Determination of BAY 93820 Residues in Plant and Animal Tissues by Alkali Flame Gas Chromatography

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A specific gas chromatographic procedure is described for the determination of residues of BAY 93820 (*O*-methyl phosphoramidothioate *O*-ester with isopropyl salicylate), a promising new cotton insecticide, and its oxygen analog in plant and animal tissues. After initial extraction, the parent compound is separated by solvent partitioning from the oxygen analog and cleaned up separately. The oxygen analog is deaminated and methylated to convert it to a more desirable derivative for gas chromatography. Following cleanup and derivatization, the parent compound and the oxygen analog are recombined for gas chromatographic analysis. Final detection utilizes the phosphorus-sensitive, alkali flame detector which allows little interference from crop extractives. Recoveries of BAY 93820 or its oxygen analog from several animal tissues and cottonseed and its products were 75 to 110%. The method is sensitive to better than the 0.02 ppm level.

BAY 93820 (*O*-methyl phosphoramidothioate *O*-ester with isopropyl salicylate) is a broad spectrum insecticide of moderate mammalian toxicity. It has proven particularly effective for the control of bollworms and boll weevils on cotton. The structural formula is shown in Figure 1.

The metabolism of BAY 93820 was studied in plants by Church and Shaw (1969) and in animals by Church (1969). In both cases, the primary toxic metabolite was identified as the oxygen analog. The residue procedure thus developed is sensitive to the parent compound (P=S) and the oxygen analog (P=O).

BAY 93820 is being developed for use primarily as a cotton insecticide. Therefore, extraction and cleanup procedures were developed for cottonseed, its products, animal tissues, and milk. Cottonseed byproducts are used as cattle feed, thus necessitating a procedure for determining residues in meat and milk. The methods of analysis for these various samples differ only in the initial extraction. Because of their differences in chemical behavior, the two compounds (P=S and P=O) are separated after initial extraction, and each extract is separately cleaned-up and prepared for gas chromatographic analysis. The separate extracts are recombined before gas chromatographic analysis to simplify the procedure and to obtain separate peaks for each compound on a single chart.

ANALYTICAL METHOD

Apparatus. Gas chromatograph, Hewlett-Packard Model 5750 equipped with a flame ionization detector modified for thermionic operation as described previously (Thornton and Anderson, 1968).

Reagents. Diazomethane reagent (Stanley, 1966). Dissolve 2.3 g of KOH in 2.3 ml of distilled water in a 125-ml Erlenmeyer flask. Cool the flask to room temperature and add 25 ml of ethyl ether. Cool the flask in an ice bath. Working in a hood, add 1.5 g of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in small portions over a period of a few min to the flask. Agitate the flask thoroughly after each addition. Decant the ether solution from the aqueous slurry and store at −20° C in a tightly capped bottle until ready for use.

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Florisil, PR grade, 60 to 100-mesh. Heat the Florisil in an oven at 130° C for 24 hr, seal the container, and cool it to room temperature. Deactivate the Florisil by adding 7% water (7 ml, H_2O + 93 g of dried Florisil), mix it well, and allow it to equilibrate in a tightly stoppered bottle for 24 hr.

Keeper solution, 4% (wt/vol) glycerine in acetone.

Methylene chloride, (10%) in benzene. Prepare by adding 10 volumes methylene chloride and make to 100% with benzene.

Sample Preparation. Grind frozen cotton foliage and animal tissue samples in a Hobart food chopper in the presence of dry ice, and place the samples in frozen storage overnight to allow the dry ice to sublime. Grind cottonseed samples in a Wiley mill to pass a No. 3 screen.

Sample Extraction. EXTRACTION OF COTTONSEED, COTTONSEED HULLS, COTTONSEED MEAL, AND GIN TRASH. Weigh a 25-g sample into a Soxhlet thimble. Place the thimble in a Soxhlet extraction apparatus and extract for 3 hr using 250 ml of chloroform as solvent. Evaporate the extract on a rotary vacuum evaporator in a 30° C water bath until all the chloroform is removed. Dissolve the residue in 250 ml of Skellysolve B, and transfer it to a 500-ml separatory funnel. Rinse the flask with 150 ml of acetonitrile and add to the separatory funnel. Shake the separatory funnel for 30 sec. Allow the phases to separate and drain the lower phase into a 500-ml round-bottomed flask. Repeat the extraction again with 50 ml of fresh acetonitrile. Add 1 ml of glycerine keeper solution and evaporate the solution just to dryness on a rotary vacuum evaporator at 30° C. Proceed to the methanol:water/Skellysolve B partition.

EXTRACTION OF COTTONSEED OIL. Weigh a 50-g sample of cottonseed oil into a 500-ml separatory funnel. Add 250 ml of Skellysolve B and swirl the separatory funnel to dissolve the oil. Add 50 ml of acetonitrile and shake to extract. Allow the phases to separate and drain the lower phase into a 300-ml round-bottomed flask. Repeat the extraction twice more with fresh 50-ml portions of acetonitrile. Add 1 ml of keeper solution and evaporate the combined extracts just to dryness on a rotary vacuum evaporator at 30° C. Proceed to the methanol:water/Skellysolve B partition.

EXTRACTION OF COTTON FOLIAGE. Weigh a 50-g sample into a 1-quart blender jar. Add 300 ml of chloroform and blend the mixture at high speed for 3 min. Filter the extract through a 32-cm Whatman No. 2V fluted filter paper. Collect

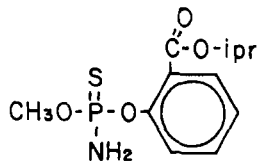

Figure 1. Structure of BAY 93820

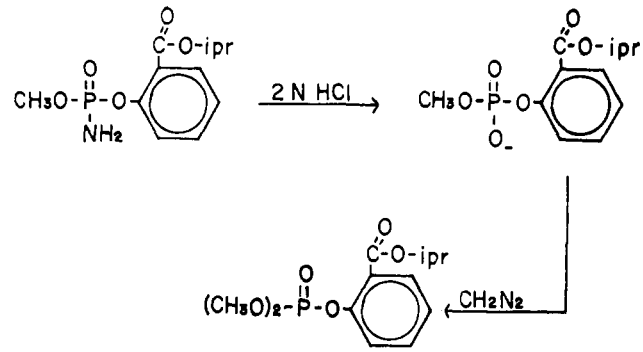

Figure 2. Derivatization sequence of BAY 93820 oxygen analog

150 ml of filtrate in a graduated cylinder. (If filtration is slow, cover the funnel with a clean folded cloth towel to reduce evaporation.) Add 1 ml of keeper solution and evaporate the solution just to dryness on a rotary vacuum evaporator at 30° C. Proceed to the methanol:water/Skellysolve B partition.

Extraction of Animal Tissues (Except Fat). Weigh a 50-g sample of chopped animal tissue into a 1-quart blender jar. Add 50 g of powdered anhydrous sodium sulfate, 10 g of Hyflo Super-cel, and 200 ml of acetonitrile, and blend the mixture for 2 min at high speed. Filter the sample with vacuum through a Whatman No. 541 filter paper covered with a $^1/_4$-in. layer of Hyflo Super-cel in a Büchner funnel. Return the filter cake to the blender and reblend it with 200 ml of Skellysolve B for 2 min. Filter as above. Rinse the blender with 100 ml of fresh Skellysolve B and wash the filter cake with the rinse. Transfer the combined filtrate to a 1000-ml separatory funnel, using 3 to 5 ml of fresh acetonitrile to complete the transfer. Shake the separatory funnel for 30 sec, allow the phases to separate, and drain the lower phase into a second 500-ml separatory funnel containing 250 ml of fresh Skellysolve B. Shake the second separatory funnel, allow the phases to separate, and drain the lower phase into a 500-ml round-bottomed flask. To the first separatory funnel add 50 ml of fresh acetonitrile and repeat the entire two-stage extraction. Add 1 ml of keeper solution to the flask and evaporate the combined acetonitrile extracts just to dryness on a rotary vacuum evaporator at 30° C. Proceed to the methanol:water/Skellysolve B partition.

Extraction of Fat Tissues. Weigh a 50-g sample of finely chopped fat tissue into a 1-quart blender jar. Add 200 ml of Skellysolve B and blend the mixture for 2 min at high speed. Filter the sample with vacuum through a Whatman No. 541 filter paper covered with a $^1/_4$-in. layer of Hyflo Super-cel in a Büchner funnel. Return the filter cake to the blender and blend with 200 ml of acetonitrile for 2 min. Filter as before. Rinse the blender with 100 ml of fresh Skellysolve B and wash the filter cake with the rinse. Transfer the combined filtrate to a 1000-ml separatory funnel using 3 to 5 ml of fresh acetonitrile to complete the transfer. Complete the partition as described for animal tissues above.

Extraction of Milk. Mix the milk sample thoroughly to disperse the cream. Weigh 100 g of milk into a 1-quart blender jar. Add 250 ml of acetone and blend the mixture for 3 min. Filter with vacuum through Whatman No. 541 filter paper in a No. 3 Büchner funnel. Rinse the blender jar with 50 ml of acetone and wash the filter cake with the rinse. Transfer the filtrate to a 1000-ml separatory funnel. Rinse the filter flask with 250 ml of chloroform and add to the separatory funnel containing the acetone filtrate. Shake the separatory funnel for 30 sec. Allow the phases to separate and drain the lower phase through a 32-cm Whatman No. 2V fluted filter paper into a 1000-ml round-bottomed flask. Repeat the partition using 100 ml of fresh chloroform. Evaporate the combined chloroform extracts using a rotary vacuum evaporator at 30° C. (NOTE: For the next series of extractions use Skellysolve B and acetonitrile which have been preequilibrated with each other.) Dissolve the residue from the previous steps in 50 ml of Skellysolve B and transfer to a 125-ml separatory funnel. Rinse the flask with 15 ml of acetonitrile and add the rinse to the separatory funnel. Shake the separatory funnel for 30 sec. Allow the phases to separate and drain the lower phase into a second 125-ml separatory funnel containing 20 ml of fresh Skellysolve B. Shake the second separatory funnel for 30 sec. Allow the phases to separate and drain the lower phase into a 100-ml round-bottomed flask. Repeat these extractions using 10 ml of fresh acetonitrile. Evaporate the combined acetonitrile extracts to dryness on a rotary vacuum evaporator at 30° C. Proceed to the methanol:water/Skellysolve B partition.

Methanol:Water/Skellysolve B Partition. Dissolve the residue from the previous steps in 125 ml of Skellysolve B and transfer it to a 250-ml separatory funnel. Rinse the flask successively with 20 ml of methanol and 80 ml of 25% aqueous sodium chloride solution, and add the rinses to the separatory funnel. Shake the separatory funnel for 30 sec, allow the phases to separate completely, and drain the lower, aqueous phase into a second 250-ml separatory funnel. Reserve the Skellysolve B phase in the first separatory funnel for the Florisil column steps. To the aqueous phase in the second separatory funnel add 25 ml of chloroform. Shake the funnel for 30 sec, allow the phases to separate, and drain the lower phase into a 100-ml round-bottomed flask. Repeat the extraction twice with fresh 25-ml portions of chloroform. Evaporate the combined chloroform extracts just to dryness on a rotary vacuum evaporator at 30° C.

Deamination (P=O Fraction Only). (Start a 5 μg oxygen analog standard at this point.) Dissolve the residue (P=O) from the chloroform extraction above in 5 ml of acetone. Add 20 ml of 2*N* HCl solution and swirl the flask to mix the solutions. Stopper tightly. Place the flask in a 60° C water bath for 30 min. Cool to room temperature, add 20 ml of water, and transfer the solution to a 125-ml separatory funnel. Rinse the flask with 25 ml of chloroform and add the rinse to the separatory funnel. Shake the funnel for 30 sec, allow the phases to separate, and drain the lower phase through a Whatman No. 541 filter paper into a 100-ml round-bottomed flask. Repeat the extraction with a fresh 25-ml portion of chloroform. Rinse the filter paper with 5 ml of chloroform. Evaporate the chloroform solution just to dryness on a rotary vacuum evaporator at 30° C.

Methylation (P=O Fraction Only). To the residue from deamination, add 2 ml of diazomethane reagent. Stopper the flask and let it stand for 10 to 15 min at room temperature. Evaporate the solution just to dryness on a rotary vacuum evaporator at 30° C. Hold this extract for combination with the P=S fraction.

Table I. Recovery of BAY 93820 and Its Oxygen Analog From Plant and Animal Tissues

Sample	Compound Added	Ppm Added	Recovery, %[a]
Cottonseed	BAY 93820	0.1	89
Cottonseed	BAY 93820 Oxygen Analog	0.1	82
Cottonseed Hulls	BAY 93820	5.0	94, 91
Cottonseed Hulls	BAY 93820 Oxygen Analog	5.0	78, 79
Cottonseed Meal	BAY 93820	5.0	84, 86
Cottonseed Meal	BAY 93820 Oxygen Analog	5.0	77, 74
Cottonseed Oil	BAY 93820	0.1	107, 98
Cottonseed Oil	BAY 93820 Oxygen Analog	0.1	115, 87
Cotton Gin Trash	BAY 93820	10.0	94, 92
Cotton Gin Trash	BAY 93820 Oxygen Analog	10.0	78, 78
Cotton Foliage	BAY 93820	10.0	93, 91
Cotton Foliage	BAY 93820 Oxygen Analog	10.0	84, 81
Bovine Brain	BAY 93820	0.05	90, 102
Bovine Brain	BAY 93820 Oxygen Analog	0.05	96, 84
Bovine Fat	BAY 93820	0.05	84, 99
Bovine Fat	BAY 93820 Oxygen Analog	0.05	116, 101
Bovine Fat	BAY 93820	0.02	87, 119
Bovine Fat	BAY 93820 Oxygen Analog	0.02	118, 83
Bovine Heart	BAY 93820	0.05	88, 92
Bovine Heart	BAY 93820 Oxygen Analog	0.05	80, 97
Bovine Kidney	BAY 93820	0.05	89, 107
Bovine Kidney	BAY 93820 Oxygen Analog	0.05	82, 120
Bovine Liver	BAY 93820	0.05	100, 94
Bovine Liver	BAY 93820 Oxygen Analog	0.05	100, 89
Bovine Liver	BAY 93820	0.02	101, 122
Bovine Liver	BAY 93820 Oxygen Analog	0.02	103, 103
Bovine Steak	BAY 93820	0.05	74, 93
Bovine Steak	BAY 93820 Oxygen Analog	0.05	86, 91
Bovine Steak	BAY 93820	0.02	100, 114
Bovine Steak	BAY 93820 Oxygen Analog	0.02	134, 127
Bovine Milk	BAY 93820	0.005	70, 87
Bovine Milk	BAY 93820 Oxygen Analog	0.005	113, 104

[a] Recovery percents are from separate determinations.

Florisil Column (P=S Fraction Only). Tamp a plug of glass wool into the bottom of a 20 × 400 mm chromatographic column. Fill the column to the reservoir with Skellysolve B. Slowly add 6 g of Florisil (deactivated with 7% water). Allow the Florisil to settle. Top the column with about 1 to 2 in. of granular sodium sulfate and drain the solvent to the top of the sodium sulfate. Drain the Skellysolve B containing the P=S fraction from the separatory funnel into the chromatography column. Pass it through the column at a rate of 2 to 4 drops per sec; discard the eluted Skellysolve B. Elute the column with 250 ml of 10% methylene chloride in benzene at a rate of 2 to 4 drops per sec; add the first few ml in portions to wash all the residue into the column. Collect the eluant in a 300-ml round-bottomed flask. To the column eluate add 1 ml of keeper solution and evaporate the sample on a rotary vacuum evaporator at 30° C to less than 4 ml. Transfer the residue quantitatively to the flask containing the deaminated, methylated P=O fraction, completing the transfer with several 1 to 2 ml washes of acetone. Evaporate the solution just to dryness on a rotary vacuum evaporator at 30° C.

Gas Chromatographic Analysis. Dissolve the combined residue from the previous steps in 5 ml of acetone (2 ml for milk samples). In the case of the standard, add 5 μg of BAY 93820 standard in 5 ml of acetone to the flask containing the derivatized oxygen analog standard. Inject aliquots of the sample or standard solution into the gas chromatograph maintained at the following conditions: column—1 ft × 4 mm i.d. borosilicate glass column, packed with 10% DC 200 solution coated on 80 to 100 mesh Gas Chrom Q; gas flows—helium carrier gas, 70 ml per min; air, 425 ml per min; hydrogen, adjust hydrogen flow after other gases are set so that approximately a ½ full scale peak results from a 5 ng standard injection with a noise level of 1% or better; temperatures—column 195° C, injection port 225° C, detector, 230° C.

Identify the BAY 93820 or oxygen analog peak by retention time and measure the area of the peak produced on the recorder strip chart with a polar planimeter. At the gas chromatographic conditions employed, BAY 93820 and the oxygen analog retention times are 4.2 and 2.3 min, respectively.

Calculate the parts per million of residue in a sample by comparing the response obtained from the unknown to the response obtained for a known amount of BAY 93820 or oxygen analog in a composite standard. Quantitation of P=S and P=O is therefore separate using the corresponding standard peak. Include appropriate factors for sample size, aliquots, and dilutions.

DISCUSSION

BAY 93820 can be easily isolated for gas chromatographic analysis by simple cleanup procedures. The oxygen analog, however, is markedly more polar and is very difficult to pass through the gas chromatographic column without serious loss by adsorption. It was found that BAY 93820 (P=S) and the oxygen analog (P=O) were completely separated by the methanol: water/Skellysolve B partition described in the procedure. This allowed the P=S portion to be further cleaned-up on a Florisil column, while the P=O was derivatized to give a more desirable compound for gas chromatography.

This derivatization involved deamination with 2*N* HCl (Garrison and Boozer, 1968) followed by treatment with diazomethane to form the *O,O*-dimethyl phosphate derivative, as shown in Figure 2. It was necessary to separate the P=S

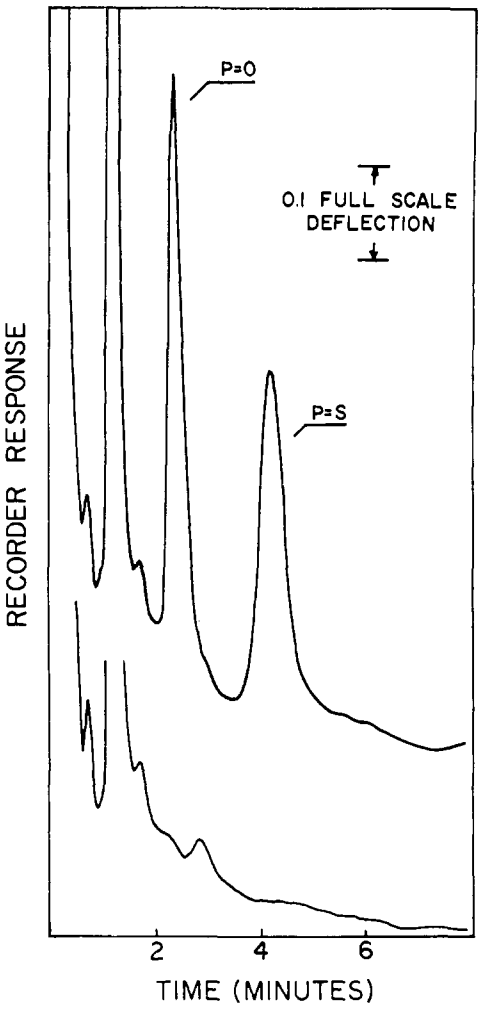

Figure 3. Gas chromatograms of control and 0.1 ppm recovery of BAY 93820 and oxygen analog from cottonseed

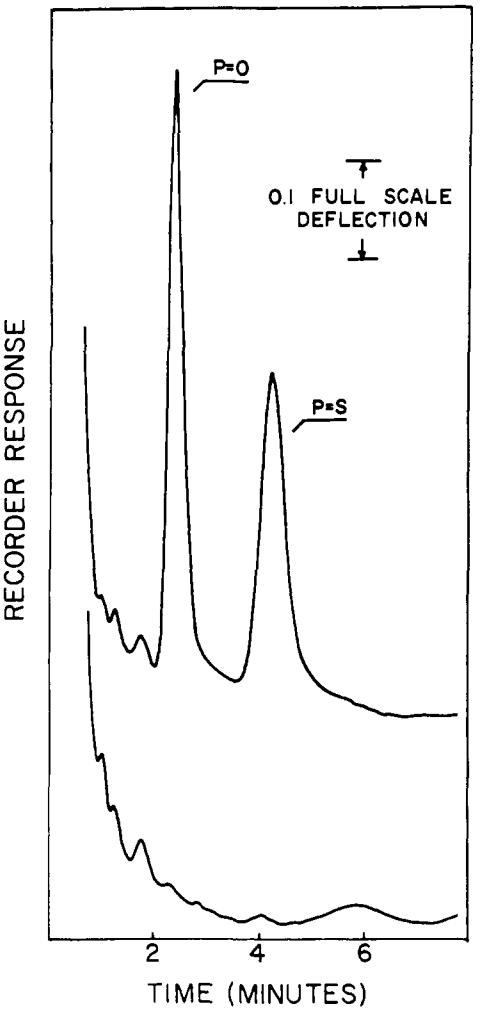

Figure 4. Gas chromatograms of control and 0.1 ppm recovery of BAY 93820 and oxygen analog from bovine heart tissue

before derivatizing the P=O, since the P=S was not stable in the presence of 2*N* HCl and decomposed instead of being deaminated. Time, temperature, and concentration studies were carried out to determine optimum conditions for deamination. Methylation of the deaminated P=O was almost instantaneous using diazomethane dissolved in ether. Some loss of the derivative was noted after methylation when the excess diazomethane and ether were removed with an air or nitrogen jet; however, no loss occurred when a rotary vacuum evaporator was used. The oxygen analog derivative was easier to chromatograph and was better separated from the parent compound than was the intact P=O. The individually treated P=S and P=O fractions were combined just prior to gas chromatographic analysis to simplify the procedure.

Solvent evaporations through the procedure were carried out at 30° C because of the volatility of BAY 93820 and its oxygen analog. BAY 93820 was the more volatile of the two; therefore, glycerine keeper was also added during its evaporation to reduce losses.

The solution coating technique (Applied Science Laboratories, 1967) was used to prepare the gas chromatographic column packing in order to obtain a column without tailing or adsorption. Also, the column was "no flow" conditioned at 250° C for 2 hr followed by flow conditioning at operating conditions for at least 4 hr before use. Several columns were prepared with identical results, indicating the procedure to be reproducible.

A standard curve was run to determine linearity of response in the gas chromatograph for BAY 93820 and the oxygen analog derivative. Response was linear over a 200-fold range for each, from 0.5 to 100 ng. Sample extracts containing residues in excess of 100 ng should be diluted and reinjected in order to ensure that the response falls along the linear portion of the plot.

Recovery experiments were run on a variety of samples by adding known amounts of BAY 93820 and oxygen analog at the initial extraction step. Several initial extraction procedures were used, since a single technique was not satisfactory for the wide variety of samples involved. A Soxhlet extraction was necessary to obtain complete recovery of the oxygen analog from dry and oily samples such as cottonseed and its byproducts, especially when these samples were fortified and allowed to stand for a time before extraction. A simple blender extraction was found to be sufficient for wet crops. In all cases, extractions were devised to obtain maximum pesticide recovery and minimum extraction of interfering pigments and oils. Recoveries were run at the 0.1 ppm or lower for cottonseed, cottonseed oil, animal tissues, and milk. Higher levels were used on cottonseed byproducts, since higher

Table II. Retention Times of Various Organophosphates Tested For Interferences

Compound	Ppm Level Tested	Conditions Used	Relative Retention Time[a]
BAY 93820	0.1	Standard/Validation	1.00
BAY 93820 Oxygen Analog	0.1	Standard	0.51
BAY 93820 Oxygen Analog	0.1	Validation	0.50
CO-RAL	1.0	Standard	9.5
Diazinon	0.75	Validation	0.33
Dimethoate	0.02	Standard	No Peak
Dimethoate Oxygen Analog	0.02	Standard	No Peak
Dioxathion (Delnav)	1.0	Standard	0.47, 0.81
Dioxathion (Delnav)	1.0	Validation	0.43
DI-SYSTON	0.75	Standard	1.49
DI-SYSTON Sulfoxide	0.75	Validation	1.30, 1.78
DI-SYSTON Sulfone	0.75	Standard	1.56
EPN	0.5	Standard	No Peak
Ethion	2.5	Standard	2.65
Famphur	0.1	Standard	No Peak
Famphur Oxygen Analog	0.1	Standard	No Peak
Fenthion	0.1	Standard	0.93
Fenthion	0.1	Validation	0.90
GUTHION	0.5	Standard	5.60
Imidan	0.2	Standard	No Peak
Malathion	4.0	Validation	0.76
Malathion Oxygen Analog	4.0	Validation	0.65
Merphos	0.25	Standard	No Peak
Ronnel	0.1	Standard	0.72
Thimet	0.5	Standard	0.35, 0.86
Thimet	0.5	Validation	0.25, 0.90
Thiol SYSTOX	0.75	Validation	1.33
Thiol SYSTOX Sulfoxide	0.75	Validation	1.33
Thiol SYSTOX Sulfone	0.75	Standard	3.00
Thiono SYSTOX	0.75	Validation	0.73
Thiono SYSTOX Sulfoxide	0.75	Validation	0.65
Thiono SYSTOX Sulfone	0.75	Validation	0.75
Trithion	0.2	Standard	3.72

[a] Retention time relative to BAY 93820 as 1.00 for both sets of conditions.

residues may be tolerated. In general, recoveries were in the 75–120% range as given in Table I. Representative chromatograms are shown in Figures 3 and 4.

The injection of 5 ngs of either the P=S or P=O derivative produced a peak with an area of 1.0 in.^{2} or larger. Since an area of 0.1 in.^{2} may be readily measured with a polar planimeter, the sensitivity of the method is at least 0.01 ppm (0.5 μg of P=S or P=O in a 50 g sample) in the absence of interfering peaks. Control values for cottonseed, cottonseed oil, and animal tissues were generally less than 0.01 ppm. Sample size and aliquots were adjusted to achieve greater sensitivity in the case of milk samples. Control values for milk were generally less than 0.002 ppm.

A validation procedure was also used in this method. Sample preparation was identical to that used in the standard procedure; however, gas chromatographic analysis was carried out using a more polar column. Detector response for BAY 93820 or the oxygen analog derivative was the same on both columns; thus, sensitivity for the validation procedure was the same as for the standard method. BAY 93820 and the oxygen analog derivative were separated from coextractives equally well on the validation column as on the standard column, so that either column could be used interchangeably. The validation gas chromatographic conditions were: column, 1 ft × 4 mm i.d. borosilicate glass column, packed with 5% OV-17 solution coated on 80- to 100-mesh Gas Chrom Q; helium carrier gas, 75 ml per min; column temperature, 200° C. All other conditions were identical to the standard method. Retention times for BAY 93820 and the oxygen analog derivative on the OV-17 column were 4.7 and 2.3 min, respectively.

To determine the specificity of the method for BAY 93820, an interference study was conducted. Because of the relative specificity of the alkali flame detector to phosphorus, only phosphorus-containing compounds were tested as possible interferences. All organophosphorus compounds registered for use on cotton, milk, and meat were checked at the highest registered level (NAC News, 1969). Compounds registered at a 0 tolerance were tested at the 0.1 ppm level. All compounds were carried through the procedure individually, starting at the methanol:water/Skellysolve B partition. Compounds having a retention time closer than 1 min to BAY 93820 or the oxygen analog derivative at the standard conditions were reinjected on the validation column. Relative retention times are shown in Table II. In all cases these chemicals can be differentiated by gas chromatography at either the standard or the validation conditions. The residue method is therefore specific for BAY 93820 and its oxygen analog in the presence of all these other registered organophosphorus pesticides.

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