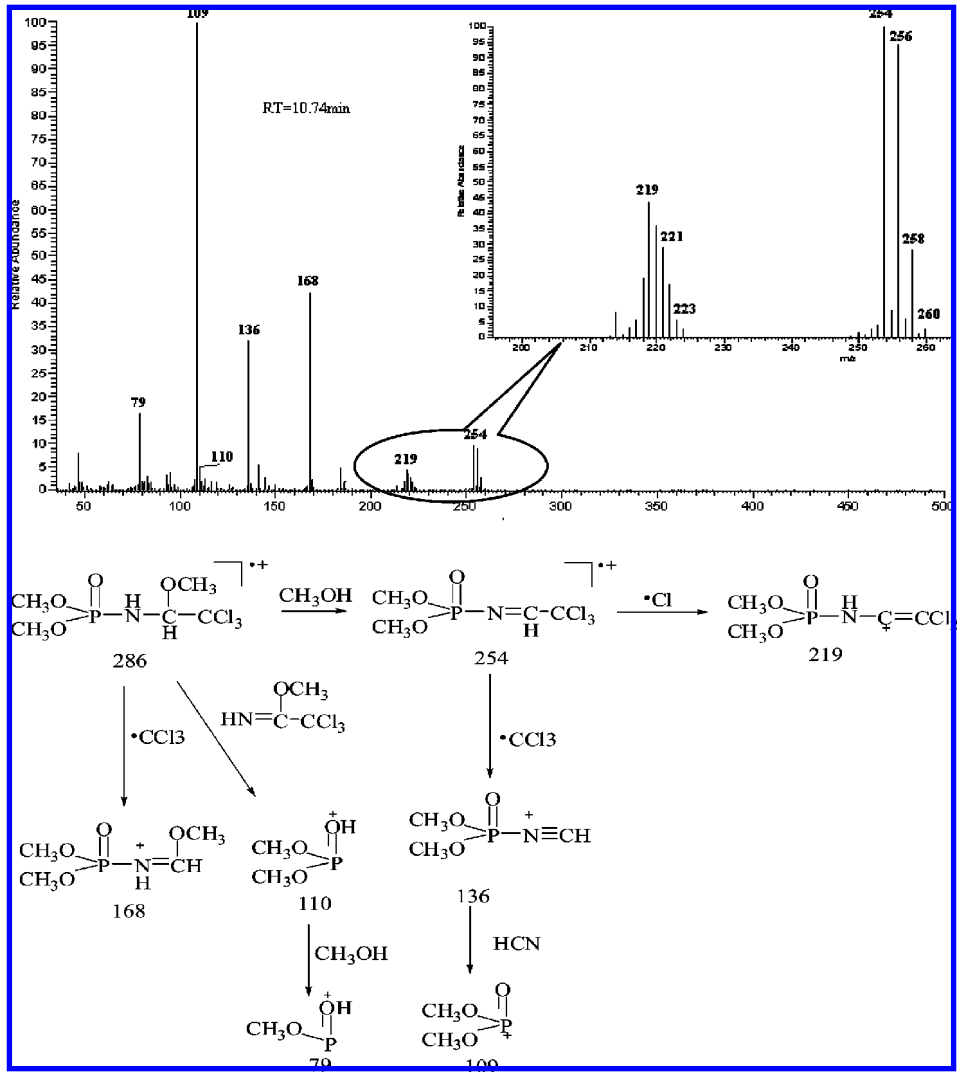


Figure 5. Mass spectrum of compound **4** and its probable interpretations.

toxicological points of view. Concentrations of the key products (product of the main reaction or high anti-AChE material) were further determined in the CP EC by HPLC as previously suggested.

Characterization of the Main Products in the Heated CP−Methanol Solution. In consideration of the decomposition of CP at the injection port of the GC, the individual products were preliminarily isolated, extracted, and then subjected to GC-MS analysis. HPLC analysis on the semipreparative C_{18} column indicated five compounds other than the original chemical CP (**Figure 2**). By GC-MS, compound **1** was confirmed as Me through a good agreement of retention time and mass spectra of compound **1** with those of the authentic sample (**Figure 3**). The chemical structure of compound **2** was also initially assumed through a comparison of the data from the GC-MS of the extracted substance with those of a standard sample of CSP (**Figure 4**). As shown in **Figure 3**, the mass spectra of both compound **2** and CSP were virtually identical, however, with that of spermine (SP, *O*,*O*-dimethyl phosphoramidothioate; **Figure 1**), which may be attributable to their consistent thermal degradation in the GC. Uniform retention time measured by HPLC (**Figure 3**) further manifested that CSP is compound **2**. The structure of compound **3** corresponds to CP. Compound **4** was tentatively assigned as *O*,*O*-dimethyl-[(2,2,2)-trichloro-1-methoxyethyl]phosphoramidate (MCPA; **Figure 1**) on the basis of its MS fragmentation pattern. The mass spectrum of compound **4** and its probable interpretation are shown in **Figure 5**. The structure of compound **5** was concluded to be the same as that of compound **6** because both total ion current chromatograms showed three peaks and the retention times as well as the fragmentation patterns of each corresponding peak were identical. As an example, **Figure 6** gives the mass spectra of compound **5** and the probable interpretation. The fragment ion data suggested that peaks 2 and 3 were assigned as MCP, whereas the structure of peak 1 was considered to be *O*,*S*-dimethyl 2,2,2-trichloroethylidenephosphoramidothioate (CD-PAT; **Figure 1**), a thermal breakdown product of MCP in the GC. Data from the GC-MS provided evidence that compounds **5** and **6** are two diastereomers of MCP. Confirmation was further accomplished by comparison with the synthesized material.

Mechanisms of Instability of the Formulated CP. The use of HPLC to isolate a minor amount of products in the CP−methanol RM is generally a rapid and convenient method. However, because of the low sensitivity of the UV signal, we obtained only four products. **Table 3** contains data for the amount of the various products and CP in the total CP−methanol mixture. Also included are data for the inhibitory potential of the product (or CP) itself to AChE and their effects on the anti-AChE activity of the RM. On the basis of the results shown in **Table 2**, it can be calculated that four chemicals, that is, Me, CSP, MCP, and CP, accounted for about 90% of the total amount of the RM. Furthermore, supposing that the enzyme

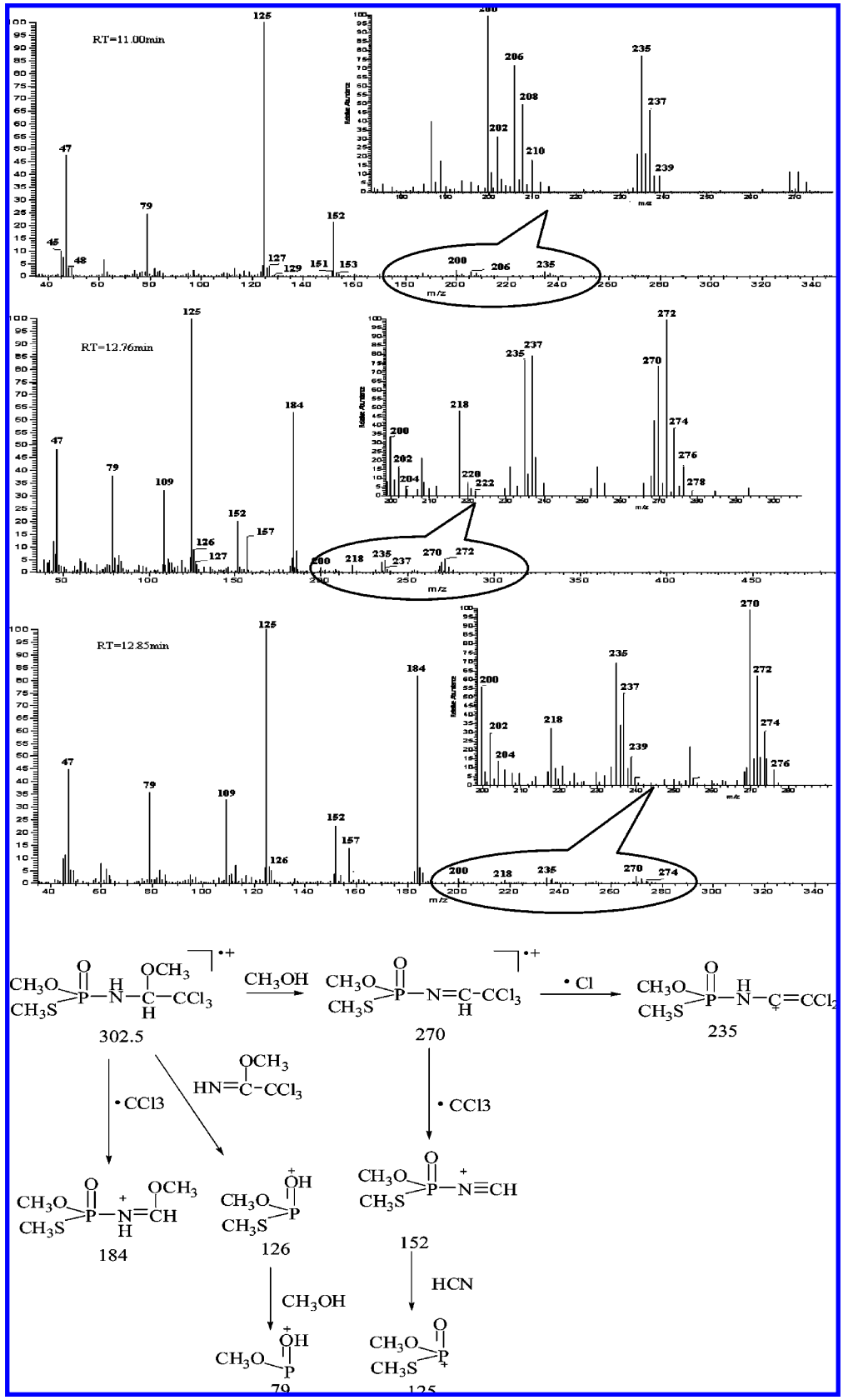


Figure 6. Mass spectra of compound **5** and its probable interpretations. Three peaks with retention times, respectively, at 11.00, 12.76, and 12.85 min are obtained in the GC spectra of compound **5**.

inhibitory actions of all compounds are independent, these four compounds contributed about 97% of the inhibition of AChE. Consequently, other products, including MCPA and those not identified, seem to be less important to the composition or toxicological aspect of this research and therefore were not pursued in the present study.

Similar changes in both composition and anti-AChE potential found in the CP EC solution were identified again after heating

Table 3. Effects of the Decided Products and CP on Acetylcholinesterase Inhibitory Potential of the Total Reaction Mixture

time (days)		compounds				IC_{50} of the mixture[a,b] (mg L^{-1})
		Me	CP	CSP	MCP	
0	IC_{50} (mg L^{-1})	0.36	9.19	>50.00	39.65	
	amount[c] (%)	ND	98.23	1.56	ND	9.19
	toxic contribution[d] (%)	0	100.00	CN	0	
7	amount (%)	2.95	5.55	2.42	78.75	9.03
	toxic contribution (%)	74.00	5.45	CN	17.93	
14	amount (%)	3.37	1.90	1.40	82.58	8.27
	toxic contribution (%)	77.42	1.71	CN	17.22	

[a] Mixture is the aliquot taken from the CP−methanol mixture warmed at 50 °C. [b] IC_{50}: = concentration of inhibitor leading to half-inhibition of EE-AChE activity in 30 min. [c] Amount % = concentrations of samples/5 mg L^{-1}. Quantification of the products and CP was accomplished by comparing the area of the peaks with that of the standards. ND, not detected. [d] Contribution % = (amount % × IC_{50} of mixture at the corresponding time)/IC_{50} of the pure product. CN, can be neglected.

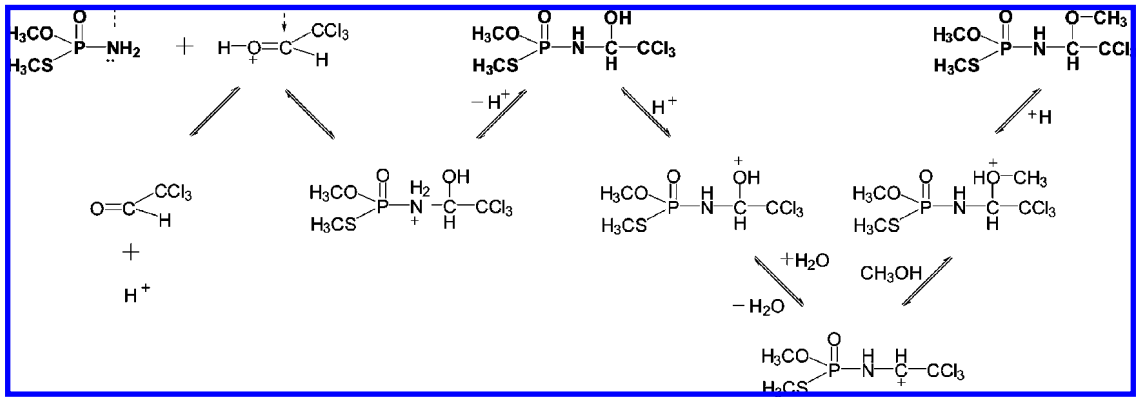


Figure 7. Possible transformation mechanisms of CP to MCP and Me in CP EC.

of the purified CP in anhydrous methanol. On the one hand, the content of CP greatly dropped. Storage of CP in methanol for 7 days resulted in a breakdown of 94.3% to other compounds, whereas there was 98.2% decomposition after 14 days. On the other hand, the anti-AChE potential of the stored CP−methanol solution was increased, but much milder than in CP EC (IC_{50} at 9.19 mg L^{-1} at 1 day to 8.27 mg L^{-1} at 14 days). Meanwhile, MCP was formed with a mass percent up to 82.6%, suggesting that the majority of CP was reacted with methanol by nucleophilic addition, incorporating the methoxyl group into the molecule in place of the hydroxyl group (**Figure 7**). This similar reaction between CP and methanol also can be extrapolated to the formulation, because peaks relevant to MCP were then successfully checked out in the stored CP EC (data not shown). By quantitative analysis, it can be estimated that about 80% of the disappeared active ingredient of CP EC has been changed to MCP. In theory, the anti-AChE potential of MCP should be weaker than that of CP because the anti-cholinesterase activity of OPs in vitro has been manifested to be closely related to their hydrophilicity (*18*). In the present study, we found a credible result that the IC_{50} of MCP was about 4.3 times larger than that of CP (**Table 3**). For this reason, contribution of MCP to the anti-AChE activity was only about 18%, even though its contribution to total mass was >80%, implying that MCP must not be a potentiator. By contrast, another product Me was about 25.5 times more active toward inhibiting AChE than CP (**Table 3**), leading to a small quantity of Me greatly responsible for the AChE inhibitory activity of the CP−methanol RM. For example, 3% of the total mass contributed >75% of the AChE inhibitory potential of the RM (**Table 2**). We further estimated that if the mass percent of Me in CP EC increased to 4.1% after storage, the anti-AChE activity would be potentiated to 3.5 times, whereas the actual amount of Me in CP EC determined by HPLC was 3.8%. Thus, it can be considered that theformation of Me is probably the main factor for enhancement of the anti-AChE activity of CP EC during storage. A previous study concerning two isomers of CP, that is, $(CH_3O)_2P(S)NHCH(OH)CCl_3$ and $(CH_3O)_2P(O)NHCH(OH)CCl_3$, showed that these two chemicals could reversibly decompose to $(CH_3O)_2P(S)NH_2$ and chloral or $(CH_3O)_2P(O)NH_2$ and chloral (*19*), respectively. As a result, we believe that decomposition of CP is one of the mechanisms for theformation of Me in CP EC (**Figure 7**). CSP is the major impurity of the analytical standard of CP. As shown in **Table 2**, the concentration of CSP first increased and then decreased, meaning that it was unstable in the RM. Moreover, the contribution of CSP to the anti-AChE activity was neglected. We therefore never measured its concentration in CP EC.

In conclusion, these results provide two new pieces of information. On the one hand, 30% EC, the exclusive formulation of CP used in China, is advised to be limited for sale for two reasons: first, the active ingredient, CP, is unstable and easily decreases and, second, Me, a highly toxic and banned insecticide, will form during storage. On the other hand, MCP, the newly formed organophosphorus compound, seems to be stable in this type of formulation because its amount was constant during storage. As we mentioned, instability was one of the key factors in stopping application of CP EC in pest control. Stability of MCP may make it a good substitute for CP. Furthermore, although the inhibitory potency of MCP to the target enzyme measured in vitro was only one-fourth of the paternal insecticide (**Table 3**), the comparable in vivo activities of 30% emulsion in water of MCP with those of 30% CP EC toward both *Leucania separate* and *Pluteua xylostella* (*14*) additionally imply its application future. Also, because instability of CP in EC is closely responsible for the hydroxylic organic

solvent methanol (**Figure 7**), it will be of interest to reformulate CP in a less hydrophilic environment.

ABBREVIATIONS USED

AChE, acetylcholinesterase; ATCh-I, acetylthiocholine iodide; CDPAT, *O*,*S*-dimethyl-2,2,2-trichloroethylidenephosphoramidothioate; CP, chloramidophos; CSP, *O*,*O*-dimethyl-(2,2,2-trichloro-1-hydroxyethyl)phosphoramidothioate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EC, emulsifiable concentrates; EE-AChE, AChE from *E. electricus*; IC_{50}, concentration of inhibitor leading to half-inhibition of AChE activity in 30 min; Me, methamidophos; MCP, *O*,*S*-dimethyl-[(2,2,2)-trichloro-1-methoxyethyl]phosphoramidothioate; MCPA, *O*,*O*-dimethyl-[(2,2,2)-trichloro-1-methoxyethyl]phosphoramidate; OPs, organophosphorus pesticides; RM, reaction mixtures; SP, *O*,*O*-dimethyl phosphoramidothioate.

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