Routine Quantitative Residue Determinations of S-[(2-Methoxy-5-oxo-Δ^2-1,3,4-thiadiazolin-4-yl) methyl] *O,O*-dimethyl phosphorodithioate (Supracide) and Its Oxygen Analog in Forage Crops

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Methods applicable to routine determination of residues of unchanged Supracide, *S*-[(2-methoxy-5-oxo-Δ^2-1,3,4-thiadiazolin-4-yl) methyl] *O,O*-dimethyl phosphorodithioate, and its oxygen analog in forage crops are presented. The unchanged Supracide is determined by microcoulometric gas chromatography with a limit of detectability of 0.05 p.p.m. Extraction is done using petroleum ether and clean-up with aluminum oxide columns. The oxygen analog is detected on thin-layer chromatography plates by fly head cholinesterase inhibition, with a limit of detectability of 0.01 p.p.m. Extraction of the oxygen analog is done using acetone. No clean-up of this extract is needed before application to the thin-layer chromatography plate. Efficacy of the extraction method for weathered residues is discussed. The methods have been used to determine residues resulting from various levels of application of Supracide to forage crops.

The registration of new pesticides depends partially upon the accumulation and evaluation of rather large numbers of residue analyses of field-treated samples. It is of utmost importance that the methods of analysis used are reliable, reproducible, and have sufficient sensitivity and specificity to determine accurately the intact pesticide and its biologically active metabolites. Methods that meet these requirements are presented for intact Supracide (referred to hereafter as GS-13005) and its oxygen analog (GS-13007), which was found by Cassidy (1969b) to be the only cholinesterase-inhibiting residue on alfalfa.

Methods for determining GS-13005 residues have been described by Eberle (1967), using a combination of thin-layer chromatography and electron-capture gas chromatography for the analysis of apples, cherries, grapes, and cotton foliage. Cassidy (1969 a, b) reported extraction and separation procedures useful in metabolism studies that are not applicable to large numbers of routine samples of forage.

Methods of analysis for routine determination of GS-13005 and its oxygen analog in forage are presented herein. GS-13005 is determined, after extraction and clean-up, by microcoulometric gas chromatography. The clean-up procedure, gas chromatographic separation, and sulfur detection cell contribute to the specificity of the method. Routine application of the method allows detection of residues as low as 0.05 p.p.m. The oxygen analog (GS-13007) is determined by spotting crude sample extracts on thin-layer chromatographic plates, developing in a suitable solvent system, and detection by the inhibition of fly head cholinesterase. Separation on the thin-layer chromatography plate and cholinesterase inhibition properties lend specificity to this method, which will detect residues of GS-13007 as low as 0.01 p.p.m.

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PROCEDURE FOR GS-13005

Materials. REAGENTS. Aluminum oxide (alumina) Basic, Activity Grade I, Woelm, Alupharm Chemical Co., New Orleans, La. Activity Grade V is prepared from this for use according to directions on the package.

APPARATUS. Glass clean-up columns, 18 mm. I.D. × 200 mm. long, equipped with porous adsorbent support and 150-ml. solvent reservoir.

GAS CHROMATOGRAPHS. Dohrmann Model No. G-100 chromatograph-furnace or Micro-Tek Model MT 220 equipped with a Dohrmann combustion unit. These were used with a Dohrmann T-300 microcoulometric titration cell (sensitive to SO_2), a Dohrmann C-200 coulometer, 1 MV recorder, and quartz or glass injection ports as supplied by the manufacturer. The columns used were 5% SE-30 on Diatoport S or Gas Chrom Q packed-in-glass or aluminum tubing, 4 feet × ¼ inch.

Extraction and Clean-Up. Chop a representative 300- to 400-gram sample of crop in a Hobart food cutter and transfer a 100-gram sample to a 32-ounce wide mouth jar equipped with a plastic cap. Several untreated check samples are fortified with known amounts of GS-13005 and carried through the entire procedure along with treated samples. Fortified amounts should be in the range of expected values of treated samples.

Two hundred and fifty milliliters of petroleum ether (b.p. 30° to 60° C.) are added and the samples are shaken for ½ hour on a mechanical shaker. Place a polyethylene liner in the cap to avoid dissolving the

extraneous material from the lid and also to avoid loss of solvent. After specified extraction period, decant the solvent and dry with anhydrous sodium sulfate.

Dried forage requires a partitioning step before the samples are ready for column chromatography. A 50-ml. aliquot (20 grams) is transferred to a separatory funnel and 50 ml. of petroleum ether are added. GS-13005 is then extracted, using two 25-ml. portions of acetonitrile. The combined acetonitrile portions are washed with 50 ml. of fresh petroleum ether. Evaporate the acetonitrile to dryness, using a flash evaporator at 45° C.

Green forage samples do not require the acetonitrile partitioning step. To prepare the green forage extract for column chromatography, evaporate a 50-ml. aliquot (20 grams) to dryness using a flash evaporator. Samples are now ready for column chromatography.

Alumina (Grade V) can be used for column chromatography clean-up of either green or dried forage. The columns are packed by pouring 25 grams of alumina into a column containing a plug of glass wool at the bottom. After gentle tapping to eliminate channeling and to insure uniform packing, another plug of glass wool is placed on top of the alumina.

The column is developed in the following way. The evaporated sample is dissolved in 2 ml. of benzene, transferred to the column, and allowed to penetrate. The flask is washed with two 5-ml. portions of *n*-hexane, which are transferred to the column and allowed to penetrate. When the last *n*-hexane wash has just entered the adsorbent surface, 90 ml. of *n*-hexane are added, allowed to pass through the column, and discarded. When the last of the hexane has just entered the adsorbent, begin collecting the eluate in a clean 250-ml. flask and continue the elution with 100 ml. of 5% ethyl ether in hexane. The eluate is collected and evaporated to dryness using a flash evaporator.

Cassidy (1969 a, b) has reported the use of Florisil for clean-up of alfalfa and milk samples for GS-13005 and GS-13007 residues.

The residue remaining after evaporation is transferred with ethyl ether into a tapered 15-ml. centrifuge tube. The ethyl ether is evaporated to dryness using a gentle stream of air and a warm water bath. A known volume of benzene is added to the tube so that a 5-μl. injection will produce approximately half scale recorder deflection. The tube is shaken or placed in a vortex mixer to insure complete solution of GS-13005.

Gas Chromatography. The following conditions were used: column temperature, 190° C.; injection port temperature, 210° C.; combustion furnace temperature, 820° C.; column nitrogen flow, 80 cc. per minute; oxygen flow, 80 cc. per minute; purge nitrogen flow, 25 cc. per minute; range, 100 ohms; bias voltage, 160 millivolts; chart speed, 0.5 inch per minute. The elution time for GS-13005 under these conditions was 4 minutes. The peak response for GS-13005 was 6 square inches per microgram, and 0.2 μg. gave ½ full scale deflection; 0.05 μg. was detectable in 1 gram-equivalent of forage.

Standardization of the gas chromatograph was done by determining the areas by triangulation of peaks obtained from known amounts of GS-13005. The amount of sample injected was chosen so that the GS-13005 determined was bracketed by the standards which were injected. From the value so obtained, recoveries of the GS-13005 from the fortified samples were calculated. Typical recoveries found for GS-13005 added to alfalfa, clover, and mixed grasses are shown in Table I. Satisfactory recoveries are shown for 0.05, 0.10, and 0.50 p.p.m. of added GS-13005 in alfalfa, grass, and clover. Typical chromatograms are shown in Figure 1 for check alfalfa and alfalfa fortified before extraction with 0.1 p.p.m. of GS-13005.

Table I. Typical Data for Recovery of GS-13005

Crop	Sample Weight, Grams[a]	Added		Recovered			
				Total		Net	
		μg.	P.P.M.	μg.	P.P.M.	P.P.M.	%
Alfalfa	1.0	0.00	0.00	0.00	0.00	—	—
	1.0	0.05	0.05	0.05	0.05	0.05	100
	1.0	0.10	0.10	0.10	0.10	0.10	100
	1.0	0.10	0.10	0.11	0.11	0.11	110
	0.2	0.10	0.50	0.10	0.50	0.50	100
Alfalfa	1.0	0.00	0.00	0.04	0.04	—	—
	1.0	0.05	0.05	0.08	0.08	0.04	80
	1.0	0.10	0.10	0.11	0.11	0.07	70
	0.2	0.10	0.50	0.09	0.45	0.41	82
Grass	1.0	0.00	0.00	0.00	0.00	—	—
	1.0	0.05	0.05	0.04	0.04	0.04	80
	0.5	0.05	0.10	0.06	0.12	0.12	120
	0.4	0.20	0.50	0.19	0.48	0.48	96
Clover	1.0	0.00	0.00	0.00	0.00	—	—
	1.0	0.05	0.05	0.04	0.04	0.04	80
	1.0	0.10	0.10	0.08	0.08	0.08	80
	0.2	0.10	0.50	0.07	0.35	0.35	70

[a] Represents weight injected into gas chromatograph.

EFFICIENCY OF EXTRACTION

A comparison of the efficiency of the extraction of GS-13005 from alfalfa using petroleum ether was made with that using the more polar solvent system, 9-to-1 acetone-water.

The following procedure was used for the acetone-water extraction. Fifty grams of the chopped alfalfa were blended in a high speed blender (Omni-Mixer) with 200 ml. of the mixed solvent for 1 minute. The solid material was then filtered off through a Büchner funnel using Whatman No. 1 filter paper. An aliquot of 100 ml. (equivalent to 25 grams of alfalfa) was transferred to a separatory funnel containing 50 ml. of water. The solution was then extracted two times, using 100 ml. of methylene chloride each time. The combined methylene chloride extracts were dried with sodium sulfate and then evaporated to dryness. The analysis was continued as described above for the petroleum ether extractions.

Weathered residues on samples of alfalfa were extracted by both procedures. The results of the analyses (Table II) show that petroleum ether extracts the unchanged GS-13005 residues as efficiently as does the acetone-water mixed solvent. There is variation between the results obtained by the two methods. However, this variation is both plus and minus, and is due to differences in sampling and analysis.

Other studies reported by Cassidy (1969a) using C^{14}-labeled GS-13005 showed that petroleum ether was as efficient in recovering unchanged GS-13005 from

weathered residues on alfalfa as was acetone or methanol.

STABILITY DURING FROZEN STORAGE

Practically all of the samples analyzed in this laboratory are stored in a freezer for a few weeks before they are extracted. The possibility of losses of the GS-13005 during this storage period was investigated.

Samples of green alfalfa and green clover which had been treated in the field were ground, mixed well, and placed in glass jars. The samples were analyzed initially and stored frozen over a period of 8 weeks. There was no significant loss in the GS-13005 during this storage period.

PROCEDURE FOR GS-13007

Fly Head Homogenate. This is prepared in batches using 48 flies (*musca domestica*). The flies are beheaded and the heads are placed immediately in an ice-cold tissue grinding tube. The tissue grinder used has a Teflon pestle, 55-ml. capacity, and a grinding area of 125 × 22 mm. (Scientific Glass, No. IT 4990). A buffer solution (pH 7.2) is made up of 12.5 grams of Na_2HPO_4 and 2.72 grams of KH_2PO_4 in 1 liter of water. Twelve milliliters of this buffer are added and the heads are homogenized for 10 minutes while the grinding tube is kept in an ice-water bath. The homogenate is then centrifuged in a clinical centrifuge and the supernatant liquid is decanted for use.

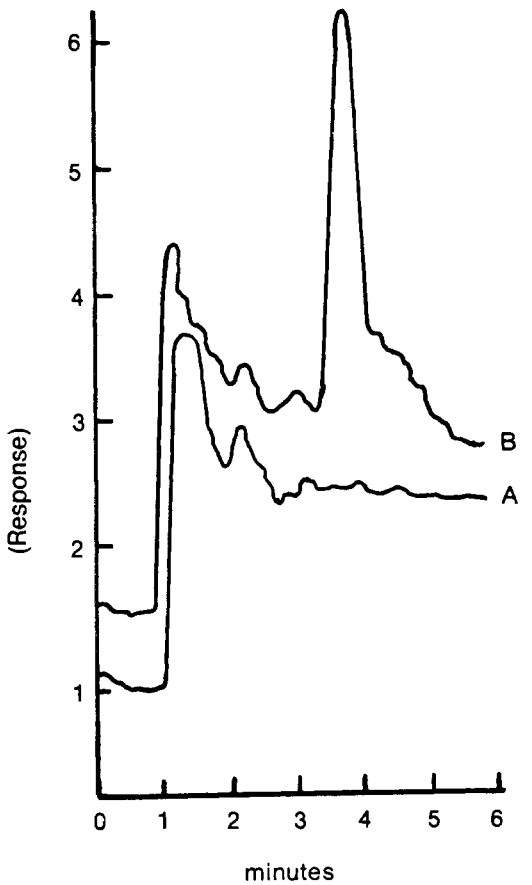

Figure 1. Typical gas chromatogram of GS-13005 added to alfalfa

A. Check alfalfa. B. Check plus 0.10 p.p.m. GS-13005. Equivalent of 1 gram of alfalfa injected

Table II. Recovery of Weathered Residues of GS-13005 From Alfalfa. Comparison of Extraction Methods

GS-13005 Dosage, Lbs. per Acre	Interval, Days	GS-13005 Found, P.P.M. Pet. ether extraction	GS-13005 Found, P.P.M. Acetone-H_2O extraction
0.5	2	15.0	10.0
0.5	9	4.6	3.5
0.5	16	0.59	0.43
0.5	23	0.34	0.26
0.5	28	0.25	0.23
1	7	6.0	5.7
1	14	0.43	0.84
1	21	0.30	0.22
2	14	3.4	3.7
2	21	0.65	1.0

Substrate solution. Twenty-five milligrams of 1-naphthyl acetate (Eastman, No. 2380) are dissolved in 10 ml. of absolute alcohol. Fifty milligrams of Azoene Fast Blue R R Salt (Alliance Chemical Corp., Newark, N. J., reagent grade) are dissolved in 40 ml. of sodium tetraborate buffer (pH 9.2, Hartmann Feddon Co., Philadelphia, Pa., made up as directed and then diluted 1 to 1 with distilled water). The two solutions are mixed and filtered through glass wool. These solutions must be prepared just prior to use because they decompose rapidly.

PREPARATION OF SAMPLES

Twenty-five grams of green or dried forage are chopped in a Hobart food cutter. One hundred milliliters of acetone are added to green forage and 200 ml. to dried forage. The mixture is placed in a blender (Osterizer) for 5 minutes. It is then filtered, under vacuum, through Whatman No. 1 filter paper on a Büchner funnel. Four milliliters of acetone extract corresponds to 1 gram of green forage and 8 ml. corresponds to 1 gram of dried forage. No clean-up of this extract is required.

THIN LAYER CHROMATOGRAPHY PROCEDURE

Silica gel G (E. Merck) plates, 250 microns thick, are prepared. The plates are activated at 110° C. and cooled in a desiccator. They are then washed with a developer consisting of acetonitrile–water–ammonium hydroxide (45:9:1) by allowing the solvent to ascend to the top of the plate. The elution is continued for an additional 15 minutes. The plates are air-dried for 10 minutes and reactivated at 110° C. for 30 minutes in a drying oven. The plates are held in a desiccator until used. Because of the highly sensitive method of detection, extreme care must be taken to avoid contamination. Plates which have not been washed as described after coating should *not* be used for this analysis.

Forty microliters of the green forage extract and 80 μl. of the dried forage extract (corresponding to 10 mg. of forage) are spotted directly on the prepared plate by the use of a microliter syringe. The spots are allowed to dry at room temperature. The plate is developed in a saturated acetonitrile–water–ammonium hydroxide (45:9:1) chamber to 15 cm.

The plate is air-dried for 10 minutes, sprayed with

fly head homogenate, and held at room temperature for 15 minutes to allow inhibition to take place. The plate is then sprayed with the substrate solution (made fresh prior to use). After about 10 minutes, GS-13007 begins to appear as white spots on a pink background. If there is not enough contrast between the spots and the background after 1 hour, the plates may be sprayed again with fresh substrate solution. Maximum spot intensity develops after about 2 hours. GS-13007 has an R_f value of about 0.85 under the conditions used.

Despite efforts to standardize the fly head preparation, some day-to-day variation in the color and intensity of the background is noticeable. Other solvent systems have been used successfully to develop GS-13007 thin-layer chromatograms using this detection system; however, best results have been obtained on forage samples using the described procedure.

TYPICAL RESULTS

A typical chromatogram obtained from the analysis of alfalfa treated with GS-13007 is shown in Figure 2. The equivalent of 10 mg. of alfalfa was spotted on the plate for each sample. The first sample is untreated alfalfa. The second sample is the untreated alfalfa with 0.01 p.p.m. GS-13007 added before extraction. A white spot appears in this sample, representing 100 pg. of GS-13007 in the 10 mg. of alfalfa applied. Sample 7 on this plate represents 100 pg. of standard GS-13007. This spot is readily apparent. Comparison of this spot and the spot from the fortified samples shows excellent recovery of the GS-13007 added to the alfalfa. The other samples (2,3,4,5, and 6) are alfalfa samples taken at various intervals after treatment with GS-13005. There is a continued decrease in GS-13007 content with length of time after application of the GS-13005. The early samples have more GS-13007 than can be evaluated by comparison with the fortified sample.

For evaluation of the amount of GS-13007 present, smaller amounts of the treated alfalfa samples were spotted, as shown in Figure 3. Table III shows the amount of alfalfa spotted, the amount of GS-13007 found in terms of picograms, and the parts per million of GS-13007 in the samples.

Table III. GS-13007 Residues in Alfalfa Determined by Thin-Layer Chromatography

Sample	Amount of Sample Spotted		GS-13007 Detected	
	μl.[a]	Mg.[b]	Pg.	P.P.M.
1-day check fortified with 0.01 p.p.m. GS-13007	40	10	100	0.01
0 days	2	0.5	200	0.40
7 days	8	2	200	0.10
14 days	30	7.5	150	0.02
21 days	40	10	80	0.01

[a] μl. of extract solution.
[b] mg. of original alfalfa in volume spotted.

ANALYSIS OF FIELD-TREATED SAMPLES

Residue data was obtained from the analysis of forage crops grown in California, Indiana, South Dakota, Utah, and Virginia. The ranges of values and average values for residues found in alfalfa at the different levels of application are shown in Table IV. Rates of application ranging from ¼ to 2 pounds of active ingredient per acre were used. Detectable residues are found 4 weeks after application at all application rates. The amounts found are, in general, proportional to the amount of GS-13005 applied.

The residue half-life (RL_{50}), defined as the length of time required for dissipation of 50% of the compound on plant materials (Gunther and Blinn, 1955) was computed from the average values between 7 and 14 days. It was 3.5 days for both the ½- and the 1-pound rates of GS-13005 on alfalfa. The half-life

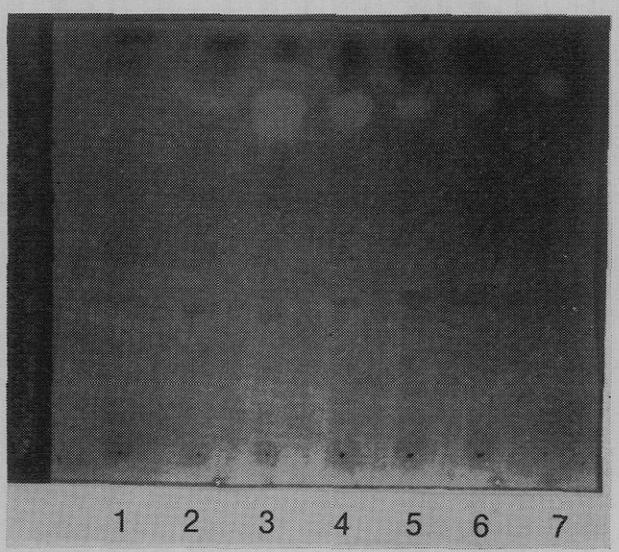

Figure 2. Thin-layer chromatograms of GS-13007 in alfalfa; 10 mg. alfalfa equivalent spotted

1. Check (1-day interval) 2. Check plus 0.01 p.p.m. (100 pg.) GS-13007. Treated samples: 3. 0 days; 4. 7 days; 5. 14 days; 6. 21 days; 7. standard GS-13007, 100 pg.

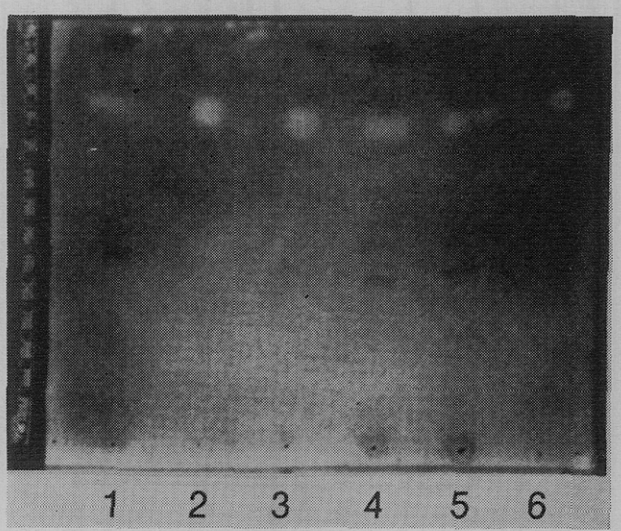

Figure 3. Thin-layer chromatograms of GS-13007 in alfalfa; varying amounts of alfalfa equivalent spotted

1. Check fortified with 0.01 p.p.m. (100 pg.) GS-13007, 10 mg. alfalfa spotted. Treated samples: 2. 0 days, 0.5 mg. alfalfa spotted; 3. 7 days, 2.0 mg. alfalfa spotted; 4. 14 days, 7.5 mg. alfalfa spotted; 5. 21 days, 10.0 mg. alfalfa spotted; 6. standard GS-13007, 100 pg.

Table IV. Resumé of GS-13005 Residues in Alfalfa

Application Rate, Lb./A.[a]	Interval, Days	GS-13005 Found (P.P.M.) Green Alfalfa Range	Green Alfalfa Average	Dry Alfalfa Range	Dry Alfalfa Average
0	0	<0.05-0.22	0.13	<0.05-0.21	0.10
	1, 2 or 3	—	—	—	—
	7 or 8	[b]	<0.05	[b]	<0.05
	14 or 15	[b]	<0.05	[b]	<0.05
	21 or 23	[b]	<0.05	[b]	<0.05
	28 or 29	[b]	<0.05	[b]	0.17
1/4	0	[b]	20.0	—	—
	1, 2 or 3	1.5-5.5	3.5	—	—
	7, 8 or 9	1.0-2.3	1.5	—	—
	14 or 15	0.18-0.37	0.26	—	—
	21 or 23	0.09-0.12	0.11	—	—
	28 or 29	0.08-0.12	0.10	—	—
1/2	0	7.0-41.0	24.0	6.5-11.0	8.8
	1, 2 or 3	3.6-14.0	8.4	[b]	0.29
	7, 8 or 9	0.27-5.2	2.3	0.53-0.60	0.56
	14, 15 or 16	0.08-1.1	0.53	0.07-0.22	0.15
	21 or 23	0.08-0.36	0.24	<0.05-0.25	0.13
	28 or 29	0.07-0.26	0.14	[b]	0.13
1	0	13.0-74.0	43.0	14.0-28.0	21.0
	1, 2 or 3	14.0-36.0	25.0	[b]	9.0
	7, 8 or 9	1.5-9.1	6.3	0.70-1.8	1.4
	14, 15 or 16	0.44-3.4	1.5	0.38-0.90	0.67
	21 or 23	0.19-0.64	0.42	0.05-0.68	0.28
	28 or 29	0.17-1.1	0.64	[b]	0.25
2	0	[b]	250.0	—	—
	1 or 3	86.0-210.0	148.0	[b]	4.7
	7 or 8	14.0-64.0	33.0	[b]	1.3
	14 or 15	3.0-12.0	6.9	[b]	2.1
	21 or 23	0.80-4.2	1.9	[b]	1.0
	28 or 29	[b]	0.70	[b]	1.5

[a] Based on active ingredient.
[b] One value only.

values found for the one clover series were 6 days for the ½-pound-per-acre application and 4 days for the 1-pound application.

One of the alfalfa studies was done using three applications at ½- and 1-pound rates at 30-day intervals. The residues found in these samples show that build-up of GS-13005 does not occur as a result of repeated application, since these values fall within the range of the single applications. Three applications were also used in a clover study at approximately monthly intervals. The clover values are again in or near the range of the alfalfa values. One study was done using wettable powder at the ½-pound-per-acre rate, instead of the 40% emulsifiable solution. These residue values fall within the range of values found for the emulsifiable solution. The GS-13005 residues found in dried forage are generally lower than those found in the green forage. The green forage has a moisture content of 60 to 80%, while dried forage contains 10 to 15%. The GS-13005 residue does not concentrate as a result of the loss of moisture.

Determinations of GS-13007 were made on selected samples (Table V). Detectable amounts of GS-13007 are found in both green alfalfa and clover. The amounts, however, are of a different order of magnitude than those of unchanged GS-13005. In general, GS-13007 represents only a few percentages of the GS-13005 residue. There is a constant decrease of the amount of GS-13007 residue with length of time after application. The residue half-life (RL_{50}) found for GS-13007 on alfalfa was 2 days for the ½-pound rate and 3½ days for the 1-pound rate. Similar half-life values for clover were 4 days for the ½-pound rate and 6 days for the 1-pound rate. At no time was there an increase or accumulation of GS-13007, indicating that if it is a degradation product, it is transient in nature. Although conversion of GS-13005 to GS-13007 could occur mainly during application of the spray, this has not been demonstrated in the laboratory.

Table V. Residues of GS-13007 in Alfalfa and Clover

Treatment[a]	Interval, Days	GS-13007 Found, P.P.M. Alfalfa, No. 1350 Green	Alfalfa, No. 1350 Dried	Clover, No. 1361 Green	Clover, No. 1361 Dried
1/2 lb. GS-13005 per acre	0	0.40	0.08	0.40	0.10
	7	0.06	0.04	0.05	0.04
	14	<0.01	0.01	0.015	0.03
	21	<0.01	0.01	<0.01	0.02
1 lb. GS-13005 per acre	0	0.40	0.20	0.10	0.40
	7	0.10	0.20	0.05	0.26
	14	0.02	0.08	0.025	0.05
	21	0.01	0.02	0.01	0.07

[a] Three applications each at the stated rate at approximately 30-day intervals.

CONCLUSIONS

The residues of GS-13005 and GS-13007 found on field-treated alfalfa tend to confirm the results reported by Cassidy (1969a) on radioactive studies. Residues remaining after various time intervals indicate that GS-13005 has a dissipation curve normal for organophosphorus pesticides. GS-13007 (oxygen analog) residues do not increase after spraying, and no cholinesterase-inhibiting metabolites other than GS-13007 were found.

The methods presented for determining GS-13005 and GS-13007 are reliable, reproducible, and detect the significant residues that occur on alfalfa. Large numbers of forage samples have been analyzed in the laboratories of the authors with consistent success. The limits of detection of 0.05 p.p.m. for GS-13005 and 0.01 p.p.m. for GS-13007 are sufficient to detect levels of residue that are actually found in field samples.

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