Mono- and Polyclonal Antibodies to the Organophosphate Fenitrothion. 2. Antibody Specificity and Assay Performance

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The performance and specificities of antibody-based assays for fenitrothion were studied. Fenitrothion could be detected in grain in assays with either polyclonal or monoclonal antibodies using either immobilized antibody or immobilized hapten–protein conjugates. Most assay formats and antibodies distinguished fenitrothion from structurally similar organophosphates, and variable extents of cross-reaction with metabolites were observed. The concentration of fenitrothion in wheat grain samples was measured in three assay formats with two antibodies, and in each case good correlations were obtained with the results obtained by gas–liquid chromatography. For routine use, a format detecting 3 ng and suitable for analysis over the range 100 ppb–20 ppm in grain was preferred.

INTRODUCTION

The preceding paper (McAdam et al., 1992) described the development of several enzyme immunoassays for the organophosphate fenitrothion (FN) that were sensitive to low nanogram amounts of this compound. Our purpose for developing these antibodies followed from the widespread use of fenitrothion as a grain protectant (Desmarchelier et al., 1977; Snelson, 1987). With many grain storages being treated annually with FN, there is a need for less expensive high-throughput laboratory methods for determination of FN residues.

In this paper, several aspects of the performance of these assays are investigated, including (1) cross-reaction with analogs of FN, FN metabolites, other stored-grain pesticides, and other organophosphates, (2) effects of solvents required for FN extraction from grain, and (3) effects of coextractives in grain matrices on assay performance. Our results indicate that ELISA assays based on these antibodies can accurately quantify fenitrothion in wheat samples at commercially relevant (0.1–20 ppm) levels.

MATERIALS AND METHODS

Antibodies, Fenitrothion Conjugates, and ELISA Methods. The preparation and properties of the antibodies and FN–HRP and FN–protein conjugates used in this study, as well as the ELISA methods used, are described in the preceding paper (McAdam et al., 1992). Standard curves for each assay are also shown in that paper.

Grain Samples. In initial work, samples (500 g) of pesticide-free wheat were spiked with small amounts (0.5–5 mL) of an ether solution of FN to yield approximately 3, 9, and 14 ppm in the grain. The samples were stored at 20 °C in the dark for 9–12 weeks before analysis. Such "aging" is necessary for the pesticide to achieve constant and reproducible distribution within and between grains and to exhibit extractability properties similar to those of field samples (Sharp et al., 1988). Samples of grain treated commercially at receival with fenitrothion and stored for 3–18 months at the various elevators were obtained from the Australian Wheat Board, Melbourne.

Extraction. Previous work has indicated that virtually quantitative extraction of FN from whole wheat could be obtained by standing the grain in 2.5 volumes of methanol for 36–48 h with intermittent (3 × 5 min at 100 rpm) shaking (Desmarchelier et al., 1977). This result was confirmed by GLC in the current study (not shown), and this extraction method was therefore used.

Test Compounds. Pesticide standards were purchased from Chem Service (West Chester, PA). FN metabolites were synthesized as follows: Demethylfenitrothion [*O*-methyl *O*-(3-methyl-4-nitrophenyl) phosphorothioate] and fenitrothion *S* isomer [*O*,*S*-dimethyl *O*-(3-methyl-4-nitrophenyl) phosphorothioate], were synthesized according to the method of Eto et al. (1968). These products were purified by column chromatography and analyzed by ^{1}H NMR. Fenitro-oxon was prepared by reaction of fenitrothion (3.8 mmol) in 15 mL of ethanol–5 mL of water with bromine (18.8 mmol in 10 mL of ethanol) for 1 h at room temperature. The mixture was extracted in ethyl acetate, and the organic layer was washed (water, 0.1 M $Na_2S_2O_5$, H_2O, brine), dried over $MgSO_4$, and then concentrated to an oil. Silica column chromatography (eluted with chloroform) gave the product as an oil (650 mg, 69% yield). Proton NMR (200 MHz): δ 8.04 (d, $J_{H_{5,6}}$ = 9.5 Hz, H_5), 7.21 (m, 2 H, $H_{2,6}$), 3.91 (d, $J_{P,H}$ = 11.5 Hz, OCH_3), 2.63 (s, CH_3); infrared (1280 cm^{-1}) P=O. 3-Methyl-4-nitrophenol was obtained from Aldrich (Milwaukee, WI).

RESULTS

Solvent Effects on Assays. The functional limit of detection for fenitrothion in grain matrices will also depend on the maximum amount of solvent that the assay will tolerate, which in turn affects the dilution factor for the grain extracts. Fenitrothion may be extracted from grain using either nonpolar water-immiscible solvents (e.g., hexane) or water-miscible solvents such as acetone, acetonitrile, ethanol, or methanol (Sharp et al., 1988). A solvent was considered suitable at 10% final concentration if it did not significantly change the ELISA control absorbance or alter inhibition by free FN. With assays using anti-YNF bound to the solid phase, solvents such as acetone (10%) or methanol (5–20%) did not reduce ELISA control values but did reduce the inhibitory potency of FN. Others, such as 20% 2-propanol, affected both parameters. Previous studies indicated that methanol was an efficient extractant, especially if grain samples were exposed to the solvent for 36–48 h (Desmarchelier et al., 1977). Immunoassays were less affected by methanol than by other solvents (Table I). Therefore, 10% methanol was routinely used for subsequent work. Use of methanol had the advantage that for confirmatory gas chromatographic analyses the same extract may be injected directly into the instrument.

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Table I. Solvent Effects on Immunoassay for Fenitrothion

	antibody							
	ONF polyclonal, solution		YNF polyclonal, solid phase		YPF polyclonal, solution		YPF MAb 10/20, solid phase	
solvent, %	C^a	I_{50}^b	C	I_{50}	C	I_{50}	C	I_{50}
methanol, 5	nt	nt	97	1.3	100	0.02	99	0.02
methanol, 10	nt	nt	93	1.4	97	0.02	97	0.02
methanol, 20	130	0.38	91	1.2	85	0.03	87	0.01
ethanol, 5	nt	nt	88	1.0	96	0.04	66	0.02
ethanol, 10	84	1.8	89	0.2	86	0.03	58	0.02
ethanol, 20	42	>100	71	0.1	69	0.06	20	0.005

[a] C, absorbance. % of solvent-free control. [b] I_{50}, concentration yielding 50% inhibition (ng/mL).

Table II. Cross-Reactions of Antibodies to Fenitrothion[a]

	substitution position					antibody							
											YPF MAb 10/20		
compound	R_1R_2	R_3	R_4	R_5	X	ODF solution	ONF solution	YNF solid phase	YPF solid phase	YPF solution	solid phase	solution	OPF MAb 8/24 solution
analogs													
fenitrothion	CH_3	H	CH_3	NO_2	S	100	100	100	100	100	100	100	100
aminofenitrothion	CH_3	H	CH_3	NH_2	S	50	200	340	–[b]	–	–	–	0.03
methylparathion	CH_3	H	H	NO_2	S	70	140	4	8	1	3	1.5	0.2
fenthion	CH_3	H	CH_3	CH_3	S	100	100	40	–	0.05	–	–	–
cythioate	CH_3	H	H	SO_2NH_2	S	–	10	20	–	–	–	–	0.01
parathion	C_2H_5	H	H	NO_2	S	50	10	0.06	2	1	0.6	0.5	8
paraoxon	C_2H_5	H	H	NO_2	O	–	–	–	0.3	0.02	0.3	0.3	–
dicapthon	CH_3	Cl	H	NO_2	S	50	110	1	2	0.4	0.3	0.3	0.01
dichlorfenthion	C_2H_5	Cl	H	Cl	S	–	–	–	–	–	–	–	–
fensulfothion	C_2H_2	H	H	$CH_2SC_6H_5$	S	–	–	10	–	–	–	–	2
grain protectants													
chlorpyrifos-methyl	CH_3	(*O*-3,5,6-trichloro-2-pyridyl)			S	10	–	0.1	–	–	–	–	–
pirimiphos-methyl	CH_3	(*O*-2-diethylpyrimidin-4-yl)			S	7	–	0.07	–	–	–	–	–
azinphos-methyl	CH_3	(*S*-3,4-dihydro,4-oxo-1,2,3-benzotriazin-3-ylmethyl)			S	–	–	0.08	–	–	–	–	–
metabolites													
fenitrooxon	CH_3	H	CH_3	NO_2	O	–	–	10	10	2	20	20	0.5
demethylfenitrothion	CH_3H	H	CH_3	NO_2	S	–	–	10	2	0.01	6	4	0.3
fenitrothion *S* isomer	$CH_3(O)CH_3(S)$	H	CH_3	NO_2	O	–	–	10	20	5	30	30	0.5
3-methyl-4-nitrophenol		H	CH_3	NO_2		–	–	–	0.08	0.01	0.06	0.06	–

[a] Data shown are cross-reactions of individual antibodies relative to fenitrothion (100%), calculated as follows: cross reaction = I_{50}(fenitrothion)/I_{50}(analog) × 100. [b] No significant cross-reaction (<0.01%) was seen.

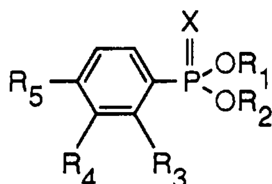

Figure 1. Key for structures of fenitrothion analogs (see Table II).

Matrix Effects. The effects of coextractives in whole grain were studied by comparing the absorbance curves for a FN dilution series prepared in methanol (then diluted to 10% in PBS–BSA–Tween) and a series of FN standards prepared in a methanol extract of grain, before similar dilution in assay buffer. Using whole wheat grain, detectable matrix effects were not found with any assay format.

Specificity of Antibodies Obtained. The specificity of each antibody in each assay format was assessed using four types of compounds (Table II; Figure 1): (1) fenitrothion analogs, (2) related *O,O*-dialkyl phosphorothioate insecticides, (3) fenitrothion metabolites, and (4) other classes of pesticide. None of the assays detected structurally unrelated pesticides, such as dieldrin (an organochlorine), carbaryl or propoxur (carbamates), or bioresmethrin (a pyrethroid), even at very high concentrations (0.1 mg/mL). However, a diversity of cross-reactions was seen. Some antibodies (e.g., YPF, YPF MAb10/20, and OPF MAb8/24) bound only to FN and weakly to one or two close analogs, while others (e.g., ODF, ONF, YNF) bound to a variety of fenitrothion analogs containing either a substituted 4-nitrophenol moiety or groups of similar size and inductive properties in the 4-position. Some general findings were the following.

(1) Antibodies prepared to FN haptens coupled through the phosphate ester (OPF, YPF) tended to be more specific than those prepared by coupling through the (derivatized) aromatic nitro group. This was likely due to the groups farthest from the point of conjugation (the unmodified 3-methyl-4-nitrophenol group), in the first case, and the *O,O*-dimethyl phosphorothioate ester (common to this class of organophosphate insecticides), in the second case. Surprisingly, binding to certain antibodies of the second type was affected markedly by substitutions distal to the phosphate ester; for example, antibody YNF bound much less well to methylparathion (lacking only the 3-methyl group of FN) than to FN itself. In no cases were antibodies produced that acted as very broad specificity *O,O*-dialkyl phosphorothioate probes. Suedi and Heeschen (1988) developed such broad specificity probes by use of an aliphatic ester. Not surprisingly, antibodies of the second type bound better to aminofenitrothion than FN, since antibodies were actually raised to a derivative of this compound rather than to FN. However, antibodies raised

to phosphate group-coupled compounds did not recognize amino-FN. Apart from the least specific and lowest affinity antibody (ODF), no binding to other organophosphates (including malathion, chlorpyrifos-ethyl, azinphos-methyl, dimethoate, temephos, dichlorvos) was seen. Greater reactivity was seen with certain antibodies and the FN metabolites fenitro-oxon and fenitrothion *S* isomer; however, these account for less than 2% of the metabolites of FN on aged grain. Reaction of each antibody with the major metabolites, demethylFN and 3-methyl-4-nitrophenol) was too low to lead to significant inaccuracies in determinations of FN on aged grain (Snelson, 1987). In contrast, the parathion antiserum of Ercegovich et al. (1981) did recognize the metabolite 4-nitrophenol.

(2) Where the same antibody was useable in both the solid-phase antibody and the solution-phase antibody formats, the solid-phase antibody format exhibited slightly broader cross-reaction.

(3) Different monoclonal and polyclonal antibodies, prepared with the same immunizing hapten chemistry, exhibited differing specificities as well as affinities.

Analysis of Whole Wheat Samples. Sets of field-treated wheat samples were analyzed using four assays: (1) immobilized polyclonal antibody to ONF, (2) immobilized polyclonal antibody to YPF, (3) immobilized monoclonal antibody to YPF (MAb YPF 10/20), and (4) immobilized antigen (OA-P-FN) with HRP-labeled MAb YPF 10.20. Results obtained were correlated with gas chromatographic data obtained on the same samples.

In preliminary experiments, the first assay distinguished wheats containing 3, 9, and 14 ppm of fenitrothion but did not accurately quantify FN in field samples with smaller differences in FN. This can be explained from the low slope of the standard curve, which would make quantitation imprecise. In contrast, analyses of sets of wheat samples using each of the other three assays gave results which agreed well with GC analyses results of the grain extracts (Figure 2A–C). Assay 4 gave slight underestimates. Results obtained with the polyclonal antibody (YPF) correlated well with those of the monoclonal antibody (YPF 10/20) in the same assay format ($n = 19$, $r = 0.984$, $P < 0.0001$; MAb result = 1.08 × PAb result). This slight overestimate with the MAb was not seen in separate analysis of larger sample sets. Similarly, results with the monoclonal antibody used on the solid phase correlated with those obtained with this antibody in solution (Figure 2D).

Precision of the Assay. The intra-assay repeatability and between-assay reproducibility of the monoclonal antibody (YPF 10/20) assay formats were studied (Figure 3). In the central portion of the standard curves, coefficients of variance were less than 10%.

GENERAL DISCUSSION

The most suitable FN ELISA for laboratory analysis of grain was based on a monoclonal antibody immobilized on the microwell. This assay was not affected by methanol solvent and was sensitive and dynamic. The corresponding polyclonal antibody prepared using the same site of conjugation and used in the same assay format had a flatter concentration–response curve, but it cross-reacted less than the MAb with FN metabolites. This finding may be of application in residue analysis of wheats containing highly aged FN residues or for analysis of environmental samples. The differences in cross-reaction with other organophosphates and between antibodies may also be of use in environmental assays (e.g., in water testing) where greater reaction of analogs may be required. Kinetic

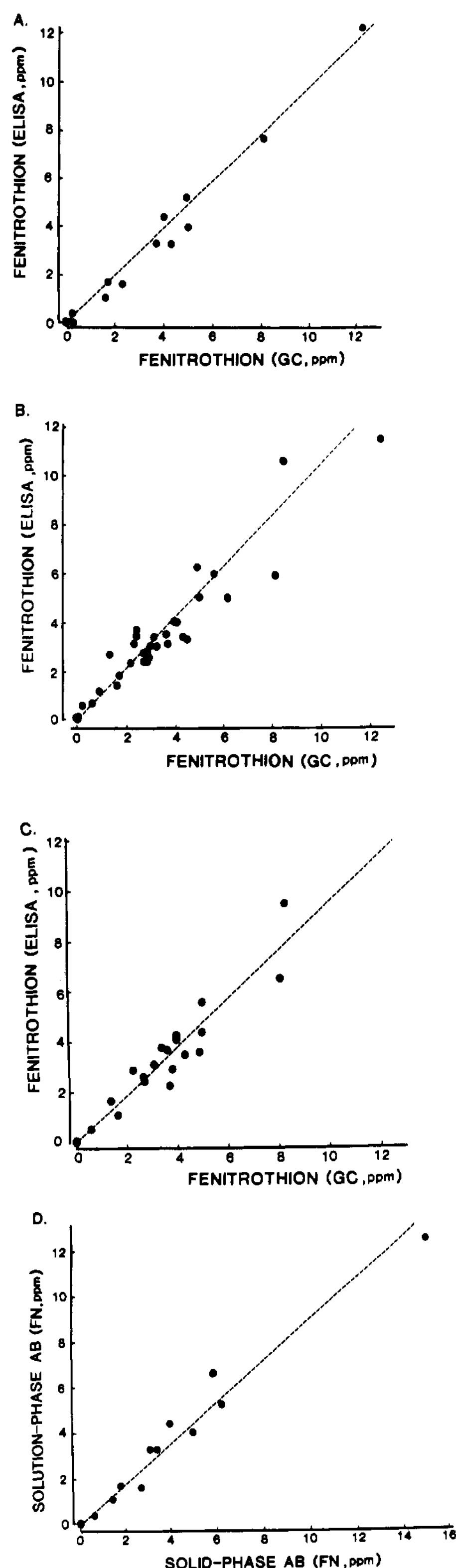

Figure 2. Relationship between fenitrothion residues determined by gas chromatography for different assays/antibodies: (A) polyclonal antibody YPF on solid phase; (B) monoclonal antibody PF10/20 on solid phase; (C) monoclonal antibody PF10/20 in solution; (D) comparison of results obtained with PF10/20 in solution and on solid phase. Regression data are as follows, fitted through the origin: (A) $n = 21$, $r = 0.984$, slope = 0.943; (B) $n = 33$, $r = 0.982$, slope = 1.055; (C) $n = 14$, $r = 0.993$, slope = 0.973; (D) $n = 12$, $r = 0.987$, slope = 0.981.

differences may also make other assay formats more suitable in "field" tests where brief (under 5 min) incubations are needed (Beasley, Skerritt, Hill and Desmarchelier, unpublished results). In conclusion, we have dem-

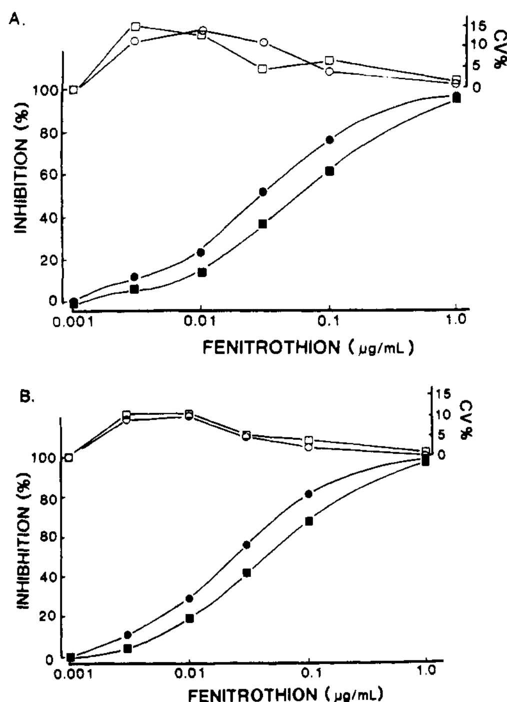

Figure 3. Precision of fenitrothion determination by ELISA with MAb OPF 10/20: (A) intra-assay (n = 3) mean standard curve and coefficient of variation of inhibition [solid-phase antibody (●, ○); solid-phase antigen (■, □)]; (B) interassay (n = 8) mean standard curve and coefficient of variation of inhibition.

onstrated the accuracy and precision of a simple ELISA for the analysis of fenitrothion in wheat samples commercially treated and stored rather than simply "spiked" in the laboratory. Unlike many other agrochemical immunoassays, this assay is intended for routine quantitative or semiquantitative analysis of a commodity where the compound is purposely present much or most of the time. This compares with the use of immunoassay to screen environmental samples for a small proportion of positives, which are then analyzed in detail. The fenitrothion antibody tests are currently being extended to wheat milling fractions and other crops such as barley (Edward, Hill, and Skerritt, unpublished results), baked wheat products, and environmental samples.

ABBREVIATIONS USED

FN, fenitrothion; HRP, horeradish peroxidase; Y/ODF, IgY or ovalbumin coupled to fenitrothion by diazotization; K/Y/ONF, KLH, IgY, or albumin coupled to fenitrothion by 6-carbon spacer arm; Y/OPF, spacer arm coupled to fenitrothion through the phosphate group.

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