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RESIDUE ANALYSIS OF TRIFLUMIZOLE AND ITS METABOLITE IN CROPS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

A procedure for high-performance liquid chromatographic (HPLC) determination of triflumizole and its metabolite (Met) in crops was examined. Triflumizole and Met in sample crops were extracted with methanol and re-extracted into methylene chloride. After clean-up of the extract on a Florisil column, triflumizole and Met were determined by HPLC with UV detection at 238 nm. The HPLC column was packed with Nucleosil 5 C_{18} (ODS, 5 μm) and the eluent was acetonitrile–3 m*M* carbonate buffer (7:3, v/v, pH 9.0). The detection limit was 0.01–0.02 ppm and the recoveries from spiked crops (0.5 ppm) were 73–99% for triflumizole and 74–94% for Met. Triflumizole and Met were determined simultaneously and the method was shown to be applicable to residue analysis of these compounds in crops sprayed with Trifmine® in fields.

INTRODUCTION

Triflumizole, (*E*)-4-chloro-α,α,α-trifluoro-N-(1-imidazol-1-yl-2-propoxyethylidene)-*o*-toluidine, is a new fungicide for control of scabs and rusts on fruit trees and powdery mildews on many crops, introduced by Nippon Soda Co. under the trademark Trifmine®[1]. It was reported that triflumizole was metabolized primarily to (*E*)-4-chloro-α,α,α-trifluoro-N-(1-amino-1-yl-2-propoxyethylidene)-*o*-toluidine (Met) in plant materials and that Met was also formed by photolysis[2]. The chemical structures of triflumizole and Met are shown in Fig. 1.

This paper reports a method for the determination of triflumizole and Met in crops by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Reagents and materials

The HPLC column, Nucleosil 5 C_{18} (Macherey Nagel & Co.) was obtained

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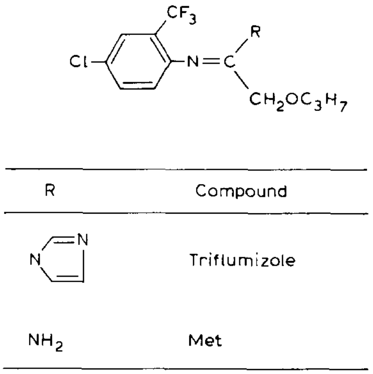


Fig. 1. Chemical structures of the compounds referred to in the text.

from Senshu Scientific. Florisil (PR grade, Floridin) was obtained from Wako Pure Chemical Industries. Before use, it was deactivated with 5% water. Carbonate buffer (3 m*M*, pH 9.2) was prepared as follows: 0.8 ml of 0.2 *M* aqueous sodium carbonate and 9.2 ml of 0.2 *M* aqueous sodium bicarbonate were mixed and diluted to 660 ml in water. Pure standard triflumizole and Met were obtained from Nippon Soda. The organic solvents were of pesticide-residue-analytical grade (or their equivalent) and all other reagents were of analytical reagent grade.

Apparatus

A TRI-ROTAR chromatograph with an UVIDEC-100-V, UV spectrophotometer (Japan Spectroscopic) was used for HPLC. Also used was an Hitachi Model 323 spectrophotometer.

Sample preparation and extraction

Brown rice and barley (whole grain). About 500 g of sample were ground in a coffee-mill. A 10-g aliquot was placed into a 300-ml flask, and 30 ml of water and 80 ml of methanol were added. Then the contents were blended with an homogenizer (Polytron®) for 1–2 min, and the flask was shaken mechanically for 30 min.

Other crops. About 1 kg of crops were homogenized with a mixer. A 20-g aliquot was placed into a 300-ml flask, 80 ml of methanol were added and the flask was shaken mechanically for 30 min. In the cases of grape, cherry and tomato samples, the homogenate (20 g) was treated with 5–10 ml of 0.2 *M* aqueous sodium carbonate to bring the pH to 5–7 before extraction with methanol.

Samples used for the stability study of the compounds in frozen samples were prepared as follows: crop homogenate (20 g) was placed in a 300-ml flask and spiked separately with 10 μg of triflumizole and Met. The flask was stoppered, shaken thoroughly and then stored in a freezer at −20°C. After storage, the sample was thawed in a water-bath at 22°C for 10 min, then analyzed immediately.

Clean-up

The crude extract in the 300-ml flask, after being shaken, was filtered through a filter-paper under suction. The filter cake and the flask were washed with 30 ml of methanol and the washing was filtered. The filtrates were pooled and transferred to a 500-ml separating funnel. To the funnel, 5 g of sodium chloride and 100 ml of water were added and triflumizole and Met were extracted twice with 80 ml of methylene chloride by shaking for 5 min. Then, the methylene chloride extract was dried with anhydrous sodium sulphate, the extract was filtered through a filter-paper and the filtrate was evaporated to dryness under reduced pressure in a water-bath at $<40°C$.

A slurry of 10 g of Florisil in hexane was poured into a glass tube (300 mm × 15 mm I.D.) and 5 g of anhydrous sodium sulphate were added on top of the adsorbent. The dried sample residue was dissolved in two 5-ml portions of acetone–hexane (5:95, v/v) and placed on top of the column. Then the column was washed with 50 ml of the same solvent. After discarding the eluate, the column was eluted with 100 ml of acetone–hexane (10:90, v/v). The eluate was collected in two separate fractions: the first 40 ml containing Met, and the next 60 ml containing triflumizole.

Each eluate was evaporated to dryness under reduced pressure in a water-bath at $<40°C$. The dried residue was dissolved in 2.0 ml of acetonitrile and a 10-μl aliquot (equivalent to 50 or 100 mg of the sample) was subjected to HPLC.

HPLC determination

The HPLC conditions were: column, Nucleosil 5 C_{18} (200 mm × 6.0 mm I.D.); eluent, acetonitrile–3 m*M* carbonate buffer (7:3, v/v), pH 9.0 with phosphoric acid; flow-rate, 1.0 ml/min; column oven, 40°C; detection wavelength, 238 nm; sensitivity, 0.008 absorbance units full scale.

Standard solutions of triflumizole and Met were prepared as follows: 100 mg of pure standard material were accurately weighed into a 100-ml volumetric flask and the flask was filled up to the mark with acetonitrile (1 mg/ml). This solution was diluted in acetonitrile to provide standard solutions of 0.2, 0.5, 1.0 and 2.0 μg/ml for each compound.

A 10-μl aliquot of the standard solutions was subjected to HPLC. Calibration graphs were prepared by plotting the peak heights against the amounts of triflumizole and Met injected. The amount of triflumizole or Met in the sample extract was determined by comparing the peak height with each calibration graph.

RESULTS AND DISCUSSION

HPLC conditions

Using a reversed-phase column (Nucleosil 5 C_{18}, ODS, 5 μm), a few modifiers added to acetonitrile were examined as eluents for the HPLC determination of triflumizole and Met (Table I). Water resulted in long and non-reproducible retention times for Met together with tailing peaks. These results suggested that Met, which is basic in nature, might be tightly adsorbed on residual silanols of the silica-based ODS packing. A subsequent study showed that a column the residual silanols of which are fully endcapped with trimethylsilyl groups, *e.g.*, Inertsil ODS (5 μm; Gasukuro Kogyo, Japan) gave a reproducible retention time and a sharp peak for Met using the same eluent. Carbonate buffer (eluent pH: 9.0) suppressed the ionization of Met, giving good peak shapes and sensitivity, and also sufficient resolution from

TABLE I

EFFECT OF MODIFIER ON HPLC DETERMINATION

20 ng/10 μl of triflumizole and Met were injected. HPLC conditions as in the text. Eluent: acetonitrile–modifier (7:3, v/v).

Modifier	*pH*	*Retention time (min)*		*Peak height (cm)*	
		Triflumizole	*Met*	*Triflumizole*	*Met*
Water	6.4	13.8	13.8–18.6	8.2	0.2–0.6
(20 m*M*) Methanesulphonic acid	1.5	4.6	3.8	14.0	5.3
(3 m*M*) Carbonate buffer	9.0	12.0	8.5	9.4	8.6

triflumizole. On the other hand, methanesulphonic acid, a ion-pair reagent for basic compounds, caused a relatively high UV background and lower sensitivity for Met under the proposed HPLC conditions.

UV absorption spectra (Fig. 2) showed that triflumizole and Met both have an absorption maximum at $\lambda_{max.}$ = 238 nm. Further study revealed that, at lower pH, $\lambda_{max.}$ of Met is shifted to shorter wavelengths, resulting in a lower HPLC response to Met in the eluents of lower pH (Table II).

It is known that silica gel deteriorates in alkaline solution, but Nucleosil 5 C_{18}, which was used in this study with acetonitrile–3 m*M* carbonate buffer (7:3, v/v, pH 9.0) as eluent, functioned well for at least 4 months without deterioration. The concentration of the carbonate buffer was minimized to prevent deposition of salts in the HPLC line. Furthermore, the line was purged with a buffer-free solvent, acetonitrile–water (7:3, v/v), after use.

The proposed HPLC method allowed separation of triflumizole and Met (Fig. 3) and other co-extractives, and also provided adequate sensitivity. The retention

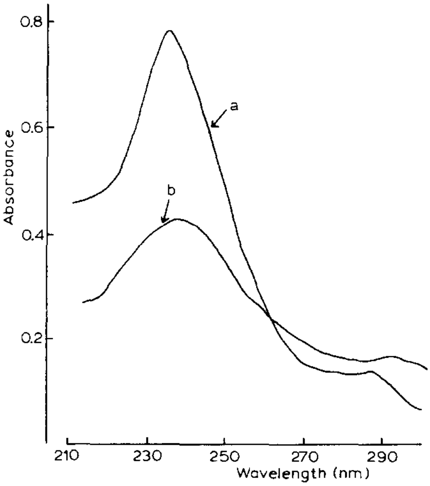


Fig. 2. UV absorption spectra of 10 μg/ml solutions of triflumizole (a) and Met (b) in acetonitrile–3 m*M* carbonate buffer (7:3, v/v, pH 9.0).

TABLE II

EFFECT OF ELUENT pH ON HPLC DETERMINATION

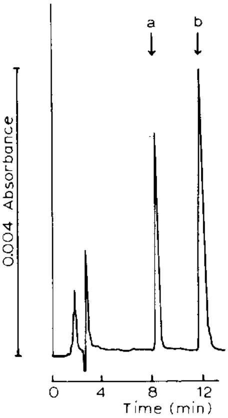
Eluent: acetonitrile–3 m*M* carbonate buffer (7:3, v/v), pH 3.0, 5.0, 7.0, 9.0 with phosphoric acid. Other HPLC conditions as in the text. 20 ng/10 µl of triflumizole and Met were injected.

pH of eluent	*Capacity factor, k'*		*Relative sensitivity*	
	Triflumizole	*Met*	*Triflumizole*	*Met*
3.0	2.8	1.0	87	50
5.0	3.4	2.5	91	39
7.0	3.3	2.4	94	59
9.0	3.3	1.8	100	100

times of triflumizole and Met were 12.0 and 8.5 min, respectively and the minimum detectable quantity was 1 ng (signal-to-noise ratio: 3) for each compound. The calibration graph between the amount and the peak height was linear over the range 2–20 ng with a correlation coefficient of greater than 0.999 for each compound. The reproducibility of the determination was also good: coefficients of variation for five determinations of each standard solution were less than 1.3% for the peak height and less than 0.9% for the retention time.

Extraction and clean-up

In the development process it was found that the pH of the sample homogenate was critical to the recovery of each compound. To elucidate the problem, 20 ml of 0.2 *M* aqueous sodium carbonate (pH 3, 5, 7 or 9 with phosphoric acid) instead of the sample homogenate were spiked with 10 µg of triflumizole and Met, then analyzed



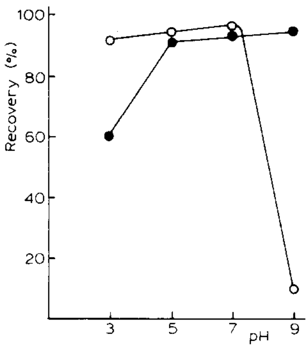


Fig. 3. Chromatogram of standard solution containing triflumizole (20 ng, peak b) and Met (20 ng, peak a). The HPLC conditions are described in the text.

Fig. 4. Effect of pH on recovery. A 20-ml volume of 0.2 *M* aqueous sodium carbonate (pH 3, 5, 7, 9 with phosphoric acid) was spiked with 10 µg of triflumizole and Met, then subjected to the whole analytical procedure. ○, Triflumizole; ●, Met. (The decrease in recovery of triflumizole at pH 9 is caused by the unfavourable extraction step into alkaline methanol.)

by the proposed procedure. It was found (Fig. 4) that the recovery of triflumizole was lower from higher pH solution (pH > 7.0) and that of Met was, on the contrary, lower from lower pH solution (pH < 5.0). These results might be due to the chemical properties of the compounds; triflumizole is unstable in alkaline methanol. It is sufficiently stable in the pH 9.0 eluent however to permit elution without losses. Some losses of triflumizole occur during shaking of the sample (pH > 7.0) with methanol, and Met is basic in nature, resulting in poor recovery in the extraction from lower pH solution into methylene chloride (Table III). From the above results, the pH of the sample homogenate should be in the range 5–7 for the extraction steps; it should be checked before analysis and adjusted, when necessary. In this experiment, the samples of grape, cherry and tomato required pH adjustment; 5–10 ml of 0.2 *M* aqueous sodium carbonate were added to the sample homogenate to adjust the homogenate to pH 5–7.

Florisil (deactivated with 5% water) and silica gel were evaluated for removal of co-extracted sample constituents. The former was more effective. In order to eliminate the interferences still remaining in the eluate, the eluate from the Florisil column [100 ml of acetone–hexane (1:9, v/v)] was collected in two separate fractions, the first 40 ml containing Met and the next 60 ml containing triflumizole.

When interfering peak(s) still appeared near the Met peak and disturbed its quantitative analysis, the following clean-up procedure was found to be useful: after clean-up on the Florisil column, Met in the eluate was extracted with 1 *M* hydrochloric acid, and re-extracted into methylene chloride after neutralization of the acid phase.

Recovery and detection limit

Known amounts of triflumizole and Met were added to the crop homogenates and determined by the proposed procedure. The recoveries were 73–99% for triflumizole and 74–94% for Met (Table IV). The detection limits of triflumizole and Met were 0.02 ppm for barley and brown rice, and 0.01 ppm for the other crops: the minimum detectable quantity was 1 ng for both compounds and 50 or 100 mg of the sample were chromatographed. The method was also reproducible (Table IV). Typical chromatograms are shown in Fig. 5. The peaks of triflumizole and Met were completely separated from interfering peaks.

TABLE III

EXTRACTION FROM AQUEOUS SOLUTION INTO METHYLENE CHLORIDE

Triflumizole and Met (each 10 μg) were added to 150 ml of aqueous solution (pH 3.0, 5.0, 7.0, 9.0) and extracted twice with 80 ml of methylene chloride by shaking for 5 min.

*pH of the aqueous solution**	*Extraction (%) into methylene chloride*	
	Triflumizole	*Met*
3.0	96	71
5.0	97	97
7.0	95	97
9.0	94	97

* 0.1 *M* Acetate buffer, adjusted to pH 3.0, 5.0, 7.0, 9.0 with hydrochloric acid or aqueous sodium hydroxide.

TABLE IV

RECOVERIES

Samples were spiked with 0.5 ppm triflumizole and Met.

Crop	*Compound*	*Recovery* (%)*	*Crop*	*Compound*	*Recovery* (%)*
Water-melon	Triflumizole	83 ± 2.5	Sweet pepper	Triflumizole	77 ± 0.3
	Met	87 ± 7.1		Met	82 ± 2.2
Apple	Triflumizole	84 ± 4.7	Pods (immature)	Triflumizole	73 ± 2.7
	Met	74 ± 4.5		Met	83 ± 1.3
Cucumber	Triflumizole	78 ± 0.4	Japanese pear	Triflumizole	87 ± 4.1
	Met	88 ± 3.4		Met	87 ± 6.9
Peaches	Triflumizole	80 ± 5.5	Japanese persimmon	Triflumizole	89 ± 3.2
	Met	87 ± 3.7		Met	86 ± 2.7
Grape	Triflumizole	81 ± 3.5	Egg-plant	Triflumizole	78 ± 1.8
	Met	92 ± 6.1		Met	88 ± 1.4
Strawberry	Triflumizole	93 ± 1.7	Barley (whole grain)	Triflumizole	77 ± 4.0
	Met	74 ± 2.4		Met	90 ± 9.2
Tomato	Triflumizole	76 ± 4.3	Brown rice	Triflumizole	87 ± 4.9
	Met	83 ± 9.1		Met	94 ± 3.3
Cherry	Triflumizole	99 ± 0.3			
	Met	84 ± 4.0			

* Mean ± S.D. (n = 3–5).

Stability of the compounds in crop homogenates frozen during storage

The stabilities of pesticide chemicals in environmental samples during storage have become of interest in recent years[3–10], because in residue analysis, samples often cannot be analyzed immediately after sampling. They have to be stored under cold conditions in an intact state or after homogenization. The stabilities of triflumizole and Met were, therefore, examined when crop homogenates were stored at −20°C.

Table V indicates that a maximum of 71% of triflumizole decomposed when frozen during storage (11–252 days), while Met was relatively stable (decomposition: maximum 37%). It was shown that the decomposition of triflumizole increased with

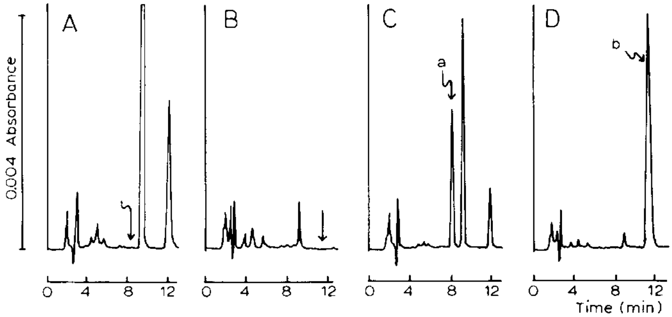


Fig. 5. Chromatogram of cherry extracts. (A) and (B), Control; (C) and (D), spiked with 0.5 ppm Met (a) or triflumizole (b). Samples of 100 mg (A and B) or 40 mg (C and D) were chromatographed. For operating conditions see text.

TABLE V

STABILITIES OF THE COMPOUNDS IN CROP HOMOGENATES FROZEN DURING STORAGE

Samples were spiked with 0.5 ppm triflumizole and Met.

Crop homogenate	*Storage period* (days)*	*Decomposition** (%)*	
		Triflumizole	*Met*
Grape	28	<5	<5
	183	70	<5
Egg-plant	154	40	14
	252	71	37
Strawberry	24	15	<5
	115	47	<5
Cucumber	11	<5	<5
	60	27	27
Cherry	215	47	<5
Apple	61	36	14
Japanese pear	40	29	8
Peaches	26	24	<5
Sweet pepper	20	12	<5
Water-melon	62	11	19
Pods (immature)	15	9	<5
Japanese persimmon	16	<5	<5

* In a freezer at −20°C.
** Mean of duplicate experiments.

storage time and was dependent on the kind of crops. Also that Met, a major metabolite of triflumizole in plant[2], was not formed in crop homogenates spiked with triflumizole during frozen storage. For analysis of triflumizole and Met in crops, it was concluded that the crops should be analyzed immediately after sampling or stored, when necessary, in an intact state in a freezer (−20°C) to minimize the decomposition.

The method proposed permitted simultaneous determination of triflumizole and Met, which have different polarities. It is simple, precise and accurate, and could be applied to the residue analysis of triflumizole and Met in crops.

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