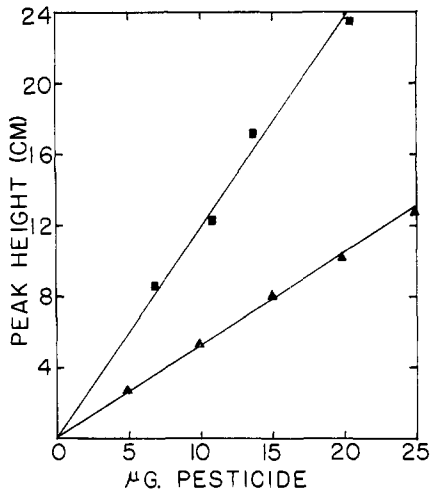

Figure 2. Standard curves of ethion (■——■) based on ethyl iodide and malathion (▲——▲) based on methyl iodide

of 48% hydriodic acid. Immediately place a piece (about 1.5 inches square) of 3-mil polyethylene on top of the vial and screw the cap on tight. The cap used is modified by drilling a $^3/_{16}$-inch hole through it and the paper liner. A silicone rubber half-septum is then fitted tightly in this hole. Immerse the sealed vial about 1 inch deep in a boiling water bath for 10 minutes. Remove and immediately transfer 10 μl. of the headspace gases in the vial into the gas chromatographic column. Plot peak height in centimeters against micrograms of insecticide in the vial.

The syringe was rinsed thoroughly with hexane between injections. Hexane was removed from the syringe by repeatedly drawing air into the syringe.

Results and Discussion

Figure 1 shows the chromatograms obtained by the method. The peak in *A* results from injection of 10 μl. of air. The peak presumably represents capture of electrons by oxygen. In *B* is shown the chromatogram of 10 μl. of the headspace from a vial containing 1 ml. of hydriodic acid which was heated (100° C.) for 10 minutes. One milliliter of acetone had been evaporated to dryness in the vial before introduction of the acid. The first peak represents oxygen. The second peak may be due to an impurity in the hydriodic acid or acetone residue. More likely it is due to the production of iodine in traces by disproportionation of hydriodic acid. Although the caps were placed on the vials as tightly as possible before the incubation step, leakage of the resulting alkyl iodides may have occurred.

Chromatogram *C* represents 10 μl. of the headspace from a vial containing pure methyl and ethyl iodides in air. The retention times for ethyl iodide evolved from ethion (containing only ethoxyl groups) and methyl and ethyl iodides from malathion (containing both methoxyl and ethoxyl groups) are identical with those in *C*.

Figure 2 shows the standard curves for ethion based on the peak height for ethyl iodide and malathion based on methyl iodide. The choice of the methyl iodide peak for developing the standard curve for malathion and of the ethyl iodide peak for ethion was to illustrate that the determination of the organophosphorus compounds could be based on cleavage of either methoxyl or ethoxyl groups. Since ethion contains only ethoxyl groups, malathion was chosen for the study as a common insecticide containing methoxyl (as well as ethoxyl) groups.

The Zeisel reaction yields alkyl iodides when applied to alcohols, ethers, and esters containing alkoxyl groups with not more than three to four carbon atoms. The method may be applicable therefore to analysis of organophosphorus insecticide residues after separation of naturally occurring interferences. It should be directly applicable to analysis of atmospheric samples.

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INSECTICIDE-ACARICIDE RESIDUES

Residue Analysis of Ethion by Cholinesterase Inhibition after Oxidation

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ETHION (*O,O,O',O'*-tetraethyl S,S'-methylene bisphosphorodithioate) is an effective insecticide and acaricide. It can be used for the control of olive scale in olive orchards. For this reason it was necessary to develop a sensitive method for residue analyses of this compound on olives.

The presently available methods for residue analysis of ethion are based on a colorimetric procedure (*4*) and cholinesterase inhibition (*5*). For the latter method, the extract or pure standard is first oxidized to an active form by dilute bromine water. This proved unsuccessful for olives since enough olefinic compounds were present in extracts which reacted preferentially with bromine water and resulted in incomplete activation of the ethion.

For this reason another procedure for the determination of trace amounts of ethion in olives was developed. Essentially, this method is based on the activation of the compound by peracetic acid, similar to the methods for Trithion and phorate (*1, 6*).

Experimental

Reagents. Barbital buffer, pH 8.0. Dissolve 7.42 grams of sodium barbital, 89.46 grams of potassium chloride, and 1.09 grams of monobasic potassium phosphate in 1 liter of distilled water. The pH is adjusted, if necessary, to 8.00 ± 0.10 with acid or base just prior to adjusting the volume to 1 liter.

Acetylcholine bromide, 0.22*M*. Dissolve 4.97 grams in 100 ml. of distilled water.

Saline solution, 0.9%. Dissolve 9 grams of sodium chloride in 1 liter of distilled water.

Glycerol solution, 10% v./v. Add 10 ml. of glycerol to absolute methanol and adjust the volume to 100 ml. with absolute methanol.

Cholinesterase solution. Mix 3 ml. of fresh horse plasma or 4 ml. of outdated human plasma with 2 ml. or 1 ml. of 0.9% saline solution, and 10 ml. of barbital buffer to result in a final volume of 15 ml. Larger amounts of enzyme

Ethion is extracted from whole olives with acetonitrile after maceration to a fine pulp in a food chopper. Interfering suspensions are removed by filtering the extract through filter paper. The concentrated extract is cleaned up on a sodium carbonate–carbon–celite column. The column is eluted with chloroform, the chloroform evaporated, and the residue dissolved in benzene. The benzene solution of ethion is activated with peracetic acid, washed free of excess acid with a saturated solution of sodium sulfate, dried over anhydrous sodium sulfate, and analyzed by the potentiometric method for residue analysis by cholinesterase inhibition.

solution can be prepared by using multiples of the 15-ml. volume. The activity of the cholinesterase solution should produce a pH change between 1.5 and 2.0 pH units when incubated with the acetylcholine bromide (0.22*M*) substrate for 60 minutes at 25° C. If this activity is not attained, the amount of plasma may be increased proportionately to the amount of saline. However, a maximum volume of plasma is 5.0 ml. since it is necessary at all times to have 10 ml. of barbital buffer per 15-ml. volume of the enzyme solution to maintain the buffered solution at pH 8.0 ± 0.1. After mixing, the enzyme solution is kept in an ice bath and prepared fresh daily.

Ethion standard, working solution. Stock solution is prepared by weighing 100 mg. of ethion analytical standard (Niagara Chemical Division, Food Machinery Corporation, Middleport, New York) and adjusting to 100 ml. with benzene. The working solution of ethion is prepared by diluting an aliquot of the stock solution so that the final concentration is 1.0 μg. of ethion per ml. of benzene. Five milliliters of this solution is oxidized and processed as described below.

Apparatus. Constant temperature shaking apparatus for 25° C. operating temperature. Labline-Dubnoff Incu-Shaker Series 54 or equivalent, equipped with a shaking platform containing 36 holes, 26 mm. in diameter, to accommodate 10-ml. Griffin beakers.

Expanded-scale pH meter. Beckman Model 76 or equivalent equipped with $2^1/_2$-inch electrodes and 36-inch shielded lead on the glass electrode.

Barnstead portable water bath with thermostat or equivalent.

Universal flash evaporator (Buchler Instruments, Batch Model FE-2) or equivalent.

Ten-milliliter Griffin beakers.

Agitating rods for Griffin beakers, stainless steel, 3 × 18 mm.

Hair dryer equipped with a glass manifold for evaporating solvents from the Griffin beakers.

Extraction. The whole olives are chopped to a fine pulp in a Hobart food chopper. Five hundred grams of the pulp are stripped for 1 hour with 1 liter of acetonitrile by tumbling in a sealed 1-gallon can at 32 r.p.m. Each can contains a stainless steel baffle to improve extraction efficiency. The resultant suspension is filtered through a 24-cm. Whatman No. 1 filter paper. One hundred milliliters of the stripping solution is taken to dryness in vacuo at 50° to 60° C. and redissolved in 10 ml. of chloroform. Each milliliter of this solution is thus equivalent to the extractives from 5 grams of plant material.

Cleanup. The column packing consists of a mixture of 30 grams of anhydrous sodium carbonate, 10 grams of Hi-flow celite, and 5 grams of Darco-G60 carbon. A glass column originally designed by Shell Chemical Company (*7*) is used (Figure 1). The column is dry-packed with a glass-wool plug, 10 grams of anhydrous sodium sulfate, and 40 grams of the packing mixture described above. While a moderate vacuum is applied, the packing is tamped with a glass rod and 50 ml. of chloroform is slowly added. As the chloroform level approaches within a few centimeters of the top of the absorbent, another 10 grams of anhydrous sodium sulfate and a glass-wool plug are added. The column is washed with 50 ml. of chloroform, and 5.0 ml. of the concentrated plant extract (equivalent to the extractives from 25 grams) is added. The column, while under vacuum, is eluted with 400 ml. of chloroform into a 500-ml. round-bottomed flask. The eluate contains only traces of pigments and foreign materials. The chloroform solution is evaporated in vacuo at 50° to 60° C. to 2 ml., and 10 ml. of benzene is added. The evaporation procedure is repeated twice to eliminate all chloroform. The volume is adjusted to 10 ml. with benzene equivalent to the extractives from 2.5 grams of plant material per ml. The time required for this purification is approximately 2 hours. Five milliliters of this solution is oxidized by a modification of the peracetic acid oxidation procedure of Patchett and Batchelder (*6*).

Grapes, apples, corn, and almond meats are extracted with benzene in the ratio of 1 gram to 2 ml. of benzene and are cleaned up as follows. An aliquot of the benzene extract equivalent to the extractives from 30 grams of crop material is evaporated to dryness in vacuo. The residue is dissolved in 50 ml. of pentane saturated with acetonitrile and extracted three times with 25-ml. aliquots of acetonitrile saturated with pentane. The pentane fraction is discarded. The

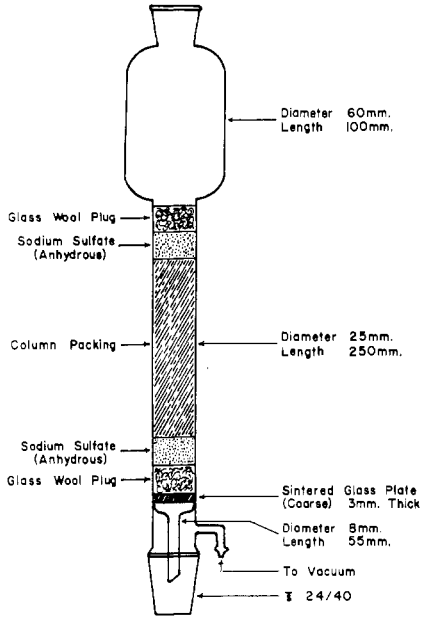

Figure 1. Chromatographic column for cleanup of crops prior to ethion analysis

Shell Chemical Co. column design (*7*)

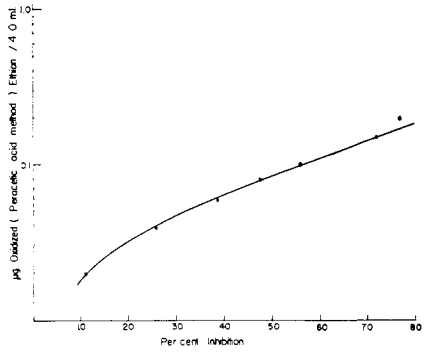

Figure 2. Standard curve for oxidized ethion by the potentiometric method using horse plasma as the cholinesterase source

pooled acetonitrile fractions are extracted with 100 ml. of pentane saturated with acetonitrile. The acetonitrile fraction is retained. The pentane is extracted four times with 20-ml. aliquots of acetonitrile saturated with pentane. All acetonitrile fractions are pooled and

evaporated to dryness in vacuo. The residue is dissolved in 6 ml. of benzene. A 5-ml. aliquot is treated as described for the activation of ethion in olive extractives.

Activation of Ethion and Olive Extractives. Five milliliters of the cleaned-up olive extractives in benzene solution are pipetted into 16- × 150-mm. glass-stoppered test tubes. The tube dimensions and other conditions were chosen for best oxidation as discussed by Patchett and Batchelder (*6*). Three milliliters of a mixture of 1 volume of 30% hydrogen peroxide and 5 volumes of glacial acetic acid are added to the benzene layer. A boiling chip is placed in the tube, and the solutions are mixed. For each analysis, at least three tubes should be prepared—a control plant sample, a treated plant sample, and a standard ethion sample. The tubes are immersed in a water bath at 75° C. and heated for 20 minutes. The tubes are then immersed in an ice bath and cooled for 5 to 10 minutes. The solutions are decanted into 60-ml. separatory funnels and the lower layers discarded. The benzene layers are washed free of acetic acid with five 1-ml. washes consisting of two 1-ml. washes with a saturated sodium sulfate solution and three 1-ml. washes of distilled water. The final solutions are dried over anhydrous sodium sulfate and filtered, and aliquots are analyzed by cholinesterase assay as described below. Five milliliters of the working standard of ethion (1.0 μg. per ml.) is oxidized and processed as described above.

Analysis by the Potentiometric Method Using Cholinesterase Inhibition. Analysis is performed using a modification of the potentiometric method (*3*, *8*). To prepare a standard curve, pipet aliquots (0., 0.02, 0.04, 0.06, 0.08, 0.10, 0.15, and 0.20 μg.) of the oxidized working standard solution of ethion into 10-ml. Griffin beakers. Pipet 0.5 ml. of the glycerol solution into each beaker. Duplicate single aliquots of the oxidized sample extracts are also treated in this manner. The glycerol reagent prevents the insecticide from going to complete dryness when the solvent is evaporated. Evaporate the solvent with a gentle stream of warm air from a hair dryer. Add a stainless steel agitator rod to each beaker after the solvent is evaporated, and rock the beakers gently on a constant-temperature, shaking apparatus at 95 cycles per minute. To beaker 1, add 3 ml. of the enzyme–buffer–saline solution and continue the addition to the remaining beakers at exactly 1-minute intervals. It is convenient to analyze 36 samples including standards in this manner. After exactly 29 minutes preincubation time, record the pH as read on the expanded-scale pH meter for beaker 1. At exactly 30 minutes, add 1 ml. of the acetylcholine bromide substrate (0.22*M*) to beaker 1 and read the pH of beaker 2. At exactly 31 minutes, add 1 ml. of the substrate to beaker 2 and read the pH of beaker 3. Continue this procedure for the remaining beakers at 1-minute intervals. The enzyme and substrate are allowed to incubate at 25° C. for exactly 60 minutes. After 60 minutes, record the pH of beaker 1 as read on the pH meter. At 1-minute intervals, record the pH of each of the remaining beakers.

Table I. Ethion Residues on Manzanillo Olives Treated for Control of Olive Scale

Days after Application	Net P.P.M.[a]	Recovery, %[b]
0	50.0	118
6	19.2	95
21	10.1	102
28	2.2	90
44	1.2	85
121	0.08	88

[a] Sensitivity of method 0.02 p.p.m.
[b] 1 p.p.m. of ethion added to olives prior to extraction.

A typical calibration curve for oxidized ethion by the potentiometric method, using horse plasma, is shown in Figure 2. The useful range of the standard curve extends from 0.02 to 0.20 μg. per 4.0-ml. volume. The standard curve is plotted on semilogarithmic paper with concentration of ethion *vs.* percent inhibition. Micrograms of pesticide per sample analyzed may be determined from the standard curve by reading percent inhibition of sample on the standard curve. Human plasma can be substituted for horse plasma as the cholinesterase source.

$$\Delta pH = pH\ (initial) - pH\ (final) \quad (1)$$

$$\%\ \text{inhibition} = 1 - \frac{\Delta pH\ (sample)}{\Delta pH\ (control)} \times 100 \quad (2)$$

$$p.p.m. = \frac{\mu g.\ found}{grams\ analyzed} \quad (3)$$

Results and Discussion

The extractives from as much as 1 gram of processed olives can be analyzed resulting in a sensitivity of 0.02 p.p.m.

The molar I_{50} value for peracetic acid activated ethion on horse plasma is $6.0 \times 10^{-8}M$ and for human plasma it is $3.7 \times 10^{-8}M$. For bromine-activated ethion, the molar I_{50} value is $4.8 \times 10^{-8}M$ for horse plasma and $2.5 \times 10^{-8}M$ for human plasma. As was shown previously (*7*), the treatment of phorate by peracetic acid presumably resulted in the conversion of thiono compounds to the phosphorothiolates. A similar mechanism may be operative in the activation of ethion. Processed olive extracts were analyzed by cholinesterase inhibition after activation with bromine water in dilutions ranging from $^1/_{10}$ to $^1/_{100}$ (*2*), but zero recovery was obtained on fortified check material at the 1 p.p.m. level. However, processed olive extracts, when analyzed by cholinesterase inhibition after activation with peracetic acid, resulted in recoveries ranging from 85 to 118% on check material fortified at the 1 p.p.m. level with ethion. The conclusion was that the bromine was reacting with olefinic compounds in the processed olive extracts. This reaction apparently interfered with the oxidation or activation of the ethion to a potent cholinesterase in vitro inhibitor. Untreated olives were found to be free from interference from natural inhibitors. Recoveries for the two possible phosphorothiolate analogs of ethion were not established at this time, since these compounds were not available in such purity as to serve as primary standards.

Table I contains data for a typical residue analysis of ethion activated by peracetic acid and analyzed by cholinesterase inhibiton. Manzanillo olives of an approximate size of 50 berries per 30 grams weight were sprayed in June with an ethion wettable powder formulation (Ethion 25 W). The trees were sprayed by hand with a Bean spray rig to the point of run-off. The spray mixture contained 2 pounds of Ethion 25 W plus 1 gallon of L-M flowable oil per 100 gallons of mixture. The fruit was picked at random after spraying and placed in a deep freezer until processed.

This method of residue analysis is applicable to other crops either without cleanup or with slight modifications in the cleanup procedure depending upon the individual crop materials. Peaches, strawberries, plums, pears, spinach, melons, and onions have been analyzed without cleanup. Almond hulls were treated similarly to olives except that the plant material was extracted with benzene in the ratio of 1 gram to 2 ml. of benzene. Grapes, apples, corn, and almond meats were extracted with benzene in the ratio of 1 gram to 2 ml. of benzene and cleaned up as described under experimental section. The method sensitivity has a limit of detection for all crops studied of 0.02 p.p.m., with the exception of 0.04 p.p.m. for almond meats.

The great advantage of cholinesterase inhibition methods for the determination of organophosphorus pesticide residues is that these methods measure a compound with a specific configuration of the molecule which is probably responsible for the toxic action toward insects and mammals. The sensitivity is significantly greater than that obtainable by most colorimetric methods. This method is particularly suitable when the insecticide undergoes metabolic changes in the plant to produce compounds with a high

inhibitory activity (*1*). A disadvantage of the inhibition methods is the lack of specificity to distinguish among different types of organophosphorus compounds.

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FUNGICIDE RESIDUES

Colorimetric Determination of Dexon Residues in Crops

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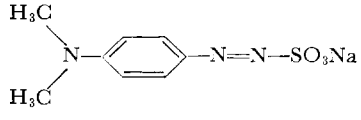
A colorimetric method has been developed for the determination of Dexon residues in plant material. The method is based on the light-catalyzed coupling of the compound with resorcinol in alkaline solution. The yellow product is extracted into benzene and measured in a spectrophotometer at 450 mμ.

DEXON (trademark, Farbenfabriken Bayer), *p*-dimethylaminobenzenediazo sodium sulfonate, is a nonmercurial fungicide for the protection of germinating seed and seedlings. It is particularly effective against those damping-off fungi in the genera *Pythium*, *Aphanomyces*, and *Phytophthora*. In the literature, this material has been referred to as Bayer 22555. Its structural formula is:

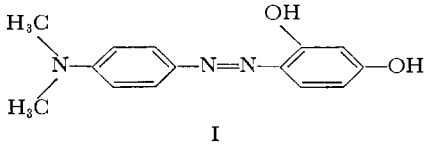


Dexon is a yellow-brown powder which dissolves in water to the extent of 2 to 3% at 25° C. to form an intensely orange-colored solution. The compound is soluble in highly polar solvents, such as dimethylformamide, but is insoluble in most organic solvents.

As is the case with many diazo compounds (*2*), Dexon is extremely sensitive to light. Dilute aqueous solutions of the compound are completely decolorized in 30 minutes or less when exposed to ordinary laboratory light conditions. However, solutions, including plant extracts, may be stabilized by the addition of sodium sulfite. Therefore, a 1% solution of sodium sulfite is used for the initial extraction of plant material.

The analytical method developed depends upon the light-catalyzed coupling of Dexon with resorcinol in alkaline solution. Light energy converts the stabilized diazo sulfonate into a labile form of the compound which then couples with the resorcinol.

The colored reaction product has been reported (*1*) to be:



I

The product (I) is yellow in acid solution, from which it may be quantitatively extracted with a nonpolar solvent such as benzene. The product (I) is stable in benzene for days.

The absorption spectrum of the yellow product (I) is shown in Figure 1. Maximum absorption occurs at 450 mμ. The molar absorptivity for the product (I) is approximately 26,800. The molar absorptivity of Dexon is approximately 16,100 with maximum absorption at 420 mμ.

As indicated above, Dexon is water-soluble and, as such, should be dialyzable. Experiments were undertaken to determine the practicability of dialysis as a process for the separation and clean-up of Dexon residues found in plant extracts. Seamless cellulose dialyzer tubing (The Visking Corp.) was selected as the dialysis membrane.

Dialysis Conditions

A study was made to determine the effect of time, temperature, and agitation on the rate of dialysis. Dexon, ring-labelled with C^{14}, was used for this purpose. One hundred fifty milliliters of sodium sulfite (1%) containing 200 to 300 μg. of radioactive Dexon were added to 100 grams of plant material (fresh corn kernels) or 100 ml. of distilled water, and blended for 2 minutes. The mixture was introduced into the dialysis tubing and dialyzed against 600 ml. of sodium sulfite (1%). The dialysis system was contained in a 2-quart, screw-capped jar covered with aluminum foil. Dialyses were conducted either in the cold (5° C.) or at room temperature (25° C.). Half of the samples for each temperature were placed on reciprocal shakers and agitated slowly during the dialysis period (120 to 140 cycles per minute).

The other half of the samples were not shaken. Aliquots (2 to 3 ml.) of the diffusate were removed at various times, and the radioactivity present was measured in a liquid scintillation spectrometer manufactured by Packard Instrument Co., La Grange, Ill. The radioassay procedure was similar to that proposed by Steinberg (*3*). The extent to which equilibrium had been reached after each interval of time was determined by comparing the radioactivity of the aliquots from the diffusate with the radioactivity of similar size aliquots from a standard solution. The standard solution was prepared by diluting a quantity of radioactive Dexon equivalent to that used for each dialysis to 850 ml. with sodium sulfite solution (1%). Thus, at equilibrium, the radioactivity of the aliquot from the diffusate should be equal to the radioactivity of the aliquot from the standard solution, and the ratio of the radioactivity of the diffusate aliquot to the radioactivity of the standard solution aliquot, reported