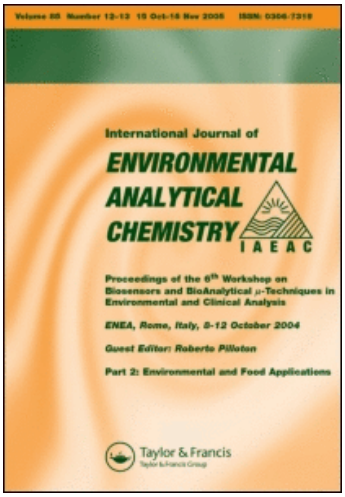

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Analysis of Fenitrothion and Metabolites in Stored Wheat

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A simple and rapid method for the analysis of fenitrothion and its metabolites, fenitrooxon, *S*-methyl fenitrothion, demethyl fenitrothion, demethyl *S*-methyl fenitrothion, 3-methyl-4-nitrophenol, and dimethyl phosphorothioic acid in stored wheat has been developed. Simultaneous analysis of the extract was conducted using FPD-GLC after derivatization with diazoethane except for 3-methyl-4-nitrophenol which was analyzed directly by EC-GLC. Recoveries of all compounds from wheat fortified at the levels from 0.1 to 5.0 ppm were greater than 90%.

The developed method was used to quantitatively determine major metabolites found in grain treated with fenitrothion and stored at 20°C for 12 months. Demethyl fenitrothion, 3-methyl-4-nitrophenol, and dimethyl phosphorothioic acid were the major breakdown products of fenitrothion found in stored wheat. Confirmation of these metabolites was carried out by chemical derivatization plus FPD-GLC and by TLC.

INTRODUCTION

Fenitrothion (*O*, *O*-dimethyl *O*-(3-methyl-4-nitrophenyl) phosphorothioate), a broad spectrum insecticide, is being developed for use as a grain protectant against insect infestations.

Following application of the insecticide to stored grain, residues may be graded into various metabolites. Many of these metabolites pose a toxicological hazard to mammals.[1,2] No method has been reported to date for the extraction and determination of fenitrothion and its metabolites which may be found in stored wheat. Shafik and Enos[3] and Shafik *et al.*[4,5] have reported methods for the simultaneous FPD-GLC analysis of six dialkyl metabolites, common to many organophosphorus pesticides, in human blood and urine using the derivatization procedure described by Stanley.[6] One of these compounds, namely dimethyl phosphorothioate, has been reported as a fenitrothion metabolite.

Greenhalgh[7] developed a method for the simultaneous analysis of fenitrothion (F) and its degradation products, amino fenitrothion (AF), fenitrooxon (FO), and *S*-methyl fenitrothion (SMF) from water. The analysis of the extract was conducted using AFID-GLC. Greenhalgh *et al.*,[8] described a procedure for F, AF, SMF and de-*S*-methyl fenitrothion (DSMF) in natural aquatic systems. Samples were extracted prior and after acidification to pH 1 with ethyl acetate. The first extract contained F and AF, and the second, DMF and DSMF. Analyses of the extracts were performed by AFID-GLC.

Recently, Volpe and Mallet[9] developed a method for the simultaneous analysis of fenitrothion and five of its metabolites, AF, SMF, formyl fenitrothion (FF), and hydroxymethyl fenitrothion (HMF) from water. Samples were passed through either XAD-4 or -7 resins and the compounds were eluted with an organic solvent such as ethyl acetate or methylene chloride. Extracts were then analyzed by FPD-GLC.

The use of high pressure liquid chromatography (HPLC) for the analysis of fenitrothion and metabolites has been reported. Abe *et al.*,[10] developed a procedure for the simultaneous analysis of F, FO, DMF, and 3-methyl-4-nitrophenol (MNP) using HPLC coupled to an ultraviolet detector (UV) at 260 nm. Cochrane *et al.*,[11] reported an analytical method for the quantitation of contaminants in technical grade fenitrothion using UV_{254}-HPLC. The chromatographic system used gave good separation of F, FO, SMF, MNP and carboxy fenitrothion.

In this study a sensitive GLC method for the analysis of fenitrothion and several of its metabolites in stored wheat is developed and metabolites found in treated wheat stored for 12 months are quantified.

EXPERIMENTAL

Reagents

1) Solvents: all pesticide grade—Caldeon Labs. Ltd., Georgetown, Ontario.

2) Potassium carbonate (3%, w/v): Three g of anhydrous potassium carbonate (Macco Reagent A.C.S.) were dissolved in 100 ml distilled water.

3) Hydrochloric acid: 4N saturated with sodium chloride.

4) Thallium (III) chloride (1%, w/v): One g of $TlCl_3 \cdot 4H_2O$ (Alfa division, U.S.A.) was dissolved in 100 ml distilled water.

5) Anhydrous sodium sulfate: Reagent grade—Fisher Scientific.

6) *p*-*N*,*N*-dimethylaminocinnamaldehyde: (Sigma Chem. Co., U.S.A.) —0.5% in ethanol:acetic acid (1:1, v/v).

7) Diazoethane: generated from *N*-ethyl-*N*-nitro-*N*-nitrosoguanidine (Aldrich Chem. Co.) (Caution-potential carcinogen, mutagen, irritant) according to the method of Stanley.[6]

8) Silica gel: 60–200 mesh Grade 950, Fisher Scientific Co.

9) Grain: Hard red spring wheat, Neepawa variety.

10) Analytical standards: fenitrothion, fenitrooxon, *S*-methyl fenitrothion, demethyl fenitrothion, demethyl *S*-methyl fenitrothion, 3-methyl-4-nitrophenol, and dimethyl phosphorothioic acid were supplied by Stauffer Chemical Co., Richmond, CA.

Apparatus

1) Gas-liquid chromatographs (GLC): A Tractor Micro Tek 220 equipped with a Melpar flame photometric detector (FPD) operator in the phosphorus mode (526 nm) and a Varian model 2440, equipped with a tritium foil electron capture (EC) detector were used. The operating conditions for FPD-GLC were as follows: 1.5 m × 4 mm (id) silanized Pyrex glass columns packed with either 5% OV-101 or 3% OV-225 on Chromosorb W HP, 80/100 mesh; temperature (°C), inlet 210; column (isothermal) 80, 120, or 175; detector 210; gas flow rates (ml/min); nitrogen (carrier) 50; hydrogen 180; air 100; oxygen 20. The operating conditions for EC-GLC were: 1.5 m × 2 mm silanized Pyrex glass column packed with 3% OV-101 on Chromosorb W AW DMCS, 80/100 mesh; temperature (°C), injector 180, column 140, detector 190; nitrogen carrier-gas flow rate 40 ml/min.

2) Rotary grinder: GS Iona Model CG8 (General Signal Appliances Ltd., Canada).

3) Glassware: Treated prior to use with Dri-film (SP5800, 15% in toluene), a silanizing reagent, to prevent adsorption of residues and standards.

4) TLC silica gel (G) plates: precoated TLC with silica gel (0.25 and 0.5 mm thickness) Brinkman Instruments, U.S.A.

Analytical method development for fenitrothion and its metabolites in wheat

(1) *Fortification of wheat*

An acetone standard solution (1 ml) containing fenitrothion (F), fenitrooxon (FO), *S*-methyl fenitrothion (SMF), *O*-demethyl fenitrothion (DMF), *O*-demethyl *S*-methyl fenitrothion (DMSMF), 3-methyl-4-nitrophenol (MNP), and dimethyl phosphorothioic acid (D, PTA) was used to fortify wheat samples (25 g, 12.5% moisture content) at the levels of 5.0, 1.0, 0.5, and 0.1 ppm. The fortified wheat samples were allowed to equilibrate for 1 hour after which they were ground for 30 sec in a coffee grinder.

(2) *Extraction of fenitrothion and metabolites*

Two extraction procedures were employed, the first to analyze all compounds when fenitrothion residue level was present in small amounts. If the latter was present in high concentration, this would alter the separation and, consequently, the quantitation of demethyl fenitrothion (to be discussed in detail later). Such a problem would be overcome by isolating neutral from acidic compounds.

(a) *Solvent extraction only*

Fenitrothion and metabolites were extracted from wheat (25 g) by shaking with acidified acetone or methanol (100 ml, 1% of 2N HCl) for 1 h. The extract was filtered quantitatively into a 250 ml round bottom flask, concentrated to 2–3 ml under vacuum at 40°C, and transferred into a 15 ml centrifuge tube to await derivatization of fenitrothion metabolites.

(b) *Extraction plus separation of the acidic metabolites*

Wheat (25 g) fortified with F, FO, DMF, SMF, DMSMF, MNP and DMPTA was extracted with acidified acetone which was concentrated to 2–3 ml as described above. The acetone extract was transferred into a 150 beaker containing 100 ml distilled water. The aqueous layer was adjusted to pH 8.5 with 3% potassium carbonate and F, FO, and SMF were removed by extracting the aqueous solution with two 30 ml portions of methylene chloride. The methylene chloride extract was dried over anhydrous sodium sulfate and concentrated as required in a water bath (45°C) using a stream of nitrogen for determination of F, FO, and SMF.

The aqueous solution was transferred to a 150 ml beaker, adjusted to pH 2 with 6N HCl, saturated with sodium chloride, and returned to the separatory funnel. DMF and DMSMF were removed by extracting the aqueous solution with ethyl acetate (2×50 ml). DMPTA was removed from the aqueous solution by extracting with ether (2×30 ml). The ethyl acetate extract was dried over sodium sulfate as before and concentrated to 2–3 ml under vacuum at 40°C. The ether was dried, concentrated under a gentle stream of nitrogen to approximately 1 ml, and then transferred into a 15 ml centrifuge tube.

(3) *Derivatization of fenitrothion metabolites*

Diazoethane (2–3 ml) was used to ethylate fenitrothion metabolites present in acetone, ethyl acetate, or ether extracts. The centrifuge tube was left to stand for 30 min in a well-ventilated hood. The solution was concentrated, under a gentle stream of nitrogen as required for determination of fenitrothion and metabolites.

(4) *Cleanup procedure*

The ethylated extract may be injected into the gas chromatograph without further cleanup. However, the following cleanup procedure could be used for extracts obtained from extraction procedure (a). This was found necessary when consecutive injections of the concentrated samples, which contain lipids, would alter the performance of the GLC column.

A silica gel chromatographic micro-column prepared by packing a plug of glass wool loosely into a disposable Pasteur pipet, 23 cm in length, having a tip opening *ca.* 1 mm, and top opening 5 mm inside diameter. One and half grams of silica gel were added, followed by 1 g of anhydrous sodium sulfate. The column was tapped to obtain good packing and then washed with 5 ml hexane.

Prior to column chromatography, the ethylated extract was evaporated to *ca.* 0.5 ml in a water bath maintained at 40°C, using a gentle stream of nitrogen. Distilled water (9 ml), benzene (5 ml), and sodium chloride (5 g) were added and the contents mixed on a Vortex mixer for 1 min and the layers allowed to separate. With the aid of a disposable pipet, the benzene extract was transfered quantitatively to the prewashed chromatography column and the eluate was immediately collected in a 25 ml centrifuge tube. The aqueous layer was extracted with an additional 5 ml benzene, and the benzene extract transferred to the column just as the previous extract reached the sodium sulfate layer. The sides of the columns were rinsed with 3 ml benzene. The elution of the column was continued with 10 ml of 10% (v/v) ethyl acetate in benzene. The eluate was then concentrated to 0.5–5 ml in a water bath at 40°C, using a gentle stream of nitrogen and analyzed by FPD-GLC (5–10 ul injected). MNP was quantified by analyzing the extract obtained from method (a) by EC-GLC (2–10 ul injected).

Quantitative determination of fenitrothion and metabolites in stored wheat

(1) *Adjusting moisture content*

Uncontaminated hard red spring wheat was adjusted to $12.5 \pm 0.1\%$ moisture content by adding distilled water. Moisture content was measured with a dielectric moisture meter. The grain was divided into 2 kg lots and kept for one week in polyethylene bags at room temperature (21 ± 2°C) to allow the wheat to equilibrate before being treated with the insecticide.

(2) *Insecticide treatment*

Fenitrothion emulsifiable concentrate containing 95.6% active ingredient (AI) was diluted with distilled water to provide a deposit of 12 ppm on wheat treated at a rate of 1.2 ml/kg. The moisture content of the wheat was increased by less than 0.15% during insecticide application. The insecticide emulsion

(2.4 ml) was applied to the wheat (2 kg lots) spread thinly in a galvanized iron tray 81 × 41 cm, with sides 4 cm high. The insecticide was sprayed evenly over the grain surface with a Paasche airbrush sprayer at a constant pressure of 0.52 kg/cm^2, the nozzle being held about 20 cm above the grain surface during application. Each lot of treated wheat was mixed for five minutes with a small metal shovel. The treated lots were thoroughly mixed together to ensure initial uniformity to residue levels, and samples were taken for insecticide determination. The remainder was transferred to screw-capped jars (240 ml) and stored in the dark at 20°C. Control wheat samples were transferred into the jars without being treated and were stored under similar conditions.

(3) *Sampling for residue analysis*

Four jars were taken randomly at 1, 3, 6 and 12 months after treatment for quantitative determination of fenitrothion and its metabolites.

(4) *Residue analysis*

The analytical method described above was used to analyze residue levels of fenitrothion and its metabolites. Sample injections were alternated with injections of standard mixtures. The external standard method of quantitation, using linear regression equations, was performed to calculate amounts of fenitrothion and metabolites in stored grain.

Confirmation tests

Two different techniques were employed to identify and confirm fenitrothion metabolites found in stored treated wheat.

(1) *Chemical derivatization*

Stored wheat samples treated with fenitrothion were extracted according to procedure (b). Ethyl acetate fraction, containing demethyl fenitrothion was concentrated to about 2 ml and derivatized with diazomethane. This reaction converts the metabolite to its parent compound. Aliquots of the methylated extract were injected along with injections of the parent compound standard into the FPD-GLC. Retention times for the resulting peaks were then compared.

(2) *Thin-layer chromatography*

Stored treated wheat (50 g) was extracted with acidified acetone which was cleaned up as before and then concentrated to 0.5 ml, using a gentle stream nitrogen. Standard solutions of F, FO, DMF, MNP, SMF and their mixtures, treated grain extract, and extract from the control were spotted on the chromatoplate. The plate was developed in ether:isooctane (7:3, v/v) (Rainsford[2]) to the scored line (10 cm). The plate was allowed to dry at room

temperature and sprayed first with aqueous solution of thallium chloride (1%, wt/v) followed by a 0.5% solution of *p-N-N*-dimethyl-aminocinnamaldehyde in ethanol:acetic acid (1:1, v/v). The R_f values of the separated components found in stored grain were calculated and then compared with those obtained for the standards.

TABLE I

Recovery data for fenitrothion and its metabolites from fortified wheat samples

Compound	Fortification level (ppm)	% Recovery[a]
Fenitrothion	5.0	97.8 ± 0.9
	1.0	97.2 ± 1.1
	0.5	97.0 ± 0.8
	0.1	96.7 ± 2.2
Fenitrooxon	5.0	96.9 ± 2.1
	1.0	96.5 ± 2.1
	0.5	96.1 ± 1.8
	0.1	95.8 ± 2.0
O-demethyl fenitrothion	5.0	92.4 ± 1.8
	1.0	92.1 ± 2.0
	0.5	91.8 ± 1.9
	0.1	91.0 ± 2.0
S-methyl fenitrothion	5.0	93.0 ± 1.1
	1.0	93.1 ± 1.7
	0.5	91.1 ± 1.6
	0.1	90.8 ± 2.8
3-methyl-4-nitrophenol	5.0	92.6 ± 2.3
	1.0	91.7 ± 1.8
	0.5	90.9 ± 0.9
	0.1	90.2 ± 1.3
Dimethyl phosphorothioic acid	5.0	95.6 ± 2.6
	1.0	94.8 ± 2.9
	0.5	94.2 ± 3.0
	0.1	93.9 ± 3.1
O-demethyl *S*-methyl fenitrothion	5.0	93.3 ± 1.3
	1.0	93.1 ± 2.1

[a]Average of three determinations.

RESULTS AND DISCUSSION

1. Extraction efficiency studies

Table I shows results obtained from acidified acetone extraction of three 25 g replicate samples of wheat fortified with a mixture of F, FO, SMF, DMF,

DMSMF, DMPTA, and MNP. Average recovery for all compounds was greater than 90% at the four fortification levels tested.

This rapid and convenient extraction method may be employed if the fenitrothion residue level is present in small amounts. If fenitrothion is present in high concentrations, separation, and consequently, quantitation of DMF (Figure 1) is precluded. Such a problem could be overcome by the use of the second (two step) method of extraction incorporating separation of neutral and acidic compounds (e.g. fenitrothion) from acidic metabolites (e.g. DMF) followed by separate GLC analysis.

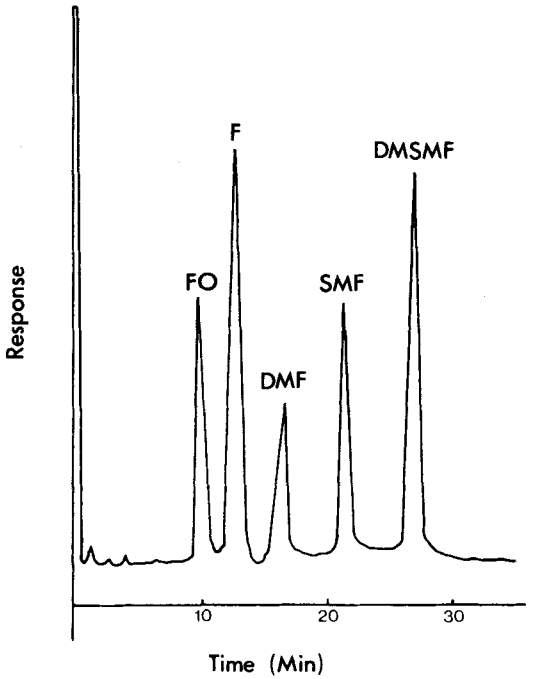

FIGURE 1 Chromatogram of fenitrothion and metabolites from fortified wheat extract.

2. Gas-liquid chromatographic analysis

The FPD-GLC response to F, FO, SMF, DMF, DMSMF, and DMPTA was linear over a range of 0.5 to 12 ng. The linear range of EC-GLC response to MNP was 0.3 to 14 ng.

Figure 1 shows typical FPD/GLC response to fenitrothion and four of its metabolites using the column at 160°C packed with Chromosorb W coated with 5% OV-101. DMPTA was resolved at 80 or 120°C on OV-101 or OV-225,

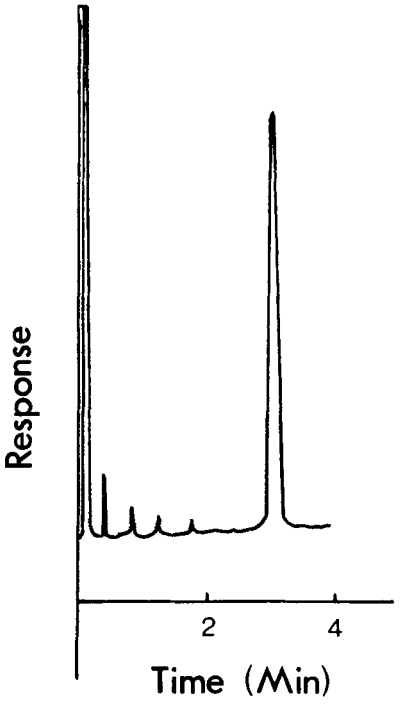

FIGURE 2 Chromatogram of MNP from fortified wheat extract.

respectively. EC-GLC response to MNP is also shown in Figure 2 using the column at 120°C packed with Chromosorb W coated with 3% OV-101. Table II presents detection limits and retention times for fenitrothion and its metabolites.

GLC analysis of extracts obtained from the second extraction procedure was performed under the same operating conditions. Three peaks resulted when the methylene chloride extract was injected. These peaks were FO, F, and SMF. Injection of the ethylated ethyl acetate extract resulted in two peaks: DMF and DMSMF (Figure 3).

3. Quantification of fenitrothion metabolites in stored treated wheat

Figure 4 presents residue levels of fenitrothion and metabolites found in wheat during 12 months storage; *O*-demethyl fenitrothion (DMF), 3-methyl-4-nitrophenol (MNP) and dimethyl phosphorothioic acid (DMPTA) were the major metabolites. The highest levels of DMF and DMPTA (2.01 and

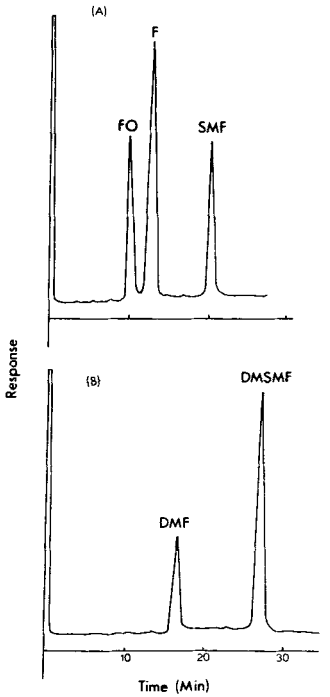

FIGURE 3 Chromatograms of fenitrothion and metabolites after separation of neutral (A) and acidic (B) compounds.

TABLE II

Retention times and limits of detection of fenitrothion and its metabolites

Compound	Retention time (min)	Limits of detection (ng)
Fenitrothion	15.5	0.05
Fenitrothion	11.5	1.0
O-demethyl fenitrothion[a]	20.0	1.0
S-methyl fenitrothion	25.6	0.5
O-demethyl *S*-methyl fenitrothion[a]	32.2	1.0
3-methyl-4-nitrophenol	3.1[b]	0.3
Dimethyl phosphorothioic acid[a]	2.8[c]	0.3

[a]Ethylated compound.
[b]3% OV-101, Column temperature 120°C.
[c]3% OV-225, Column temperature 120°C.

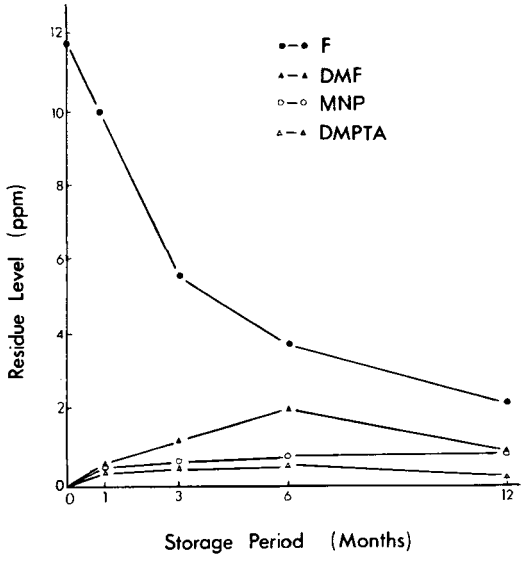

FIGURE 4 The breakdown and metabolic products of fenitrothion on wheat stored at 20°C.

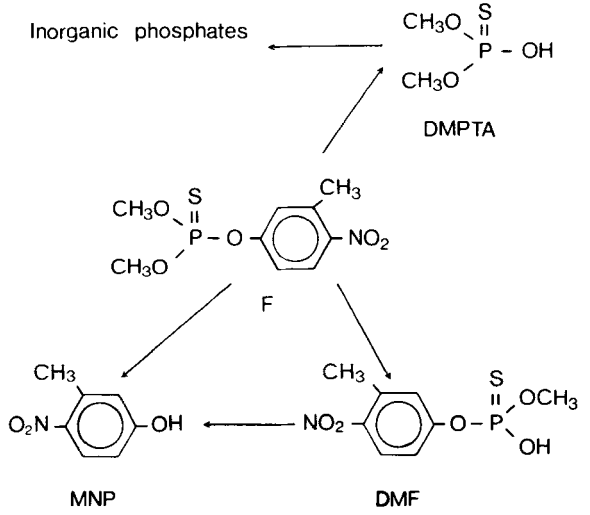

FIGURE 5 Suggested degradation pathways of fenitrothion in stored wheat.

0.55 ppm, respectively) were found after 6 months storage; they decreased to 0.98 ppm DMF and 0.21 ppm DMPTA at the end of storage. MNP showed an initial value of 0.38 ppm after one month storage but continued to increase with the duration of storage. After 12 months, MNP level reached 0.96 ppm. No fenitrooxon or *S*-methyl fenitrothion, above the detection limits of 0.01 and 0.05 ppm, respectively was detected throughout the study period.

Figure 5 suggests degradation pathways of fenitrothion in stored treated grain. It appears that production of MNP is probably from fenitrothion and *o*-demethyl fenitrothion. The maximum DMF residue level was found after 6 months storage. This level started to decrease with gradual increase of MNP residue level, presumably from the degradation of DMF as well as fenitrothin.

4. Confirmation tests

(a) *Chemical derivatization*

When aliquots of the methylated ethyl acetate fraction were injected with the fenitrothion standard, one peak was obtained. The finding indicates the conversion of the metabolite (i.e. DMF) found in the stored treated grain to its parent compound. The test confirms the results discussed earlier on the production of DMF during storage of treated grain.

(b) *Thin-layer chromatography*

Fenitrothion and metabolite standards, an extract of treated wheat, and an extract from an untreated control were chromatographed on a silica gel TLC plate. When the chromatoplate was sprayed with an aqueous solution of $TlCl_3$ followed by the solution of *p*-*N*, *N*-dimethylaminocinnamaldehyde, the separated components appeared as reddish spots against a light background.

Comparison of R_f values calculated for the standards with those obtained for the treated wheat extract confirmed that DMF, MNP and DMPTA were found in the grain sample. On a second TLC plate, half of the separated components of the treated wheat extract was covered with a glass plate before spraying. The DMF spots were located by matching the R_f values of the standard with that of DMF from the treated wheat extract, scraped off, and the compound was extracted in methanol and then derivatized with diazomethane.

Results by GLC revealed that the methylated spot had a similar retention time to fenitrothion, confirming the presence of DMF in the grain sample.

CONCLUSION

The method described in this paper for the extraction and analysis of fenitrothion and metabolites, namely FO, DMF, DMSMF, DMPTA, MNP

and SMF gives good recovery (>90%) for the four fortification levels tested. The technique was successfully utilized to quantify metabolites of fenitrothion in treated wheat stored for 12 months. DMF, MNP and DMPTA, the major breakdown products, were confirmed by TLC. DMF was further confirmed by derivatization and FPD-GLC. Fenitrothion appears to degrade in stored wheat both by hydrolysis and dealkylation.

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