Detection of Carbofuran and Other Carbamates Using Cholinesterase Inhibition with *N*-Methylindoxyl Acetate as Substrate

Robert J. Argauer* and Richard T. Brown

Beltsville Agricultural Research Center, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705

A screening test was developed to detect trace amounts of carbamate insecticide residues in water and in aqueous extracts of meat. This test considers multiple insecticides with the common ability to inhibit cholinesterase. Assay solutions containing inhibitory insecticides remain colorless upon addition of substrate, while negatives and negative controls exhibit both fluorescence and a yellow color, followed by the formation of a blue precipitate that can serve as a permanent marker for assays judged negative. Phospholipids such as lecithin interfere with the test and must be removed before assay.

Keywords: *Carbamates; cholinesterase; inhibition; N-methylindoxyl acetate; food safety*

INTRODUCTION

N-Methylcarbamate insecticides possess short-term environmental persistence. Their lack of accumulation within organisms accounted for their development as alternatives to the organochlorine insecticides used in the 1940s and 1950s to which insects were developing resistance. Improved formulations and rational application to crops have reduced economic losses from insect pests and have minimized the presence of pesticide residues in food, animal feed, and ground and surface waters draining from treated crop land.

Increasing concerns about the effect of chemicals on the environment have led to studies concerning the impact of the cholinesterase-inhibiting *N*-methylcarbamate insecticides on wildlife and other nontarget organisms (Mineau, 1991). Recently, Martin and Solomon (1991) measured plasma and brain cholinesterase of wild mallard ducklings receiving a sublethal dose of carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate). Fleming et al. (1992) studied the brain cholinesterase activity of forest songbirds exposed to aerial application of Zectran, a carbamate insecticide. Findings varied from "sublethal doses of carbofuran increased mortality of ducklings at low ambient temperatures" to "cholinesterase response was biologically unimportant to Zectran". The variation in plasma cholinesterase among 125 greenhouse workers (Lander and Hinke, 1992) and among 331 Danish schoolteachers eating crops containing cholinesterase-inhibiting chemicals (Lander et al., 1992) was measured to assess exposure. Findings included "the in-season cholinesterase depression indicated absorption by chronic percutaneous or oral uptake" to "it remains to be determined whether a subclinical but chronic intake of anticholinesterase agents in the diet can be hazardous to humans".

Many workers have used cholinesterase inhibition for detecting residues of organophosphorus and carbamate insecticides (Giang and Hall, 1951; Mendoza et al., 1968; Mendoza, 1974; Menn and McBain, 1966; Zoun and Spierenburg, 1989). Guilbault et al. (1968) evaluated 11 compounds as potential fluorogenic substrates for cholinesterase and recommended the use of *N*-methylindoxyl esters that were stable in solution and had a low rate of spontaneous hydrolysis and a high rate of enzymic hydrolysis. Moye and Wade (1976) used housefly head cholinesterase and the acetate ester in a flow reactor for detecting insecticide residues in fortified samples of cabbage.

The purpose of this study was to develop an efficient and rapid screen for detecting residues of carbamate insecticides above tolerance levels in meat using cholinesterase inhibition. Readily available chemical and biological reagents were used, while no chromatographic separations were used to remove interfering coextractives.

MATERIALS AND METHODS

Carbamate Standards. Analytical reference carbamate standards were obtained from the Environmental Protection Agency. Analytical standards of 1 mg/mL were prepared in HPLC-grade methanol and stored in the refrigerator. Fortification solutions were made from dilutions of the standards.

Substrate. *N*-Methylindoxyl acetate from Sigma Chemical Co. was weighed in a 25-mL volumetric flask and diluted to volume with 2-methoxyethanol (methyl cellosolve) to produce the 1.25 mg/mL working solution that was then stored in the refrigerator. The solution was prepared fresh weekly.

Meat Samples. Ground beef was obtained through the Meat Science and Dairy Science Laboratories (USDA, ARS, Beltsville, MD) from animals that were not exposed to carbamate insecticides.

Buffer Solution. The contents of one packet of preweighed 10 mM phosphate-buffered saline, pH 7.4 (P-3813, Sigma), was dissolved in 1 L of deionized water.

Cholinesterase Solution. Acetylcholinesterase from electric eel, type V-S, was obtained as a lyophilized powder from Sigma and stored in a freezer below 0 °C. A stock solution was prepared by adding 500 units, the contents of the supplier's vial, to a 100-mL volumetric flask and diluting with buffer solution. The solution was then stored in a refrigerator below 4 °C. The cholinesterase working solution was prepared by diluting the stock solution 20:100 with the buffer at room temperature.

Cleaning Procedure for Glass Erlenmeyer Flasks. The flasks were rinsed with methylene chloride and dried. Phosphate-buffered saline solution, pH 7.4, containing 0.05% Tween 20 (P-3563, Sigma) was added, and the flasks were ultrasonically agitated in a water bath for 5 min. The flasks were rinsed clean with deionized water and air-dried.

Spectrofluorometer. A Perkin-Elmer MPF-2A fluorescence spectrophotometer was used with a manually operated four-cuvette-capacity turntable. Fluorescence was monitored at wavelengths of 410-nm excitation and 504-nm emission. Sample sensitivity setting was 2, with excitation slit at 4 nm and emission slit at 10 nm. To provide data that may be compared with data obtained on the various spectrofluorometers used in other laboratories, a reference solution of 1 μg/mL quinine sulfate in 0.1 N sulfuric acid at these instrument sensitivity settings produced a relative fluorescence signal of 34 (350-nm excitation and 450-nm emission wavelengths). A Perkin-Elmer Lambda 3 spectrophotometer was used to measure the absorbance of a negative control solution at a wavelength of 410 nm. The absorbance increased from near 0 to 0.2 unit within 9 min after substrate was mixed with the working cholinesterase solutions.

Carbofuran Extraction from Meat. Samples consisting of 50 g of ground beef were blended with 200 mL of deionized water for 1 min and filtered through 9-cm Whatman No. 1 filter paper under a water aspirator vacuum to remove the fat and fiber. A 125-mL aliquot of the filtrate was transferred to a separatory funnel and extracted with 100 mL of methylene chloride. The lower gel-like layer was transferred to an Erlenmeyer flask and 30 g of anhydrous sodium sulfate added. A 50-mL portion of the recovered methylene chloride was decanted into a clean 125-mL glass Erlenmeyer flask. Four milliliters of the sodium sulfate-dried methylene chloride layer was considered equivalent to 1 g of ground beef since a 50% aliquot of the aqueous extract (50 g of meat and 200 mL of water) had been extracted with 100 mL of methylene chloride. Variously sized aliquots of the dried methylene chloride extracts were transferred to clean glass 125-mL Erlenmeyer flasks, and the solvent was evaporated under vacuum for the studies. Samples were fortified at various parts per million (ppm) levels by adding microgram amounts of the carbofuran standard in methanol either directly to 50 g of ground beef in the blender or to the methylene chloride extracts.

Insecticide Assay—Carbamate Standards. Three milliliters of cholinesterase working solution was added to each of four disposable acrylic plastic 10-mm fluorometer cuvettes (Wilmad Glass Co., Buena, NJ) that contained either 0 μg (the negative control) or between 0.1- and 10-μg amounts of the carbamate. The solutions were allowed to incubate for 10 min at room temperature (25 °C). After this incubation period, a stopwatch was started and, using a Hamilton syringe, 40 μL of substrate working solution was added to each cuvette. The contents of the cuvettes were mixed by briefly blowing into each through a 5-lambda disposable micropipet. The fluorescence generated within these cuvettes by the hydrolysis of the substrate was monitored between 2 and 9 min at 1-min intervals.

Insecticide Assay—Meat Samples. Three milliliters of cholinesterase working solution was added to each of four 125-mL Erlenmeyer flasks that contained either 0 μg (the negative control) or the residue from the extraction procedure. The solutions were incubated for 10 min at room temperature. After this incubation period, a stopwatch was started and 40 μL of substrate working solution added to each flask. The contents of the flasks were decanted into the disposable acrylic cuvettes, and the fluorescence was monitored.

Screening Method (in Brief) for Insecticides That Might Be Present as Cholinesterase Inhibitors in Meat. Weigh 50 g of ground meat. Blend with 200 mL of water. Filter. Extract 125 mL of filtrate with 100 mL methylene chloride in a separatory funnel. Drain lower gelatinous layer into a flask and dry with 30 g of sodium sulfate. Decant, filter, and save resultant methylene chloride extract. Transfer 0.4, 5.0, and 20 mL or other sizes of aliquots to three 125-mL clean glass Erlenmeyer flasks and evaporate contents under a water aspirator. Proceed with the assay as described.

RESULTS AND DISCUSSION

Principle. The carbamate screen described is a qualitative, colorimetric, and fluorometric assay for the detection of carbamate and other cholinesterase-inhibiting chemicals. It is based on the inhibition of the enzyme acetylcholinesterase. In the absence of insecticides that inhibit acetylcholinesterase, the enzyme hydrolyzes a colorless, nonfluorescent substrate, *N*-methylindoxyl acetate, to the fluorescent, yellow-colored *N*-methyl-3-hydroxyindole. Samples containing carbamate insecticides cause a decrease in fluorescence/color production compared to the negative control. Though applied to the analysis of meat samples, the screening system with some changes should be useful for finding these insecticides in agricultural produce and processed foods and also in surface and ground waters.

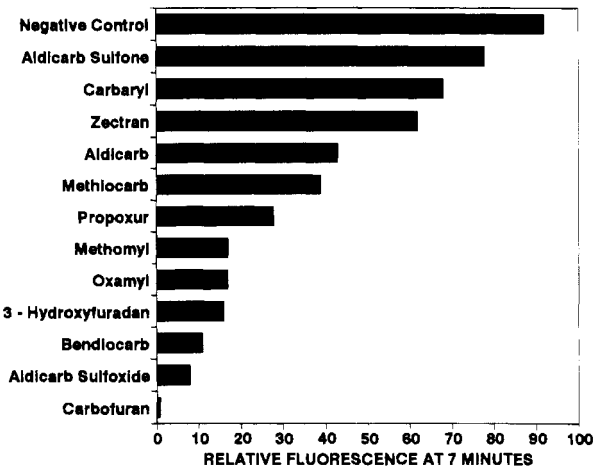

Figure 1. Relative inhibition of eel cholinesterase by 1-μg amounts of various *N*-methylcarbamate insecticides.

Number of Samples per Analysis. Due to time and equipment constraints when reading the fluorescence developed in the cuvettes, one negative control and three samples were found to be the maximum number of analyses that could conveniently be managed at one time. Nearly 10 min is required for monitoring the fluorescence generated by the kinetic hydrolysis reactions occurring in the four cuvettes positioned in the turntable within the fluorometer. However, because a deep yellow color develops within 10 min for the negative control while strongly inhibited solutions remained colorless, a simple visual comparison of this difference should permit the screening of more samples within the same period.

Relative Inhibitory Effects of Various Carbamates. Figure 1 gives results of assays performed for 12 *N*-methylcarbamate insecticides. Incubation of pesticide (1 μg/10 μL in methanol) with buffered cholinesterase for 5–10 min prior to the addition of substrate was needed to obtain consistent results. The fluorescence intensity readings observed 7 min after substrate was mixed into the buffered solutions containing cholinesterase and inhibitors were selected to indicate the relative ability of these insecticides to inhibit eel cholinesterase at a concentration of 1 μg in 3 mL of the assay solution. Carbofuran was the most inhibitory of the carbamates tested, showing inhibition at 0.025 μg in 3 mL of the assay solution. For the negative controls and the solutions not strongly inhibited, the fluorescence and the yellow color disappear after 1 h with the formation of a blue-colored coagulate in the solutions. Solutions containing carbamates at concentrations that were strongly inhibitory become slightly yellow but do not fluoresce. The blue coagulate that forms can serve as a permanent visual documentation for those samples that were not inhibitory. Various cholinesterase sources were not compared to find one that would provide a

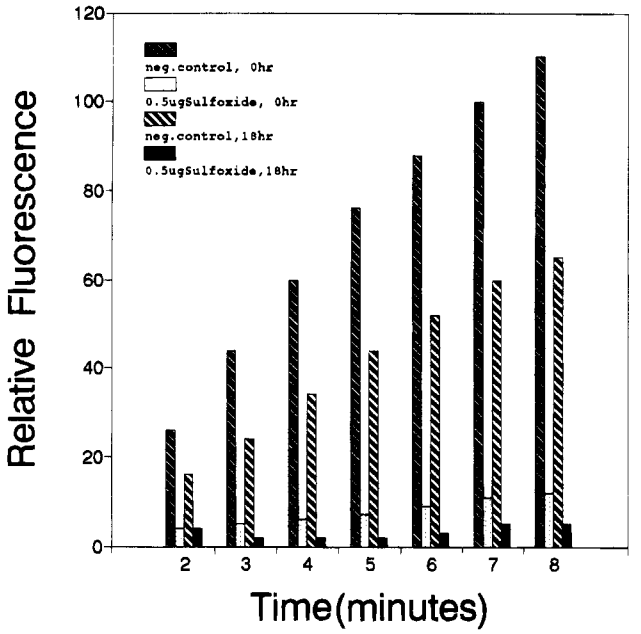

Figure 2. Inhibition of cholinesterase working solutions by aldicarb sulfoxide; newly diluted compared with dilution stored for 18 h at room temperature.

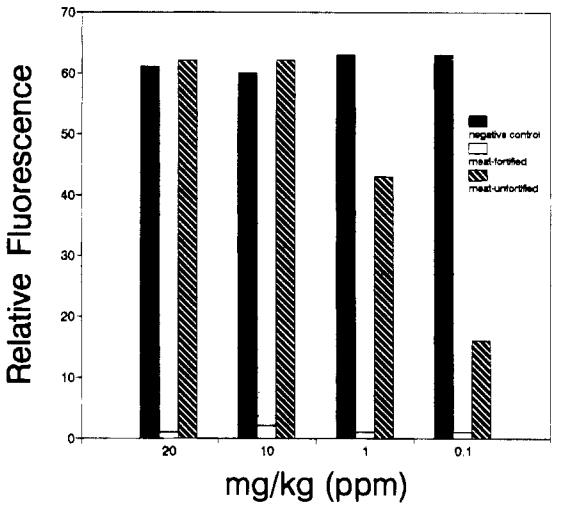

Figure 3. Ground beef samples fortified at 20, 10, 1.0, and 0.1 mg/kg (ppm) with carbofuran compared with unfortified samples. 0.2, 0.4, 4.0, and 40 mL of methylene chloride extract evaporated, respectively. Fortified meat samples contain 1-μg of carbofuran in 3-mL assay. Enzyme and substrate were added. Fluorescence reading was taken at 7 min.

uniform level of inhibition for the 12 carbamates screened. Commercial qualitative assay kits such as the Enzytec Biosensor Pesticide Detector Ticket (EnzyTec, Inc., Kansas City, MO) and InQuest OP/Carbamate Screen (Ohmicron, Newtown, PA) have relied on the blue color produced with indoxyl acetate as the substrate. The oxidation processes involve the formation of indigo blue (Holt, 1952).

Stability of Cholinesterase Working Solution. Figure 2 gives the results obtained for aldicarb sulfoxide. A working solution newly prepared by dilution of stock cholinesterase solution stored at 4 °C was compared with a working solution stored at room temperature for 18 h. The cholinesterase working solution stored at room temperature lost about 40% of its activity. Even at this lower activity level, the inhibition caused by the presence of 0.5 μg of aldicarb sulfoxide is readily differentiated from that of the negative control. Normally one would not store the working solution at room temperature for more than a few hours before completing the assay. Sadar et al. (1970) showed that the enzyme is stable in solution when kept at 4 °C and could be used for many weeks without loss of activity.

Matrix Interferences. Figure 3 gives the results obtained for ground beef fortified with carbofuran at 20, 10, 1.0, and 0.1 mg/kg (ppm) and compared with the results of an unfortified sample. Water conveniently extracts the insecticide, leaving both fat and fiber behind. The red color of the aqueous extract is caused by hemoglobin and related proteins. The methylene chloride extraction of the aqueous extract eliminates most coextractives, but some remain to interfere when concentrated. By this procedure, 50 g of ground beef yielded less than 1 mg of coextractives. The data in Figure 3 are based on four differently sized aliquots and show the effect of increasing amounts of coextractives when larger aliquots are required to improve carbamate detectability. This provides a limit to the utility of the assay. It is not known how various animal diets affect the concentration of coextractives in the meat extracts. Aqueous extracts could include amino acids, antibiotics, anticoagulants, dyes, general anesthetics, hormones, inorganic compounds, quaternary compounds, and vitamins. However, these compounds are not expected to be found in the sample preparations at high enough concentrations to influence the test.

Alternate Extraction Procedure. A sample of ground beef (50 g) was blended with 150 mL of acetonitrile. The extract was filtered, washed with 25 mL of hexane, and dried over 40 g of sodium sulfate. The solvent from a 30-mL aliquot (considered to be equivalent to 10 g of sample) was evaporated under a water aspirator vacuum, leaving 60 mg of residue in the flask. Care was exercised to remove the last traces of solvent since acetonitrile inhibits the action of cholinesterase. The assay followed. The fluorescence readings at 7 min for both unfortified and fortified meat samples were 90% below the negative control. These results show the difficulties that occur if large aliquots of the acetonitrile extracts were required to be concentrated to adjust the detection capabilities of the assay for insecticides less inhibitory than carbofuran.

To demonstrate that these coextractives contained phospholipids, a second 30-mL aliquot was concentrated and the residue dissolved in methylene chloride and spotted on a silica gel thin-layer plate. The plate was developed with a mixture of methylene chloride–methanol (65:30), dried, and sprayed with Draagendorff's reagent for detecting quaternary nitrogen compounds. Spots appeared, one at a retention time corresponding to that for lecithin (phosphatidylcholine), a mixture of diglycerides of fatty acids linked to the choline ester of phosphoric acid. A second spot, at somewhat shorter retention time, was believed to be caused by cephalin (phosphatidylethanolamine). The effect of lecithin on the assay was determined. A 1 mg/mL solution of lecithin in methylene chloride was prepared; aliquots containing 0.1, 0.5, and 1.0 mg were transferred to flasks. The solvent was evaporated, and the solutions were assayed. The fluorescence readings were 40% lower than that of the negative control, suggesting some inhibition of the cholinesterase enzyme had occurred. In the presence of both lecithin and 1.0 μg of carbofuran, the fluorescence readings suggested nearly complete inhibition. Lecithin forms a colloidal suspension in aqueous solutions, as evidenced by some

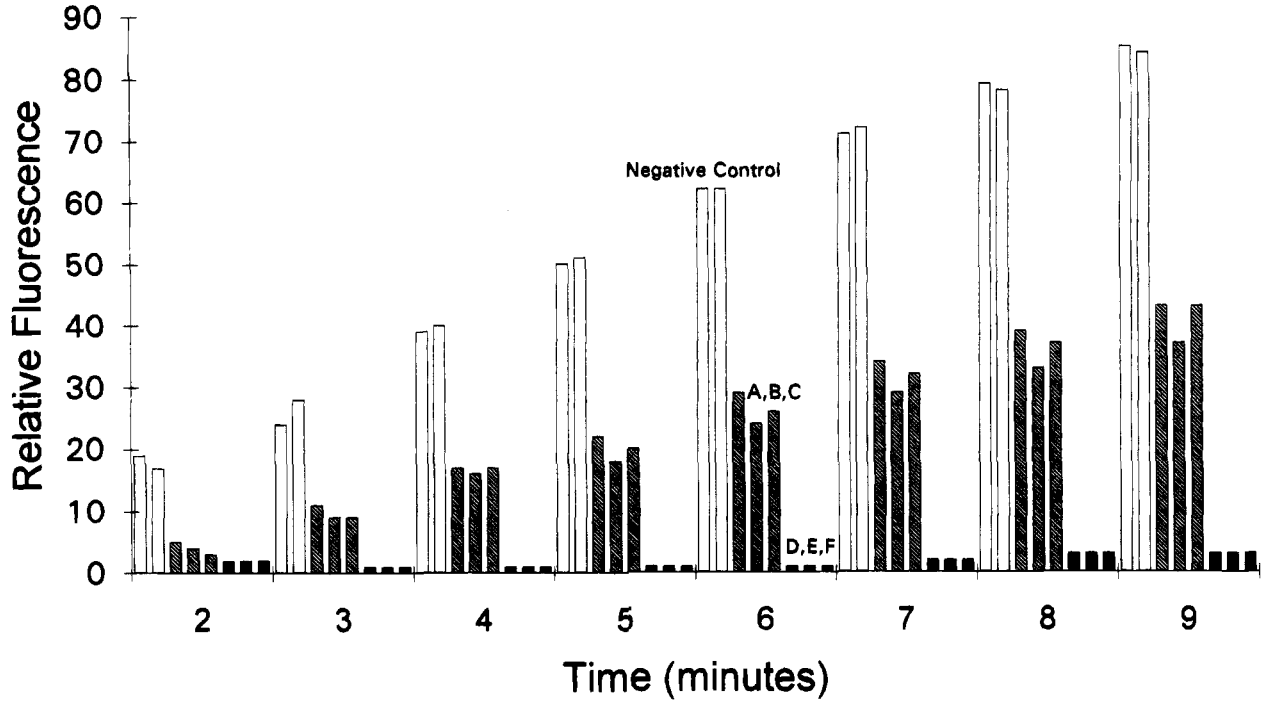

Figure 4. Stability of carbofuran in aqueous meat extracts over 4 h: aliquots of aqueous filtrate extracted with methylene chloride (A, D) immediately, (B, E) 1 h later, and (C, F) 4 h later; (A, B, C) 0.1-mL aliquot of methylene chloride evaporated and assay contains 0.025 μg of carbofuran at 100% recovery; (D, E, F) 4-mL aliquot of methylene chloride evaporated and assay contains 1.0 μg of carbofuran at 100% recovery.

turbidity in the assayed solutions containing 0.5 and 1.0 mg of lecithin.

Stability of Carbofuran in Aqueous Extract. Aqueous extracts of meat contain various types of proteins and other biochemicals that could degrade residues of carbofuran that are present. To determine if degradation of carbofuran was occurring in the extracts, 50 g of ground beef was fortified with 50 μg of carbofuran, blended with 200 mL of water, and filtered. Three 25-mL aliquots of the filtrate were extracted, each with 20 mL of methylene chloride, the first immediately after filtering and the second and third, 1 and 4 h later. Aliquots of the methylene chloride extracts were evaporated and assayed. Results given in Figure 4 show no difference in relative activity, suggesting little or no degradation of carbofuran had occurred in the aqueous filtered extract over 4 h. It is also plausible that some or all of the carbofuran could have degraded into another product over the time interval and that the degradation product possessed cholinesterase inhibitory powers similar to those of the parent. Water-soluble conjugates present as metabolites presumably were not extracted by the methylene chloride. This assay relies on a hydrolysis reaction that includes time as a variable and should not be used as the method of choice for determining percent recovery of these carbamates. A vast amount of literature is available to show that methylene chloride extracts nearly 100% of the carbamate insecticides from aqueous solutions.

Relationship between Method and Tolerance Levels in Meat. This screening test is based on the common ability of carbamate insecticides to inhibit the enzyme acetylcholinesterase. Tolerances have been established for the combined residues of the insecticide carbofuran and its metabolites in cattle meat at 0.05 ppm and for aldicarb and its metabolites at 0.01 ppm (*Code of Federal Regulations*, 1992). In addition, no tolerance in meat has been established for the other carbamates listed in Figure 1. Furthermore, as shown in Figure 1, there is nearly a 10-fold difference in enzyme inhibition between carbofuran and 3-hydroxycarbofuran, methomyl, and oxamyl. Therefore, on the basis of data in Figures 1, 3, and 4, assays that proved to be highly inhibitory (e.g., 0.4- or 4.0-mL aliquot of the methylene chloride representing 0.1 and 1.0 g of beef) would be judged to contain either (1) carbofuran or aldicarb at a concentration of at least 1 ppm, substantially above the established tolerances; (2) other carbamate insecticides at still higher concentrations; or (3) other inhibiting chemicals not yet considered in this method. Various organophosphorus insecticides are known to inhibit cholinesterase but usually require oxidation to their oxygen analogs before becoming highly inhibitory. For routine assays the number of samples expected to produce false positives would be directly proportional to the size of the aliquot selected. Because of these limitations, this assay is not sensitive enough to be used for absolute quantification.

Conclusions. A rapid method of extraction and analysis was developed for screening trace amounts of carbofuran and other cholinesterase-inhibiting insecticides that could be present in meat due to accidental or deliberate adulteration. Because the tolerance levels are set low and metabolism is high in animals, few studies have been published to suggest contamination. By following the label recommendations for the proper use of insecticides, the farmer and consumer are assured that tolerance levels are not exceeded.

This test appears to be best used as a visual screen. A simple visual comparison of samples against negative controls should permit the screening of more samples within the same period. The rapid formation initially of both fluorescence and a yellow color, followed later by the formation of a blue precipitate, can serve as a permanent visual marker for those assays that would be judged as negative. Samples found to be inhibitory will require confirmation by GC and HPLC methods of analysis that are inherently more precise and less prone

to errors. Carbamates, being polar and thermally unstable to gas chromatography, are usually determined in water and agricultural products by HPLC (Moye et al., 1977; Argauer, 1980; Krause, 1985; McGarvey, 1989; de Kok and Hiemstra, 1992; Page and French, 1992). This research should benefit regulatory agencies by providing alternate rapid screening procedures that may increase the number of samples that can be monitored.

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