Table II. Comparison of Field and Olfactometer Ratings of Esters of 6-Methyl-3-cyclohexene-1-carboxylic Acid

Ester	Field	Olfactometer
sec-Butyl	279	87
1-Ethylpropyl	231	83
Isopropyl	100	100
Butyl	98	71
Propyl	58	96
Allyl	53	107
Isobutyl	48	99
Cyclopentyl	47	79
2-Propynyl	40	83
Ethyl	38	122
2-Chloroethyl	29	86

Eleven of the compounds that appeared most effective in the olfactometer were tested in the field. They were applied to wicks in dry traps (*5*) at 2 to 3 ml. per wick in combination with 0.5% of the toxicant 2,2-dichlorovinyl dimethyl phosphate (DDVP) and exposed in infested coffee and citrus areas. (DDVP alone in traps catches nothing. There is no definite information on its repellency, except that it gave one of the highest catches of the flies of all the insecticides tried. It, therefore, is presumed not to be appreciably repellent, if at all.) Efficiency ratings of the compounds in comparison with the isopropyl ester are shown in Table II. For the purpose of comparison, the ratings obtained in the olfactometer tests are also included.

Discussion

As a fairly large group of closely related esters of 6-methyl-3-cyclohexene-1-carboxylic acid had been tested, the results were studied to determine whether any relationship existed between the chemical structure of the alcohol component of the esters and their attractiveness to the Medfly.

The olfactometer ratings indicate that the following esters tested had attractant ratings of 85 or better at the concentrations tested: ethyl, propyl, isopropyl, isobutyl, *sec*-butyl, allyl, 2-chloroethyl, and 2-bromoethyl. The most effective compounds were among those with an alcohol moiety not exceeding four carbons. In general, the attractiveness of the compounds with larger radicals falls off sharply.

In the field, esters prepared from secondary alcohols were superior to those prepared from primary ones. Thus, the isopropyl ester proved better than the propyl, and the *sec*-butyl ester was the best of those tested in the field. The 1-ethylpropyl ester, which had only an 83 attractant rating in the olfactometer, proved effective in the field.

Performance of an attractant in the field as compared with that in the olfactometer may be affected by its volatility, and this variable, along with many other factors that affect field performance, is being investigated currently.

A large number of closely related compounds proved attractive, in some degree, to the Medfly in this study. Thus far, the action of these esters has appeared to be specific to this species, but they have not been tested extensively in areas where other species of fruit flies occur.

Acknowledgment

The attractants program at Beltsville is under the direction of S. A. Hall and N. Green; the entomological program in Hawaii is under the direction of L. D. Christenson and L. F. Steiner. The olfactometer tests were conducted by Doris H. Miyashita. R. Kinoshita, S. Nakagawa, and G. Farias assisted in the field tests.

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Received for review January 14, 1958. Accepted March 18, 1958.

INSECTICIDE RESIDUES

Demeton Residues in Collards, Lettuce, and Mustard

C. H. VAN MIDDELEM and R. E. WAITES

Florida Agricultural Experiment Station, University of Florida, Gainesville, Fla.

Demeton residues were obtained enzymatically by using a cholinesterase inhibition technique. Differential quantities of acetic acid resulting from the enzyme hydrolysis of acetylcholine were measured by pH change in a buffered system. The usually recommended 21-day waiting period following the last application was inadequate when three or four foliar applications (4-ounce active) were applied once per week. The Miller Bill tolerance of 0.75 p.p.m. (established on lettuce) can be met on all three of these leafy vegetables if foliar applications, using recommended dosages, are spaced 2 to 3 weeks apart and a 21-day waiting period following the last application is allowed before harvest.

WITHIN THE LIMITATIONS of the law and under certain conditions, vegetable growers are resorting to the use of systemic insecticides for control of sucking types of insects such as aphids, mites, and mealybugs. As the normal weathering processes are not as effective in lowering toxic residues of certain systemics—i.e., in the case of conventional insecticides—considerable investigations are necessary to determine the quantities of this class of insecticide that persist on or in a raw agricultural commodity being prepared for interstate commerce. As the compound demeton (systox) was the first systemic insecticide to be cleared for commercial use on vegetables, it was chosen for this residue study. The active ingredient in demeton is a controlled mixture of isomeric organic phosphate esters. The two isomers are *O,O*-diethyl *O*-2-(ethylthio)ethyl phosphorothioate and *O,O*-diethyl-*S*-2-(ethylthio)ethyl phosphorothioate.

Since passage of the Miller Bill (Public Law 518), a number of official tolerances have been established recently for the use of demeton on certain commercial vegetables, fruit, alfalfa, and cotton. A tolerance of 0.75 p.p.m. has been established by the Food and Drug Administration on the following leafy vegetables: broccoli, brussel sprouts, cabbage, cauliflower, and lettuce. The U. S. Department of Agriculture, after examining pertinent residue data, specified that growers should maintain a 21-day waiting period between their last foliar application and harvesting demeton-treated leafy vegetables for which a tolerance has been

approved. No recommended interval has been officially established to date for the use of demeton on the important vegetable crop grouping known as "greens." The residues on collards and mustard reported here are an effort to determine whether the established interval on such vegetables as lettuce is sufficient for greens as well.

As pointed out by Von Rümker (*7*), demeton does not contain any chemical groupings in its molecule that would lend themselves as a basis for a specific chemical method for residue analyses. However, many of the organophosphatic insecticides are exceptionally active inhibitors of the enzyme cholinesterase. This inhibitory effect causes interferences in the normal mechanism of nerve impulse transmission of vertebrates and invertebrates. The function of cholinesterase is to hydrolyze acetylcholine which accumulates as a result of muscle action and is highly toxic. Therefore, this method is based on the characteristic property of the entire class of phosphatic insecticides, which is their ability more or less to inhibit the normal function of the enzyme cholinesterase. Although nonspecific, the cholinesterase inhibition technique has the definite advantage of determining total toxicity in the sample rather than only a portion thereof as is sometimes the case when a specific chemical is employed.

Michel (*5*) pioneered the simplified method of measuring the enzyme activity of human blood plasma. In Michel's method, the acetic acid produced by the splitting action of cholinesterase on the substrate acetylcholine is measured in terms of the pH change in the presence of a buffer and following a standardized time interval. Using the basic principle of Michel's method, Giang and Hall (*2*) originated certain adaptations for microdeterminations of various organic phosphorus insecticides in plant material. Hensel *et al.* (*3*) adapted the basic procedure still further in order that subsurface as well as surface residues could be determined by maceration. In most instances, no extraction was employed, but the aqueous extracts themselves were subjected to the enzymatic analyses. The subsequent demeton residue data presented here were obtained by using a technique very similar to that advocated by Hensel *et al.* (*3*).

Because of the high surface area per fresh weight of such crops as collards, lettuce, and mustard, residue studies on samples taken from demeton-treated field plots were thought very necessary. Disappearances or degradation curves were developed for such crops in an endeavor to establish the safest waiting period that could be recommended to commercial vegetable growers who might wish to use demeton on crops of this type if official tolerances were established.

Procedure

Field Sampling and Subsampling. All vegetable experiments were replicated (randomized block) three times in the field and were designed specifically for residue studies. Untreated or check plots were also replicated three times and were carried throughout the sampling and analytical phases as treated plots. The collards and mustard were cut at random from four row plots until enough plant material was obtained to fill a 25-pound paper bag. Ten heads of lettuce were selected at random from the inner two rows of the normal four-row plot. Once harvested, the plant material was immediately brought into the laboratory and cut into approximately $^1/_2$-inch segments. The material was then well mixed and a representative 1000-gram sample was selected and placed in a sealed polyethylene bag. All demeton-treated samples were immediately placed in a freezer room (0° F.) and maintained in frozen state until analyzed.

Laboratory Preparation and Analysis. The residue samples were removed from the freezer long enough for a 150-gram segment to be cut from the frozen 1000-gram original composite sample. This material was allowed to thaw thoroughly in Mason pint jars—care being taken not to lose any of the liquid. After the sample thawed, 75 ml. of distilled water was added to the sample to facilitate the cutting action. The sample was macerated in a Model 5 Hamilton Beach Blendor for 3 minutes, and the slurry was filtered through four layers of clean cheesecloth. After the macerate had been squeezed dry, the resultant plant juices were aliquoted.

Following the neutralization of 35 ml. of the aqueous extract with 1*N* sodium hydroxide, the solution was transferred to 50-ml. volumetric flasks, 7.5 ml. of blood plasma was added, and the mixture was made to volume with distilled water. After the solution was mixed, the 50-ml. volumetric flasks were placed in a constant temperature, serological water bath for 70 minutes. During this period, the varying amounts of demeton differentially inactivate the cholinesterase present in the plasma.

At the completion of the 70-minute incubation period, 2-ml. aliquots were pipetted from the 50-ml. volumetric flasks into 15-ml. microbeakers, each determination being conducted in duplicate. Then 1 ml. of buffer [Hensel's procedure (*3*)] was added to each beaker, mixed with a stirring flea, and placed in a 37.5° C. controlled temperature bath for 10 minutes. The beakers were then removed and the initial pH reading was made. To each beaker was added 0.5 ml. of acetylcholine substrate solution [Hensel's procedure (*3*)], mixed with a magnetic stirrer and replaced in a 37.5° C. bath for 2 hours. After this period had elapsed, the beakers were removed from the bath, one at a time, and the final pH reading was made on a laboratory Model G meter. The difference (delta pH) between the initial and final pH of the untreated sample, which represents an uninhibited system, was used to calculate all demeton-treated samples. To calculate the per cent inhibition of the demeton-treated samples, the formula presented by Hensel (*3*) was used.

Preparation of Standard Curves. From the 21.2% demeton reference concentrate, a standard solution was prepared using distilled water as the solvent. The concentration of the standard was usually in the range of 1 ml. of solution, equivalent to 30 to 40 γ of actual demeton. Known increment quantities of demeton were pipetted into six 50-ml. volumetric flasks containing 35 ml. of untreated crop filtrate. Complete standard curves or appropriate portions thereof were determined daily. Due to variations in blood plasma and plant enzymes, some displacement in the standard curves of a given crop substrate usually occurs. A typical standard curve determined from the greens and salad vegetable homogenates is shown in Figure 1.

As certain plant samples of the earlier harvests contained relatively high concentrations of toxicant, less than the usual 35 ml. of treated plant substrate was required in order to obtain a percentage inhibition that would fall on the linear portion of the standard curve. Aliquots of the original treated plant filtrate may range from 1 to 35 ml., depending on the time of harvest following the last application, the dosage level of active insecticide used, and the nature of the crop being investigated for systemic residues. If less than the normal 35-ml. volume was used when analyzing certain treated unknowns, untreated check plant extract was added to standardize on the final volume.

Results and Discussion

The residue results reported in Table I are averages of duplicate analyses conducted on composite samples from each field replicate. Control or field check samples were selected and analyzed exactly as unknowns. The apparent demeton for each corresponding control sample was deducted from the results obtained from the treated replicate plot.

Although all results are reported in parts per million of demeton, considerable metabolic and oxidative processes were effecting the original toxicant on and within the plant prior to harvest and analysis. This manuscript is not an attempt to explain all the pos-

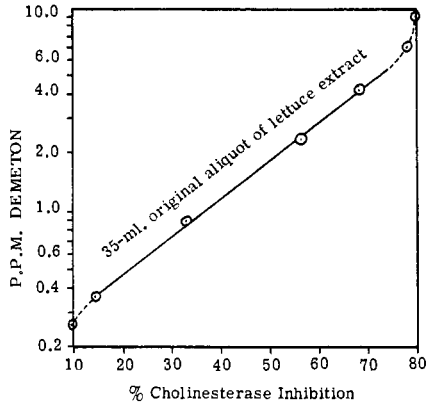

Figure 1. Typical standard curve developed from 2 ml. of aliquot of original solution composed of 35 ml. of plant extract, 7.5 ml. of human blood plasma, 1.0 ml. of buffer, and 0.5 ml. of acetylcholine chloride substrate

Points on graph resulted from addition of 10, 20, 30, 40, 60, and 80 γ of demeton to original extract volume

Table I. Demeton Residues at Intervals Following Foliar Applications

Vegetable Crop	Application Dates	Rate/ Applic. Active/ Acre, Oz.	Days from Final Applic. to Harvest	Residue, P.P.M.[a]
Lettuce[b]	May 17, 28, 1956	2	1	3.67 ± 0.18
			7	1.42 ± 0.24
			14	0.56 ± 0.09
Lettuce	May 17, 28, 1956	4	1	6.68 ± 0.13
			7	3.48 ± 0.32
			14	1.65 ± 0.09
Mustard[c]	Jan. 13, 25, 1956	2	1	2.45 ± 0.26
			7	1.05 ± 0.17
			14	0.50 ± 0.10
Mustard	Jan. 13, 25, 1956	4	1	7.07 ± 0.36
			7	2.42 ± 0.17
			14	0.66 ± 0.07
Collards[d]	Oct. 29, Nov. 5, 13, 19, 1956	4	7	12.80 ± 1.20
			14	6.06 ± 0.47
			21	2.66 ± 0.26
Lettuce	Oct. 29, Nov. 5, 13, 19, 1956	4	7	11.90 ± 1.54
			14	3.59 ± 0.56
			21	1.31 ± 0.06
Mustard[e]	Oct. 23, 30, Nov. 6, 1956	4	7	9.06 ± 0.53
			14	3.23 ± 0.22
			21	1.51 ± 0.29

[a] Average of three field replications and duplicate lab analysis.

		Rainfall, In.	Mean Temp., °F.
[b] Climatological data:	May 17–31	0.94	75.0
	June 1–10	2.64	75.8
		Rainfall, In.	Mean Temp., °F.
[c] Climatological data:	Jan. 13–31	3.02	51.7
	Feb. 1–8	0.94	55.9
		Rainfall, In.	Mean Temp., °F.
[d] Climatological data:	Oct. 29–31	0.00	67.0
	Nov. 1–30	2.66	58.6
	Dec. 1–10	0.00	54.5
		Rainfall, In.	Mean Temp., °F.
[e] Climatological data:	Oct. 23–31	0.00	66.8
	Nov. 1–7	2.25	59.8

sible changes that took place in the originally applied systemic per se, resulting in the final product that was measured by the cholinesterase inhibition technique. Metcalf (*4*) states that when demeton is primarily functional as a contact insecticide, the thiono isomer is only 0.2 to 0.3 as toxic as the thiol isomer and is therefore a weaker inhibitor. Moreover, the thiol isomer is absorbed and translocated in plants from five to 10 times as rapidly as the thiono isomer and is probably responsible for the primary systemic effectiveness of demeton as a cholinesterase inhibitor.

The demeton residues in the vegetable greens were found to be somewhat higher than in cabbage or celery tested earlier. In general, however, after applying recommended dosages of demeton and observing the proper interval between applications, a 21-day waiting period before harvesting was adequate for collards, lettuce, and mustard. Where this interval was adhered to following the last foliar application, marketable leafy vegetables contained residues below the official tolerance levels established for demeton on lettuce. However, experimental evidence in the table indicates that higher than tolerance levels of demeton may result on and in leafy vegetables if applications are made at too frequent intervals, even though the proper final interval before harvest is observed. Too frequent application of demeton is not only dangerous because of the buildup of excess residues, but it is not required to obtain satisfactory biological control.

Figures 2 and 3 illustrate demeton persistency curves on lettuce, collard, and mustard greens. Both sets of graphs plot time after last application against the logarithm of residue concentration. The official tolerance for lettuce is illustrated at 0.75 p.p.m. on both graphs for comparative purposes. Projecting the graph of the 4-ounce active dosage applied twice per month in Figure 2 shows that the 21-day interval would probably be sufficient time to reach the tolerance limit. The 21-day interval would be inadequate for weekly applications of the 4-ounce treatment on lettuce.

A 2- or 4-ounce dosage applied twice at 2-week intervals would be entirely safe for mustard greens (Figure 3) provided at least a 15-day interval is allowed following the last application. This is assuming of course that a 0.75-p.p.m. tolerance be established on the greens. Three or four 4-ounce active foliar dosages applied consecutively each week is entirely out of the question for all practical commercial usage on greens because of the resultant excessive residues.

The very high systemic and conventional pesticide residues that are found in leafy vegetables when compared with a more smooth-surfaced fruit are to be expected. There are of course, numerous factors involved in the higher residues found on crops such as the greens. One of the most important is the greater and more heterogeneous surface area that is exposed to the foliar spray. The fact that a systemic pesticide is involved in this study brings up other factors.

Some of the demeton that remained on the vegetables after the foliar spray applications probably did not become truly systemic, but merely deteriorated and volatilized more or less as a conventional organophosphatic insecticide. However, some unknown quantity of the demeton undoubtedly achieved limited penetration of the epidermal layers and became systemic, resulting in longer period for detoxification to safe levels. While leaves may not be considered normal absorptive areas of a plant, some limited absorption by a systemic can occur. Also, a foliar-applied systemic such as demeton,

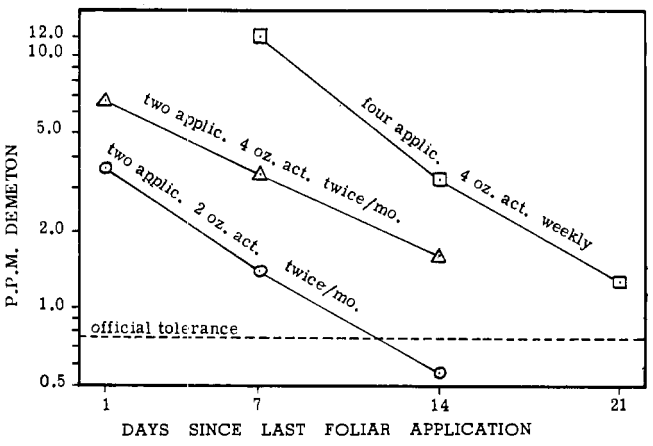

Figure 2. Demeton residue persistency on lettuce

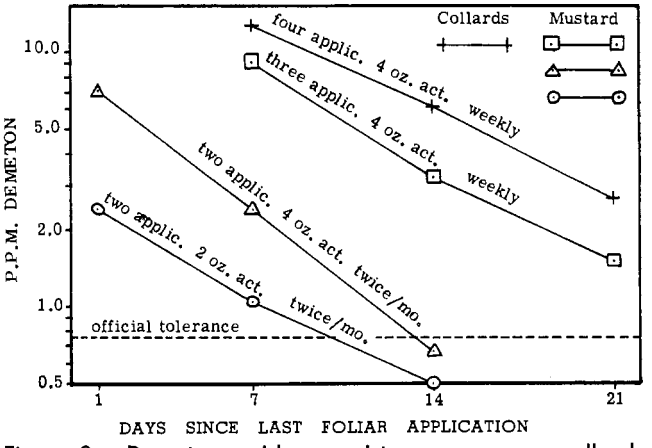

Figure 3. Demeton residue persistency curves on collards and mustard greens

when applied to leafy vegetables, deteriorates or detoxifies with time by a number of processes. Some of these processes such as evaporation, adsorption, and breakdown are probably taking place simultaneously (*1*).

As the translocation of demeton moves more preferentially through the xylem (*1*, *8*), it will move quite rapidly up into young, actively growing plants following absorption through the roots. According to Tietz (*6*), the active ingredient of demeton is translocated in the plants tested through the xylem following an application of the region of the roots, whereas following a leaf treatment it is transported chiefly in the phloem. He also states that the demeton is translocated through the medium of the transpiration stream to shoot organs growing above the ground. As a result of foliar applications, therefore, limited translocation and diffusion could occur in the leaves, primarily in the peripheral regions. There is also the possibility that some of the foliar applied systemic fell on the soil and was eventually absorbed through the roots and carried into the plant through the transpiration stream.

There are of course many intangibles and uncontrollable variables involved in any pesticide residue experiment in the field. A given crop, maturing rapidly under a comparatively high mean temperature, might be expected to absorb and translocate demeton rather readily in comparison with the same crop growing more slowly under cooler temperatures. There are also, however, the variables of sunlight, rainfall, and wind and their interactions which would tend to break down a foliar-applied systemic phosphate—as a conventional phosphate insecticide—until actual penetration of the plant itself was achieved.

In dealing with systemic insecticides applied to edible portions of leafy vegetables, the following factors should be kept in mind as far as possible excess residues remaining on the marketable crop: heterogeneity of leaf surface, very high surface to fresh weight ratio, dosage level in active ingredient, number of and interval between applications, interval or waiting period following the last application and harvest, possibility of systemic entering plant through roots as well as limited access provided by leaf contact, slower rate of detoxification and breakdown after penetration of the plant, stage and rate of plant growth at application time and the highly important, but largely uncontrollable, climatic factors.

Acknowledgment

The authors are grateful to the Chemagro Corp. of New York and Pittsburgh, Pa., for furnishing outdated, human blood plasma, as the source of cholinesterase used in this study, and 21.2% demeton standard reference.

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Received for review May 22, 1957. Accepted April 18, 1958. Divisions of Agricultural and Food Chemistry and Analytical Chemistry, 131st Meeting, ACS, Miami, Fla., April 1957. Florida Agricultural Experiment Station Journal Series No. 621.

PESTICIDE RESIDUES

Determination of Diphenylamine Residues on Apples

ROBERT B. BRUCE, JOHN W. HOWARD[1], and JAMES B. ZINK

Hazleton Laboratories, Falls Church, Va.

DIPHENYLAMINE has shown promise as an effective agent for the prevention of scald on apples. This report presents two procedures that are useful for the determination of this compound as a residue on fruit.

Methods similar to those presented here have been described. Ponomarenko (*3*) described a colorimetric procedure for the determination of diphenylamine in air by coupling with diazotized sulfanilic acid in acid solution. Clements and Harrow (*1*) presented a method for the determination of diphenylamine in dyes based on ultraviolet absorption.

Analytical Procedure

Special Reagents. Petroleum ether. Reagent grade, purified by allowing each gallon to percolate through a column 2.6 × 76 cm. of silica gel (Davison Chemical Co. 20–200 mesh). The first 100 ml. is discarded.

n-Heptane. Purify 99% *n*-heptane (Phillips Petroleum Co.) in the same manner as the petroleum ether.

[1] Present address, National Cotton Council of America, Washington, D. C.