Analysis of Piperazine, of the Piperazine Generating Fungicide Triforine, and of Triforine's Metabolites in Barley Plants

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The systemic fungicide triforine (N,N'-bis(1-formamido-2,2,2-trichloro-ethyl)-piperazine is used principally against powdery mildew, rust and other leaf diseases on cereals. The half-life of triforine in plants varies from 20 to 30 days depending on the plant species (Fuchs et al., 1976; Rouchaud et al., 1976). An analytical procedure is thus needed not only for triforine but also for its metabolites in plants, in order to have a true measure of plant's contamination. This is necessary specially because of the potential nitrosation of piperazine (an identified metabolite of triforine) in the stomach of animals, and the carcinogenic properties of the nitrosopiperazines (Mirvish, 1971). The described analytical procedures for piperazine are not useful for plant residues because they lack sensitivity, or because of interferences due to plant compounds (Fricke and Walters, 1966; Loucks and Nauer, 1967). Triforine breaks down when directly gas liquid chromatographied (GLC), but may be analysed after acidic hydrolysis and GLC of the generated chloral (Eichler, 1972). This method measures neither most of the metabolites of triforine, nor the total incorporation of triforine in the plant. The sole thin-layer-chromatography (TLC) of triforine and of its metabolites is not useful with non-radioactive compounds because the detection methods suffer from interferences by plant compounds.

We developed a reliable and sensitive procedure for the measure of the total amount of compounds containing the piperazine ring present in barley plants treated with triforine; this is the sum of the soluble compounds and of the one bound to the residue left by the extraction. We developed also a method for the specific analyses of triforine and its identified metabolites N(1-formamido-2,2,2-trichloro-ethyl)-piperazine (TF/2), and piperazine in these plants. Combined preparative TLC (PTLC) and GLC are used; new procedures are used for GLC of piperazine and chloral, the latest one being generated by a modified procedure of hydrolysis.

EXPERIMENTAL

Chemicals. Saprol, triforine, ^{3}H triforine (uniformly labelled in the piperazine ring, 105 μCi/mg) and TF/2 were from Cela Merck (Germany). PTLC were performed with activated (105°C, 24 hr) plates (Kieselgel 60 F254, 20x20 cm/2 mm, Merck). Porapak P was from Waters Assoc., and Silyl-8 from Pierce Chem. The other chemicals were of analytical grade from Merck.

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GLC and PTLC. A 5 µl "plug" of the final GLC sample is injected with a series 7005 N Hamilton syringe into a Varian Aerograph model 2700. Recorder is a Varian model A-25, 1 mV. Chart speed is 50 cm/hr. The glass columns (intern. diam. 2 mm) contain Porapak P 80- 100 mesh treated with Silyl-8 (50 µl at 150°C, 50 µl at 200 °C, and 50 µl at 250°C). Carrier gas is nitrogen at 40 ml/min. For chloral GLC, detection is by ^{3}H electron capture; the column (1.80 m) is at 150°C, injection and detection temperatures being 160°C; the retention time of chloral is 1.4 min; the detector is linear when the amount of chloral in the column is lower than 4 nanograms. For piperazine GLC, detection is by flame ionisation (H_2: 30 ml/ min; air: 300 ml/min); the column (1 m) is at 160°C, injection and detection temperatures being respectively 240 and 210°C; the retention time of piperazine is 1.6 min; the detector is linear when the amount of piperazine in the column is lower than 5 µg. The GLC sample is eventually diluted so that detection is linear. The peak height is compared with that of a reference standard, each day a calibration curve being made using standard solutions containing different concentrations of the analysed compound. Under these conditions, there is a linear relationship between peak height and the amount of analysed compound.

PTLC is performed by applying the sample as a band by means of a pipette. The plates are developed to 16 cm with solvent I (ethyl acetate) for the analysis of triforine (R_f=0.67), and with the solvent system II (n-butanol-acetic acid-water, 4:1:5 ml/ml, upper phase) for the analyses of TF/2 (R_f=0.52) and piperazine (R_f=0.26). The zones comprised between 0.69 and 0.56 (R_f units) for triforine (solvent I), and between 0.54 and 0.41 for TF/2 (solvent II) are scraped off. The R_f are controlled with standards, triforine being detected by means of iodine vapour, and TF/2 and piperazine by spraying the plates with a solution of vanillin (2 g%) in isopropanol and heating at 50°C.

Extraction procedure. For recovery studies, a representative sample of straw or grains of untreated barley plants (Hebe) is fortified directly with a known amount of the studied compound before adding the extracting solvent. Hundred g of tissue are homogenised during 5 min at 20°C with 300 ml chloroform in a Sorvall omnimixer (8,000 rpm). The residue is decanted, and homogenised three times more with chloroform (3x150 ml). The combined homogenates are centrifuged (4,500 rpm) during 15 min and filtered, giving the primary chloroform extract and the solid residue, left by the extraction, which is dried at room temperature. The primary chloroform extract is transferred into a separatory funnel (1 liter) and extracted 3 times with aqueous 0.1 N HCl (3x90 ml), giving the secondary chloroform extract and, after combination, the 0.1 N HCl extract. The secondary chloroform extract is filtered through potassium carbonate, and concentrated to 5 ml in a rotatory vacuum concentrator at 15°C, giving the concentrate A. The 0.1 N HCl extract is brought to pH 4 with aqueous 2 and 0.1 N NaOH, divided into two parts which are freeze- dried, giving the concentrate B.

Determination of the total amount of compounds containing the pi-

perazine ring present in barley plants treated with triforine. A part of the concentrate A is transferred into a 250 ml two- necked round-bottom flask, and is evaporated to dryness by a slow stream of nitrogen at 20°C. Eighty ml of 10% sulfuric acid are added, and the mixture is distilled (ground joints glass apparatus) at 170°C (oil bath) under nitrogen (20 ml/min) during 1.5 hr. The receiving flask (200 ml) is cooled in ice and contains 10 ml of distilled water . The aqueous distillate (about 80 ml) is transferred into a separatory funnel (250 ml), mixed with 30 g of NaCl, and shaken two times with ethyl formate (2x15 ml). The combined organic phases are made up with ethyl formate to a volume of 25 ml in a volumetric flask, and dried with calcium chloride. The GLC of this solution measures chloral, two moles of chloral corresponding to one of the compound which contains the piperazine ring (as in triforine).

A part of the concentrate B is dissolved into 10 ml of aqueous 2 N HCl, maintained at 100°C during 4 hr in a sealed tube, brought to pH 4 with aqueous 2 and 0.1 N NaOH, and freeze- dried. The residue is shaken (1 min) with a mixture of 0.5 ml aqueous 6 N NaOH and 5 ml chloroform. The chloroform phase is analysed by GLC for piperazine, one mole of piperazine corresponding to one of the compound which contains the piperazine ring.

The solid residue left by the extraction is heated (reflux) with 80 ml aqueous 4 N HCl during 2 hr, centrifuged (4,500 rpm, 15 min), brought to pH 4 with aqueous 2 and 0.1 N NaOH, and freeze-dried. Piperazine is analysed in the residue by GLC as with concentrate B, one mole of piperazine corresponding to one of the compound which contains the piperazine ring and which is bound to the solid residue. The results relative to the concentrates A and B, and to the solid residue are added up, giving the total amount of compounds containing the piperazine ring present in the treated plant.

Analysis of triforine, TF/2, and piperazine. A part of the concentrate A was PTLC with solvent I. The band corresponding to triforine is scraped off, hydrolysed in sulfuric acid and analysed for chloral by GLC as described above. Two moles of chloral correspond to one of triforine.

A part of the concentrate B is shaken with a mixture of 0.5 ml aqueous 6 N NaOH and 5 ml chloroform. An aliquot of the chloroform phase is injected into the GLC for the analysis of piperazine. The rest of the chloroform phase is PTLC with the solvent II. The band corresponding to TF/2 is scraped off, hydrolysed in sulfuric acid, and GLC analysed for chloral as previously described. One mole of chloral corresponds to one of TF/2.

RESULTS AND DISCUSSION

For the determination of the recoveries of triforine, TF/2, and piperazine at a definite concentration in a determined part of the plant, separately with each of these compounds 2 analyses are performed following the procedure relative to the specific determination of them, and 2 analyses following the procedure relative to the determination of the total amount of compounds con-

taining the piperazine ring in the concentrates A and B. No systematic difference is observed for both procedures, and Table 1 gives the total range of percentages of compound recovered for each group of 4 analyses. Each of the three compounds is found

TABLE 1

Recovery of triforine, TF/2, and piperazine in barley straw and grains fortified separately with varying amounts of these chemicals

Concentration of the added compound, ppm relatively to the fresh weight of tissue	0.05	0.5	2	10
Analysed part of the plant, and compound	Range of percentages of compound recovered			
1) Straw				
Triforine	82-103	91-98	88-107	85-96
TF/2	78-102	76-101	79-95	83-105
Piperazine	77-95	79-104	78-93	76-92
2) Grains				
Triforine	88-104	87-97	83-103	89-102
TF/2	81-95	85-108	80-102	86-92
Piperazine	74-93	79-95	81-96	73-99

in its own extract and not in the other one. None of these compounds degenerates into the others during the analysis; their identity is determined by PTLC with several developing solvents, and may be rapidly controlled again with new plant materials or treatment conditions (Rouchaud et al., 1976).

For the analysis of the total amount of compounds containing the piperazine ring in the concentrates A and B, and relatively to the unidentified metabolites, the procedure is based upon their quantitative hydrolysis into chloral and piperazine, what is suggested by the chemical structures of triforine, and its identified and potential metabolites, and by the hydrolytic breakdown of triforine in aqueous solution (Fuchs and Ost, 1976). More than 90 mole% of the concentrate A is triforine, what justifies the relationship of two moles of chloral for one of triforine or its metabolite in this concentrate (Rouchaud et al., 1976); if there was a discrepancy, the error would not be large on account of the apparent metabolic pathway of triforine and the extraction procedure used here. The radioactivity of the concentrate B, from ^{3}H triforine treated plant, is compared to the amount of piperazine observed in the same concentrate by the procedure for the analysis of the total amount of compounds containing the piperazine ring (2 N HCl hydrolysis); by this procedure, the recovery of the unidentified metabolites in concentrate B is higher than 70% (Rouchaud et al., 1976). In the same way, the radioactivity of the solid residue is compared to the amount of piperazine observed by the procedure for total analysis of the piperazine containing compounds (4 N hydrolysis); the recovery of the

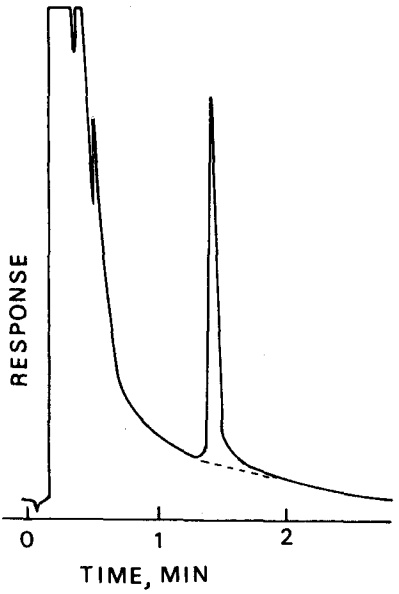

Figure 1. Chloral generated from triforine in concentrate A from shoots of triforine treated barley plants. Peak value= 4 nanograms.

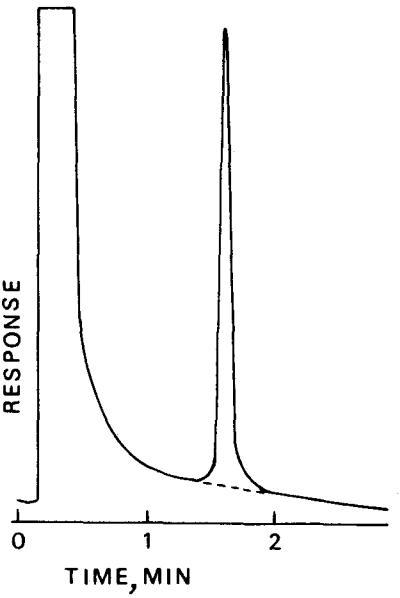

Figure 2. Piperazine from the solid residue left by the extraction of straw of triforine treated barley plants. Peak value=30 nanograms.

last procedure is 68-94% when the concentration of the piperazine bound (as such or as an other metabolite of triforine) to the residue is 0.5-5.3 ppm relatively to the total fresh weight of analysed tissue.

This analytical procedure makes possible the specific analysis of triforine and of its two identified metabolites TF/2 and piperazine present in triforine treated barley plants. It permits also the determination in the plant of the total amount of unidentified chloroform soluble compounds which contain the piperazine ring, and the total amount of the unextractable piperazine ring containing compounds bound to the solid residue. If wanted, these analyses may be partly performed. The GLC limit of sensitivity for piperazine is 0.03 ppm, and 0.01 ppm for chloral (corresponding to 0.02 ppm for triforine, and 0.01 ppm for TF/2) when gas chromatographing a 5 μl aliquot originating from 100 g of plant. Representative chromatographs illustrating the elution patterns of chloral and piperazine are shown in Figures 1 and 2. The dotted lines are relative to untreated plants. Gas chromatograms of numerous control samples of untreated plants do not show any significant peaks which interfere with those of chloral or of piperazine.

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