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New Method for Microdetermination of Triforine and Its Metabolite Using Thermal Reaction with Alcohol

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A new method for microdetermination of triforine [TF, 1,4-bis(2,2,2-trichloro-1-formamidoethyl)-piperazine] and its major metabolite [TF/2, 1-(2,2,2-trichloro-1-formamidoethyl)piperazine] is described. Thermal reaction of triforine or TF/2·HCl [1-(2,2,2-trichloro-1-formamidoethyl)piperazinium 4-hydrochloride] with several alcohols in a closed glass tube resulted in formation of highly sensitive compounds to electron capture detector (ECD) on gas chromatography (GC). The compounds were identified as *N*-(1-alkoxy-2,2,2-trichloroethyl)formamides. The reaction was applied to the residue analysis of triforine and TF/2 in several crops. The analytical method involves extraction with acetone, separation to triforine and TF/2 portions by liquid–liquid partition, thermal reaction with methanol, and analysis by GC. Minimum limits of detections were 0.005 ppm for triforine and 0.01 ppm for TF/2·HCl. Recoveries of added triforine and TF/2·HCl from peach, green pepper, and strawberry averaged 99% and 70%, respectively.

Triforine [1,4-bis(2,2,2-trichloro-1-formamidoethyl)-piperazine, Saprol] is a systemic fungicide used for controlling powdery mildew, scab, rust, monilia, and leaf spot disease on a wide range of crops (Schicke and Veen, 1969). Piperazine and 1-(2,2,2-trichloro-1-formamidoethyl)-piperazine (TF/2) have been shown to be metabolites in barley plants (Rouchaud et al., 1978).

The most widely used approach to the analysis of triforine and TF/2 metabolite has been that of acidic hydrolysis and gas chromatographic measurement of the liberated chloral hydrate (Eichler, 1972; Bourke et al., 1977; Rouchaud, 1977). Methods for the determination of piperazine by gas–liquid chromatography (Rouchaud, 1977; Newsome, 1982) are also available for the analysis of triforine and its metabolites. While triforine can be detected when it is directly introduced into gas chromatograph (GC) (Ishii, 1980; Nagayoshi et al., 1981), accurate measurements cannot be obtained because the method is based upon the determination of triforine's thermal product formed in the injection port of GC.

We developed a new method for the determination of triforine and its metabolite (TF/2) using thermal reaction of these compounds with methanol in a closed glass tube, followed by the measurement of thermal product with GC. The availability of the method for residue analysis in several crops was demonstrated by the analysis of triforine and TF/2·HCl [1-(2,2,2-trichloro-1-formamidoethyl)-piperazinium 4-hydrochloride] added to peach, green pepper, and strawberry.

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MATERIALS AND METHODS

Apparatus. A Hewlett-Packard Model 5890 gas chromatograph equipped with electron capture detector was used for all measurements. A 2.4 m × 2 mm (i.d.) glass column was packed with Ultra-Bond 20M (100–120 mesh). Column temperature was held at 160–170 °C; inlet and detector temperatures were 280 and 300 °C, respectively. Carrier flow (N_2) was 40 mL/min. Quantitation was achieved by measurement of peak heights.

Identification of the products obtained by the thermal reaction of triforine or TF/2 with several alcohols was achieved through gas chromatography–mass spectrometric analysis performed on a JEOL JMS DX-300 gas chromatograph–mass spectrometer [equipped with a dual electron impact (EI)/chemical ionization (CI) source] interfaced to a JEOL JMA DA-5000 data system. Ion source operating temperature was maintained at 200 °C with an ionizing voltage of 70 eV. All CI spectra were measured with use of isobutane. Samples were introduced through a gas chromatographic column fitted to a JEOL MS-DC05 gas chromatograph and interfaced via a glass jet separator. The GC analyses were accomplished on a 1.5 m × 3 mm

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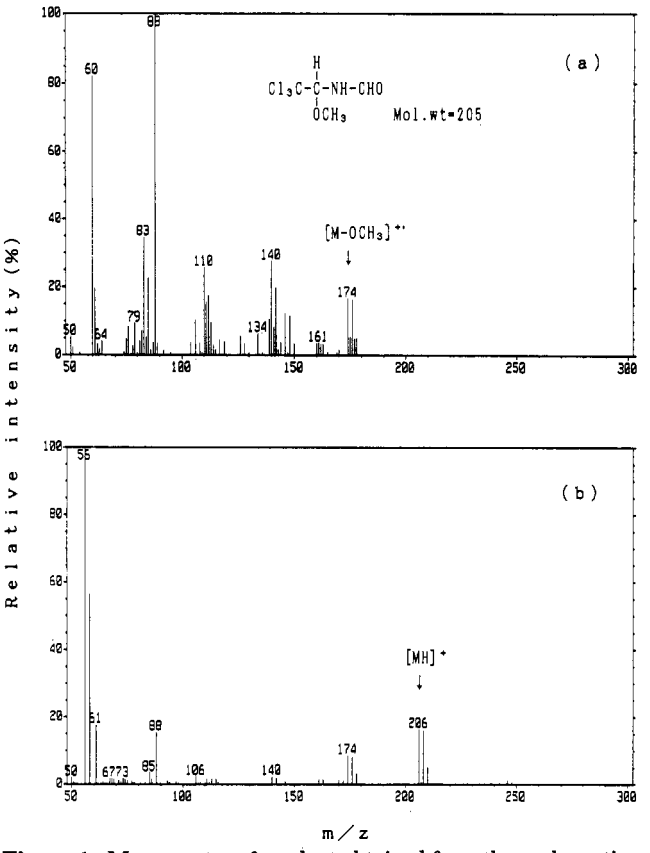

Figure 1. Mass spectra of product obtained from thermal reaction of triforine with methanol: (a) electron impact; (b) chemical ionization.

glass column packed with Ultra-Bond 20M (100–120 mesh) with helium carrier gas (30 mL/min) and a column oven temperature of 190 °C.

A 10-mL (10 cm × 10 mm (i.d.)) glass tube (used commonly for freeze-drying) was used for the thermal reactions of triforine and TF/2 with alcohols.

Reagents. Analytical standards of triforine (purity 99.6%) and TF/2·HCl (purity 99.1%) were provided by Sumitomo Shoji Ltd. *N*-(1,2,2,2-Tetrachloroethyl)formamide (TCEF), the starting material for triforine synthesis, was purchased from Tokyo Kasei Co. Each chemical was diluted with methanol to prepared 1000 ppm stock solution. Sep-Pak silica cartridge (Waters) was used for column chromatographic cleanup. All solvents were pesticide residue grade or equivalent.

Procedure. (*1*) *Thermal Reaction.* A 2-mL aliquot of sample solution (methanol containing analytical compounds) was introduced into the glass tube for thermal reaction, followed by ethyl acetate (0.05 mL) and pyridine (0.005 mL). The tube was closed by fusing the open end with a gas burner under slightly reduced pressure, and the closed tube was dipped below the surface of the sample liquid in a oil bath with temperature of 160 °C for 30 min. After the reaction, the tube was cooled to room temperature and the top of the tube was opened. A 0.1-mL portion of 5% diethylene glycol in acetone was added to the reacted solution as a keeper. The solution was transferred to a 20-mL test tube and evaporated to dryness with a rotary vacuum evaporator. The residue in the evaporation tube was dissolved in acetone to prepare a suitable concentration for GC analysis.

(*2*) *Extraction and Purification of Analytical Compounds from Crops.* Aliquots of the homogenized sample (30 g) of a crop were mixed with 100 mL of acetone and shaken for 30 min on a reciprocating shaker. The mixture

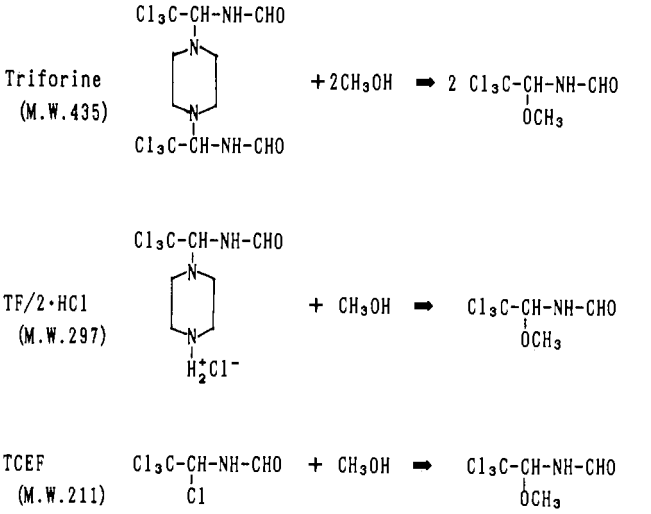

Figure 2. Proposed reaction sequence of triforine, TF/2·HCl, and TCEF with methanol.

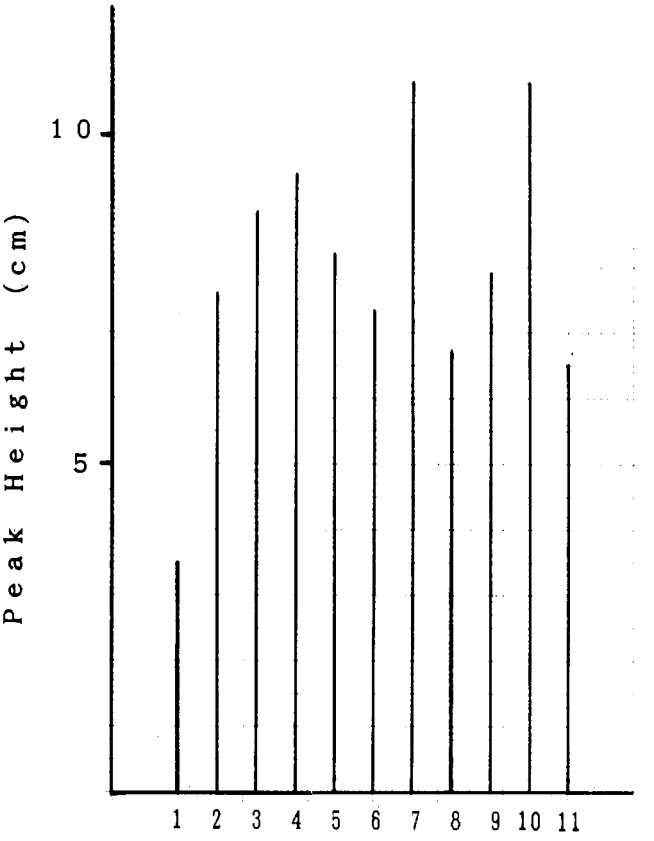

Figure 3. Reaction accelerators for reaction of triforine with methanol (reaction temperature 160 °C). Key: 1 = methanol; 2 = methanol + pyridine; 3 = methanol + formic acid; 4 = methanol + acetic acid; 5 = methanol + propionic acid; 6 = methanol + methyl acetate; 7 = methanol + ethyl acetate; 8 = methanol + *n*-propyl acetate; 9 = methanol + pyridine + methyl acetate; 10 = methanol + pyridine + ethyl acetate; 11 = methanol + pyridine + *n*-propyl acetate.

was filtered through a layer of Celite on filter paper under suction into a 300-mL round-bottom flask, and the residue was washed with 50 mL of acetone. The combined filtrate was evaporated on a rotary evaporator at 40 °C, and the concentrated solution was transferred to a 500-mL separatory funnel. A 200-mL portion of 5% NaCl was added to the solution, and the mixture was extracted twice with 100 mL of benzene. The benzene extract (Triforine fraction) was dried by passage through a bed of anhydrous

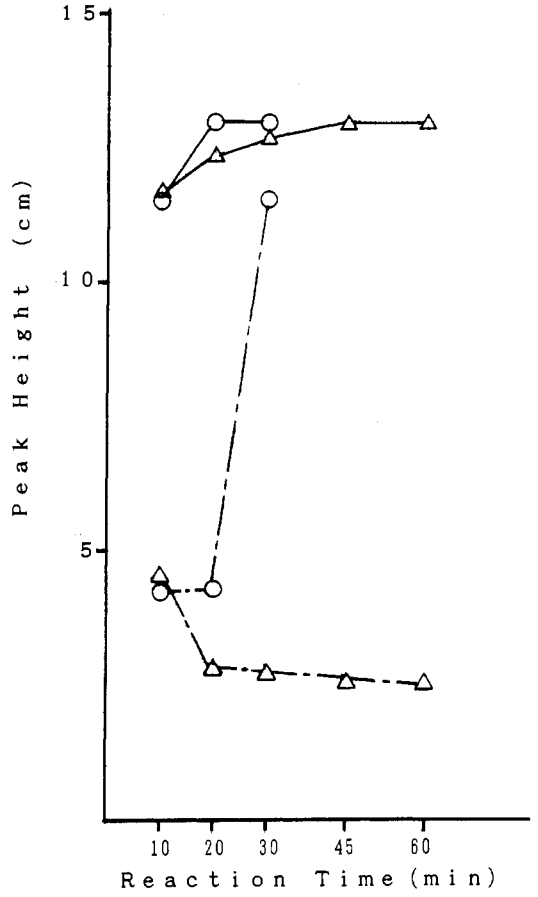

Figure 4. Effects of reaction time on yield of product obtained from reaction of triforine with methanol (reaction temperature 160 °C). Key: O—O, methanol + pyridine + ethyl acetate; Δ—Δ, methanol + ethyl acetate; O-·-O, methanol + pyridine; Δ-·-Δ, methanol.

Table I. Products Derived from Thermal Reaction of Triforine with Several Alcohols

tested alcohol	product structure	ret time,[a] min	RPH[b]
methanol	$Cl_3CCH(OCH_3)NHCHO$	1.8	1
ethanol	$Cl_3CCH(OC_2H_5)NHCHO$	1.9	0.85
propanol	$Cl_3CCH(OC_3H_7)NHCHO$	2.4	0.80
butanol	$Cl_3CCH(OC_4H_9)NHCHO$	3.5	0.54
methoxy-ethanol	$Cl_3CCH(OC_2H_4OCH_3)NHCHO$	6.0	0.32

[a] Retention time on GC analysis; column temperature 160 °C. [b] Relative peak height to methanol.

sodium sulfate, combined in a 300-mL round-bottom flask, and evaporated to dryness. The residue in the flask was dissolved in a 4 mL of 5% acetone in benzene, and a 2-mL aliquot of the solution was then transferred to a Sep-Pak silica cartridge. The chromatograph was developed by adding first 5 mL of 5% acetone in benzene and second 20 mL of 20% acetone in benzene. The first elution was discarded, and the second elution was collected in a 50-mL round-bottom flask. The eluate was evaporated to dryness, and the residue in the flask was dissolved in 3 mL of methanol. A 2-mL aliquot (equivalent to 10 g of homogenized sample) of the methanol solution was pipetted into the reaction tube and subsequently treated as the same manner as described in procedure 1.

The residual water phase (TF/2 fraction) after the extraction with benzene in liquid–liquid partition was extracted three times with adding both 5 mL of 5 N NaOH and 100 mL of ethyl acetate, and the ethyl acetate extracts

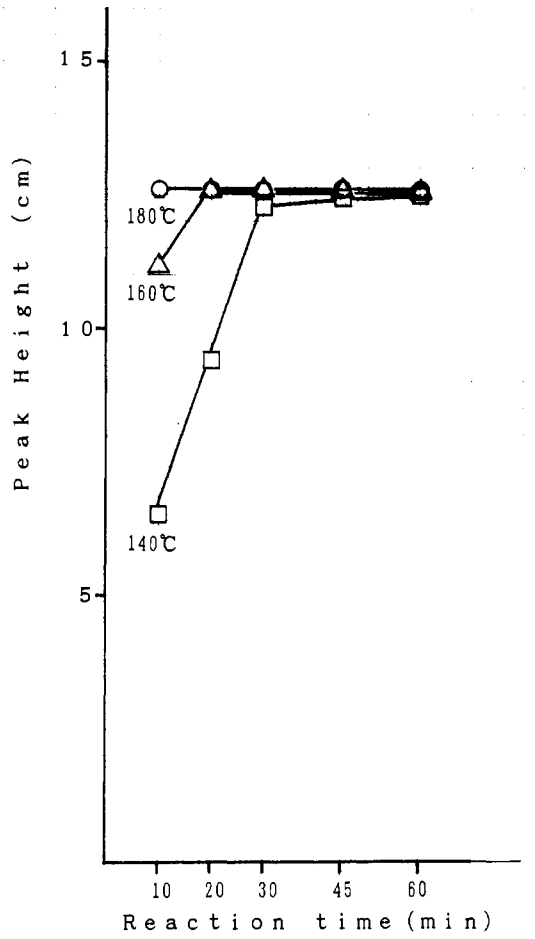

Figure 5. Effects of reaction temperature on yield of product obtained from reaction of triforine with methanol (composition

were combined in a 500-mL round-bottom flask. The combined extract was evaporated to dryness on a rotary evaporator, and the residue in the flask was dissolved in 3 mL of methanol. A 2-mL aliquot of the methanol sample solution was treated as the same manner as described in procedure 1.

Standard curves for triforine and TF/2·HCl were individually established with thermal products derived from each corresponding standard, and concentrations of triforine and TF/2 in experimental samples were determined from each corresponding standard curve.

RESULTS AND DISCUSSION

(1) Structure of Thermal Product Derived from Triforine and TF/2·HCl. A product with high response for ECD was obtained by the thermal reaction of triforine with methanol in the closed glass tube. So, the mass spectra of the product were investigated with the intent that it might provide clues for the elucidation of the structure of the product. As can be seen in Figure 1, EI mass spectra of the product show m/z 174 with a mass cluster indicative of three chlorines and m/z 140 with a mass cluster indicative of two chlorines. While ionization in the CI mode produced the pseudomolecular ion (m/z 206) with three chlorines. Since the product was formed from a triforine molecule with three chlorine atoms, it was considered that the simplest structure consistent with the mass spectral data is made up by a combination of (trichloroethyl)formamide (side chain of triforine) from triforine and a methoxy group from methanol. On the other hand, the same product was also obtained by the thermal reaction of *N*-(1,2,2,2-tetrachloroethyl)formamide (TCEF; the starting material for triforine synthesis) with methanol,

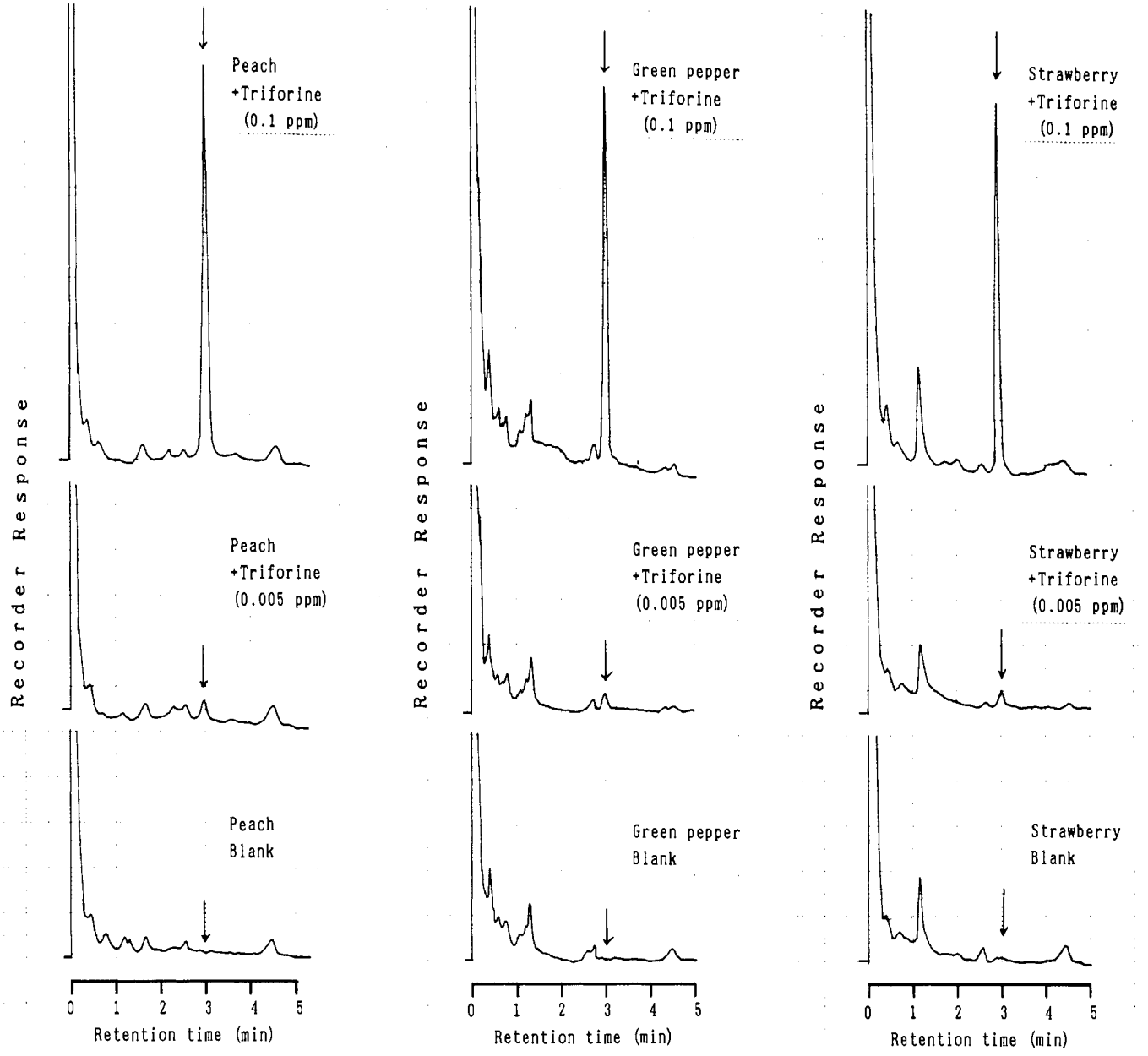

Figure 6. Gas–liquid chromatograms on analyses of spiked and nonspiked crops with triforine.

since the mass spectra and retention time on GC of the product were the same as those of the product derived from triforine. From these results, it can be concluded that the structure of the thermal product is *N*-(1-methoxy-2,2,2-trichloroethyl)formamide.

Triforine can be detected when it (dissolved in a mixture of methanol and ethyl acetate) is directly introduced into gas chromatograph (Ishii, 1980; Nagayoshi et al., 1981); however, the chemical structure of its measurable component on gas chromatogram has not been clarified until now. In this study, the component was also identified as *N*-(1-methoxy-2,2,2-trichloroethyl)formamide by comparing the mass spectra and GC retention time of the measurable component on gas chromatogram from direct GC injection with those of the above thermal product.

The product with high response for ECD was also obtained by the thermal reaction of TF/2·HCl with methanol. The mass spectra and GC retention time of the product were the same as those of the product derived from triforine. From the results thus far obtained, it was proven that an identical compound was derived from all the three compounds: triforine, TF/2·HCl, and TCEF. The proposed reaction sequence is shown in Figure 2.

The real yield of the product cannot be determined because no authentic standard of the product with known content is available. However, the relative yield of each product formed by the reaction of triforine, TF/2·HCl, and TCEF with methanol was in good agreement with the theoretical relative yield ($^2/_{435}$:$^1/_{297}$:$^1/_{211}$ = 1:0.73:1.03) of the corresponding product obtained.

Further, it was found that similar products were also obtained by reaction of triforine with several alcohols other than methanol. The kinds of tested alcohols, the structure of corresponding product obtained, retention time, and relative peak height on GC analyses are shown in Table I. By these data it is possible to select alcohols to achieve good separation from interfering peaks on GC. This is valuable for residue analysis of an analytical compound in many crops.

(2) Optimization of Reaction Conditions for Triforine with Methanol. Reaction accelerators for the reaction of triforine with methanol were studied, and the results are shown in Figure 3. Several reagents such as pyridine, acids, and acetate esters were tested. Coexistence of both pyridine and ethyl acetate in the reaction enhanced the yield of the product.

Table II. Recovery Test of Triforine and TF/2•HCl Metabolite from Peach, Strawberry, and Green Pepper[a]

	% recovery					
	triforine			TF/2·HCl		
sample	1	2	av	1	2	av
peach	98.5	102	100	66.0	69.8	67.9
strawberry	98.0	101	99.5	71.5	76.0	73.8
green pepper	97.0	97.3	97.2	68.9	70.0	69.4
average			98.9			70.4

[a] Fortification levels at 0.1 ppm for triforine and 0.4 ppm for TF/2·HCl.

Effects of reaction time and reaction temperature on yield of the product formed by the reaction of triforine with methanol were examined, and the results are shown in Figures 4 and 5, respectively. Ethyl acetate and pyridine were found to be effective for making the reaction time short as required for constant yield.

As can be seen in Figure 5, the higher the reaction temperature, the shorter the reaction time required for constant yield. However, the use of a reaction temperature above 180 °C is in danger of detonation of the closed glass tube. For these reasons, the reactive conditions with methanol were established as shown in procedure 1.

(3) Sensitivity, Reproducibility, and Linearity. The reproducibility of the thermal reaction for six replicate analyses of methanol sample solutions containing only standard triforine or TF/2·HCl was quite good. The relative standard deviation values were 2.2% (n = 6) for triforine and 2.3% (n = 6) for TF/2·HCl, respectively. Stability of the product (in acetone) at room temperature was evaluated by injection of the product to the GC again 1 month after the reaction. Relative sensitivity to an internal standard (a pesticide) was the same between the measurements just after the reaction and 1 month after the reaction. No new peaks other than that of the analyte was observed. The absolute detection limit (peak height equal to 3 times the noise) is 2 pg for triforine and 4 pg for TF/2·HCl, respectively. Both calibration plots for triforine and TF/2·HCl were linear from 0.1 to 0.5 ng.

(4) Application of Thermal Reaction to Residue Analysis. We have applied the thermal reaction to the residue analysis of triforine and TF/2 metabolite in the three crops, and the accuracy was evaluated by recovery experiment (Table II), in which known amounts of triforine or TF/2·HCl were added to peach, strawberry, and green pepper.

In triforine analyses, good recoveries were obtained in all samples. But only about 70% of TF/2·HCl was recovered. This low recovery of TF/2·HCl was probably due to incomplete recovery of the compound by the extraction with ethyl acetate in liquid–liquid partition, since the recovery of TF/2·HCl added to the ethyl acetate extract after the partition was complete. GC chromatograms on the analyses of spiked and nonspiked crops with triforine are shown in Figure 6.

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Inhibition of Hepatic Mevalonate Biosynthesis by the Monoterpene, *d*-Limonene

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The adaptive increase in avian hepatic 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity following fasting and refeeding was muted when the monoterpene *d*-limonene was fed. The suppression of the induction of HMG-CoA reductase activity was dose dependent to 100 ppm dietary *d*-limonene and additive to that of dietary cholesterol. Noninduced hepatic HMG-CoA reductase activity in rats fed a diet containing 1.0% *d*-limonene was 55% of the activity in rats fed a control diet.

Brown and Goldstein (1980) described the multivalent feedback regulation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase in which a non-sterol, post-mevalonate product(s) regulates HMG-CoA reductase independently of, and in addition to cholesterol. Among the prospective exogenous, non-sterol modulators are *d*-limonene, the initial cyclic monoterpenoid product of the mevalonate pathway of secondary plant metabolism, and oxy- and hydroxy-substituted mono- and bicyclic monoterpenes that elicited a transient decrease in rat hepatic HMG-CoA reductase mass and activity when given by gavage (Clegg et al., 1980, 1982). *d*-Limonene (Elegbede et al., 1984, 1986) and other monoterpenes (Wattenberg, 1983; Maltzman et al., 1985) act as anticarcinogens when added to diets of rats treated with a chemical carcinogen,

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