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AGRICULTURAL DIVISION
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GREENSBORO, N.C.

UPTAKE AND BALANCE OF ϕ-^{14}C-CGA-48988 AND ITS METABOLITES IN GREENHOUSE GROWN BRIGHT AND BURLEY TOBACCO

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A B S T R A C T

Bright tobacco was treated with ϕ-^{14}C-CGA-48988 at 0.25 and 0.5 lb. ai/A (transplant water treatment) and burley tobacco was treated ppi at 6.3 lb. ai/A.

For burley and bright leaves, the ppm equivalent to ϕ-^{14}C-CGA-48988 decreases with time, e.g., at 3 weeks (transplant at 0.25 lb. ai/A) bottom bright leaves had 35.3 ppm while at 12 weeks 7.8 ppm. Curing the burley or bright leaves increased the radioactivity about tenfold, e.g., to 69.3 ppm for 0.25 lb. ai/A bright cured bottom leaves. Higher levels of radioactivity were found in the bottom cured leaves than in the top cured leaves, e.g., for 0.25 lb. ai/A, bright cured top leaves had 36.6 ppm.

Metabolism of ϕ-^{14}C-CGA-48988 in tobacco results in the formation of many nonpolar and polar metabolites. At 12 weeks, mature bottom leaves from the 0.25 lb. ai/A uncured bright tobacco contained 38.4% of the total radioactivity as ϕ-^{14}C-CGA-48988. After curing, these leaves contained 34.5% as CGA-48988 and about 10% as 7 nonpolar metabolites. At 12 weeks, bright tobacco contained at least 25 unknown polar metabolites in uncured leaves. Of these, only six individually accounted for 2% or more of the total radioactivity. Twenty polar metabolites composed a total of 20% of the total radioactivity. Only one metabolite (VI) composed a significant portion (11%) of the total radioactivity in the uncured leaf.

Curing of this bright tobacco resulted in only minor differences in amounts of metabolites. Very little CGA-62826 (<1.5%) could be found in uncured or cured bright tobacco.

The metabolism picture for bright tobacco at the higher treatment rate was essentially the same.

Burley tobacco showed the same qualitative and quantitative pattern of uptake and metabolism as did the bright tobacco, indicating that the tobacco species or the type and rate of application did not influence the percentages of metabolites relative to the total radioactivity present.

Radioactivity in the 0-3" layer of greenhouse burley soil dissipated rapidly from 6.5 ppm at 0 time to 2.88 ppm by 12 weeks. The residual radioactivity at 12 weeks in the 0-3" layer contained 28.8% as ϕ-^{14}C-CGA-48988 and 2.8% as the acid metabolite (CGA-62826). Two other unknown polar metabolites (j and o) made up a total of 1.8%. Both CGA-48988 and CGA-62826 probably leach to lower depths with CGA-62826 appearing to leach faster.

At 12 weeks, nonextractables in burley soil accounted for 42.5% of the total radioactivity in the 0-3" layer. Since very little aqueous soluble metabolites were found, it is possible that the parent and its metabolites rapidly bind to the soil and limit uptake of CGA-48988 and metabolites into the tobacco at later stages of growth.

By the time mature leaves were taken, data for soil from the bright tobacco pots were similar to that from burley tobacco pots. The movement of CGA-48988 and its degradation products and the growth of the root systems account for this similarity.

INTRODUCTION

The experimental compound CGA-48988, N-(2,6-dimethylphenyl-N-(methoxyacetyl)alanine methyl ester*, is a fungicide proposed for control of Black Shank on tobacco. Application is effective either by transplant-water treatment or by preplant incorporation. Buckets of soil, each containing one tobacco plant, were treated with [U-ring-^{14}C]-N-(2,6-dimethylphenyl)-N-(methoxyacetyl)alanine methyl ester**. The burley was treated by preplant incorporation and the bright, by transplant-water. The objectives of this study were to: 1) determine the uptake and balance of ϕ-^{14}C-CGA-48988 in bright (flue cured) and burley tobacco grown to maturity in the greenhouse; 2) determine the extent of CGA-48988 metabolism in mature uncured and cured bright and burley tobacco; and 3) determine the extent of degradation and leaching of ϕ-^{14}C-CGA-48988 applied to greenhouse soil.

EXPERIMENTAL

Preparation and Transplanting of Tobacco into Greenhouse Pots: Five gallon pails were 75% filled with Georgia sandy loam soil (Table V). There were 5 buckets for 0.25 lb. ai/A transplant water treatment, 15 buckets for 0.5 lb. ai/A transplant water treatment and 8 buckets for preplant incorporation treatment. Details for preparation, planting, treatment and care of tobacco plants are given in Biological Report No. 78003 (1).

Treatment and Radioactive Dose: Coker 319 bright tobacco seedlings were treated at 0.25 and 0.5 lb. ai/A as follows: A 2" wide x 8" long x 3" deep furrow was made in each bucket of soil. For the 0.25 lb. ai/A treatment, the seedling was transplanted and to each furrow was added 125 ml of a standard solution which contained 95 mg of ϕ-^{14}C-CGA-48988 (s.a. = 29.0 µCi/mg or 8.4 mCi/mM) dissolved in 10 ml of ethanol plus 615 ml of water. Each plant received 19 mg of ϕ-^{14}C-CGA-48988. For the 0.5 lb. ai/A treatment, each seedling was transplanted and to each furrow was added 125 ml of a standard solution which contained 570 mg of ϕ-^{14}C-CGA-48988 dissolved in 10 ml of ethanol and 1865 ml water. Each plant received 38 mg of ϕ-^{14}C-CGA-48988. The above amounts were calculated on the basis of 6000 plants/acre.

* Chemical names and structures are given in Figure 1.

** Hereafter referred to as ϕ-^{14}C-CGA-48988.

After treatment, the furrows were packed up around the plants as in normal agricultural practice. All young plants were watered daily and fertilized every 6 days with 50-100 ml of Nutrileaf® (20-20-20). Older plants were fertilized with 400 ml of Nutrileaf®.

Burley seedlings (MS21X KY10 variety) were treated by preplant incorporation at 3.15 lb. ai/A broadcast (6.3 lb. ai/A - banded). A total of 357 mg of ϕ-^{14}C-CGA-48988 was mixed into 2 lbs. of carrier soil which in turn was blended into 108 lbs. of fresh soil using a Hobart mixer. Eight 5 gallon buckets were partially filled with untreated soil. The treated soil (13.75 lb. for each bucket) was then added on top to a depth of 3". Six week old burley tobacco seedlings were then transplanted into this soil. Each plant was treated with 44.8 mg of ϕ-^{14}C-CGA-48988. This amount was calculated on the basis of a 0.63 ft^2 area for the soil surface in each bucket.

<u>Sampling</u>: Bright and burley leaves were taken at 3, 6 and 12 weeks after transplanting. The last three samplings of each type of tobacco were two to six weeks apart and were primings of the bottom, middle, and top leaves respectively. Soil cores were taken at these same time intervals and divided into 0-3", 3-6", and 6-11" segments.

<u>Curing of Tobacco</u>:

<u>Bright</u> leaves were oven cured by hanging them in a 20" x 20" x 18" Blue M oven with forced air. A temperature curing program was used as follows: The leaves were cured 36-48 hours at 35°C, then the temperature raised at 5°/hour to 68°C and left for 8-24 hours. The oven was then shut off and a pan of water placed under the tobacco for 24 hours to bring it to order (regain moisture).

<u>Burley</u> leaves were air dried in the greenhouse for 3 weeks at 65-85°F and 65-80% relative humidity. Details of the curing process are given in Biological Report No. 78003 (1).

<u>Sample Preparation and Analysis</u>: Uncured or cured tobacco leaves were homogenized with dry ice in a Wiley mill (2). Samples of 100-200 mg were combusted in a Harvey oxidizer (3). Plant extractions done in accordance with AG-214 (4) gave an organic phase, aqueous phase and nonextractables. Soil samples were prepared according to AG-233 (2) and 1-2 grams of each were combusted (3). Extraction of soil was in accordance with AG-254 (5).

Thin Layer Chromatography (TLC) Analysis of Plant and Soil Extracts: The organic phases of plant samples were characterized by two dimensional TLC using first ethyl acetate (saturated tank) and then ethyl acetate/acetic acid (90/10 - v/v). The aqueous phases of plant samples were characterized by two dimensional TLC using first ethyl acetate-isopropanol-water-formic acid (65/25/10/2) and then chloroform-methanol-formic acid-water (75/20/4/2). CGA-48988 and other selected standards (Figure 1) were cochromatographed with the above extracts. Silica Gel GF TLC plates were used (Analtech, Inc., Newark, Del.). Radioactive spots were located using KODAK NO Screen x-ray film (NS-2T). Quantitation was by scraping the radioactive zones from the plates into vials, eluting with methanol (2 ml) and adding Aquasol® (New England Nuclear, Boston, Mass.).

Methanol soil extracts were characterized using the TLC systems described above.

Radioactivity Measurements: Radioassays were done in a Beckman LS-255 or Mark III liquid scintillation counter. Efficiencies were obtained by external standardization. Limits of detection and quantitation were determined in accordance with AG-276 (6).

RESULTS AND DISCUSSION

Radioactivity in Tobacco Leaves: The levels of radioactivity equivalent to ϕ-^{14}C-CGA-48988 in tobacco leaves are shown in Table I and II. The radioactivity in bright tobacco as well as burley tobacco decreases with time, e.g., at 3 weeks, 35.3 ppm for 0.25 lb./A bright tobacco and at 12 weeks, 7.8 ppm for 0.25 lb./A bright tobacco. These data suggest that radioactive metabolites of ϕ-^{14}C-CGA-48988 are being diluted by growth of the tobacco.

After curing the first primary leaves, the radioactivity increases about tenfold for both the bright and burley tobacco. This is due to a loss of water during the curing process. Extremely high levels (>100 ppm) of radioactivity are found in the cured bottom leaves of bright (0.5 lb. ai/A) and burley tobacco. The concentrations of ϕ-^{14}C-CGA-48988 and its metabolites are higher in bottom leaves than in top leaves.

Balance data for uncured as well as cured bright and burley tobacco show a decrease in organic radioactive solubles, e.g., from 67.1% at 3 weeks to 53.4% by 12 weeks for 0.25 lb. ai/A uncured bright tobacco and an increase in aqueous soluble metabolites from 27.2% at 3 weeks to 40.5% by 12 weeks. These changes indicate that metabolism of CGA-48988 is occurring rapidly to produce polar metabolites.

Nonextractable radioactivity remains low until the tobacco is cured, at which time the nonextractables approach 10% of the total radioactivity present. This increase in nonextractable radioactivity is probably due to occlusion of ϕ-^{14}C-CGA-48988 and its metabolites during curing. Polar metabolites are also higher in the middle leaves after curing than in the lower leaves, suggesting that age increases the amount of polar metabolites.

Chromatographic characterization (Figure 2A - Table I and II) of the leaf radioactivity showed that uncured bright tobacco treated at 0.25 lb. ai/A (bottom leaves) contained 38.4% of the total radioactivity as ϕ-^{14}C-CGA-48988 while cured bright bottom leaves contained 34.5%. Uncured and cured burley tobacco showed similar percentages of ϕ-^{14}C-CGA-48988.

At least 7 unknown organic soluble metabolites can be found in cured bright tobacco (Table II - Figure 2A) and compose 10-12% of the total radioactivity. These organic soluble metabolites are not prominent in uncured tobacco and their increase in cured tobacco must result from the curing process, possibly by enzymatic cleavage of conjugated CGA-48988 metabolites. Even after curing, CGA-48988 (zone C, Figure 2A) is the largest single compound in the organic phase. Curing resulted in only minor increases in the amounts of nonpolar metabolites.

Two dimensional TLC (Figure 2B - Table I and II) of the aqueous fraction revealed at least 25 polar metabolites in both burley and bright tobacco. At the most, 1.5% of the radioactivity was CGA-62826 in both uncured and cured bright and burley tobacco. Metabolites IV, V, VI, VII, VIII and XIII each were in most instances greater than 2% of the total radioactivity in the cured leaf. Anywhere from 19-21 other unknown polar metabolites, each was less than 3%, made up no more than 20% of the total radioactivity in the cured leaf. Bright tobacco treated at 0.5 lb./A and burley tobacco treated at 6.3 lb. ai/acre have exactly the same qualitative and quantitative pattern of polar metabolites, indicating that the species of tobacco, or the rate or type of application, would not affect the percentages of each metabolite relative to the total radioactivity present in the tobacco.

Using TLC and cochromatography, no evidence was found in tobacco extracts for the presence of acid, amine, amide or alcohol analogs of CGA-48988, such as CGA-67869, CGA-79353, CGA-78532, CGA-68125, CGA-37734, CGA-68124, CGA-67866, CGA-67867, CGA-67868 or CGA-72649. These metabolites would all be expected as obvious products of side chain N-dealkylation metabolism of the CGA-48988. The absence of such compounds indicates that another fundamental change occurs to the CGA-48988 molecule, possibly to the benzene ring itself or to one of the ring methyl groups.

Soil Balance and Characterization: Radioactivity in the greenhouse soil from the burley or bright tobacco pots dissipated rapidly (Tables III and IV). By 12 weeks, the burley soil was 2.9 ppm in the 0-3" layer. Some leaching was observed between 3 and 12 weeks, e.g., the radioactivity in the 3-6" and 6-11" segments increased from 1.7 to 2.7 ppm and 0.2 to 0.4 ppm respectively.

The radioactive balance data for burley soil show that the organic solubles decreased significantly by 12 weeks while the aqueous soluble metabolites remained low. The non-extractable radioactivity in the soil increased significantly to 42.5% by 12 weeks, suggesting that CGA-48988 and/or its metabolites become tightly bound to the soil in a short time.

Chromatographic characterization (Figure 3) of the radioactivity in the soil showed that parent ϕ-^{14}C-CGA-48988 decreased from 65.4% at 3 weeks to 28.8% at 12 weeks in the 0-3" layer (Table III). The 0-3" and 3-6" segments at 12 weeks contained the same amounts of parent, but the amounts for CGA-62826 differed, the 0-3" containing 2.8% of the total radioactivity as CGA-62826 while the 3-6" 6.0%. These data show that CGA-62826 may leach faster than CGA-48988. Unknown metabolites j and o were also found in the 0-3" burley soil, composing a total of 2.2% of the total radioactivity. Based on rf values (compare Figure 2A with Figure 3), these unknowns are the same as those observed in the organic solubles of uncured and cured bright and burley tobacco. Translocation of CGA-48988 as well as its metabolites from soil into tobacco is possible.

The decrease in radioactivity in the bright tobacco soil appears to be more extensive than in the burley soil. This difference is probably due to the method of treatment. Transplant water treatment resulted in an initial high concentration of ϕ-^{14}C-CGA-48988 in the furrow around the plant (which accounts for the initial high uptake). However, as the plants were watered regularly, the ϕ-^{14}C-CGA-48988 and its metabolites spread throughout the soil, reducing the amount of radioactivity in the immediate area of the plant. Occuring at the same time, the root systems of both bright and burley grow out of the treated zones. At 12 weeks, the burley and bright (0.5 lb. ai/A) soil (Table IV) have the same concentration of ϕ-^{14}C-CGA-48988 and its metabolites. This movement of CGA-48988 following a transplant-water treatment and the growth of the tobacco roots account for the similarity in the amount of radioactivity found in the burley and bright tobacco at 12 weeks.

The above data indicate that CGA-48988 is taken up rapidly from soil into tobacco plants and is partially metabolized to a multiplicity of products. The residual CGA-48988 in the soil binds rapidly to become nonextractable, possibly limiting uptake in later stages of tobacco growth.

ACKNOWLEDGEMENTS

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REFERENCES

1. [redacted], Biological Report No. 78003, "Biological Report for the Metabolism of ϕ-^{14}C-CGA-48988 in Greenhouse Grown Tobacco when Applied as a Drench to Bright Tobacco and Preplant Incorporated to Burley Tobacco."

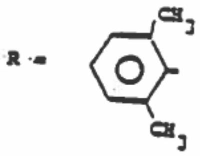
2. [redacted], AG-223, "Blending of Soils and Homogenization of Biological Materials for Radioassay and Extraction."

3. [redacted] AG-252, "Radioassay of ^{14}C in Biological Materials Using the Harvey Biological Material Oxidizer (BMO).

4. [redacted] AG-214, "Biphasic Extraction of Radioactive Metabolites from Treated Biological Material."

5. [redacted] and [redacted], AG-254, "Extraction of CGA-10832 Residues From Soil."

6. [redacted] AG-276, "Statistical Methods in the Measurement of Radioactivity."



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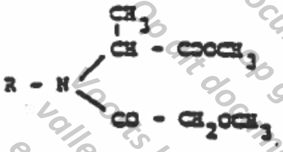
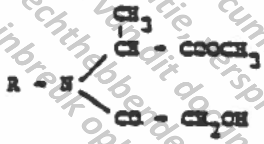
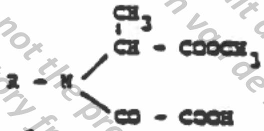
1		CGA-48988 N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-alanine methyl ester
2		CGA-67869
3		CGA-79353
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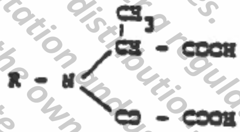
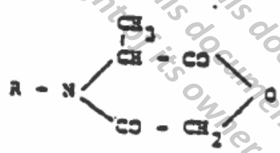
5		CGA-78532
6		CGA-68125

FIGURE 1. CHEMICAL NAMES AND STRUCTURES

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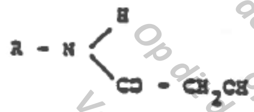
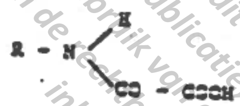
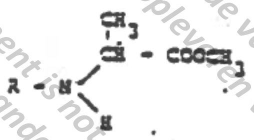
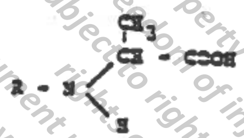
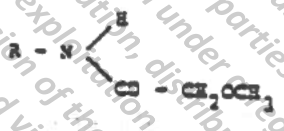
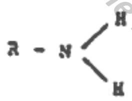
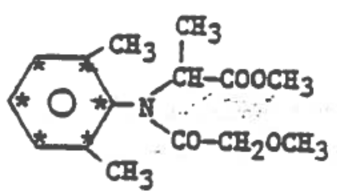
7		CGA-37734
8		CGA-68124
9		CGA-67866
10		CGA-67867
11		CGA-67868
12		CGA-72649

RADIOACTIVE COMPOUND



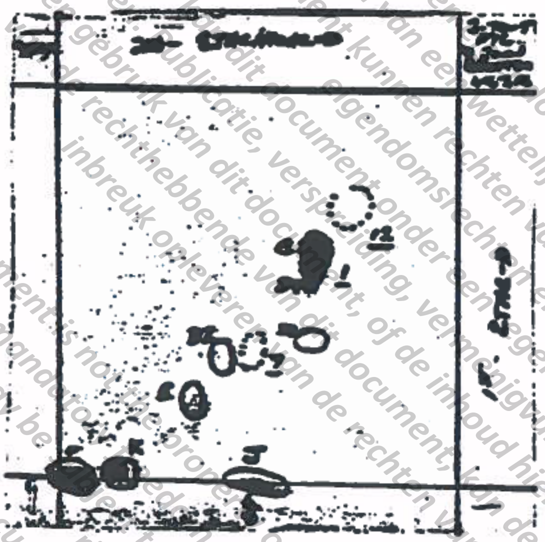
ϕ-[14]C-CGA-48988

[U-ring-[14]C] N-(2,6-dimethyl-phenyl)-N-(methoxyacetyl)-alanine methyl ester

* = ^{14}C

FIGURE 1. CHEMICAL NAMES AND STRUCTURES (Continued)

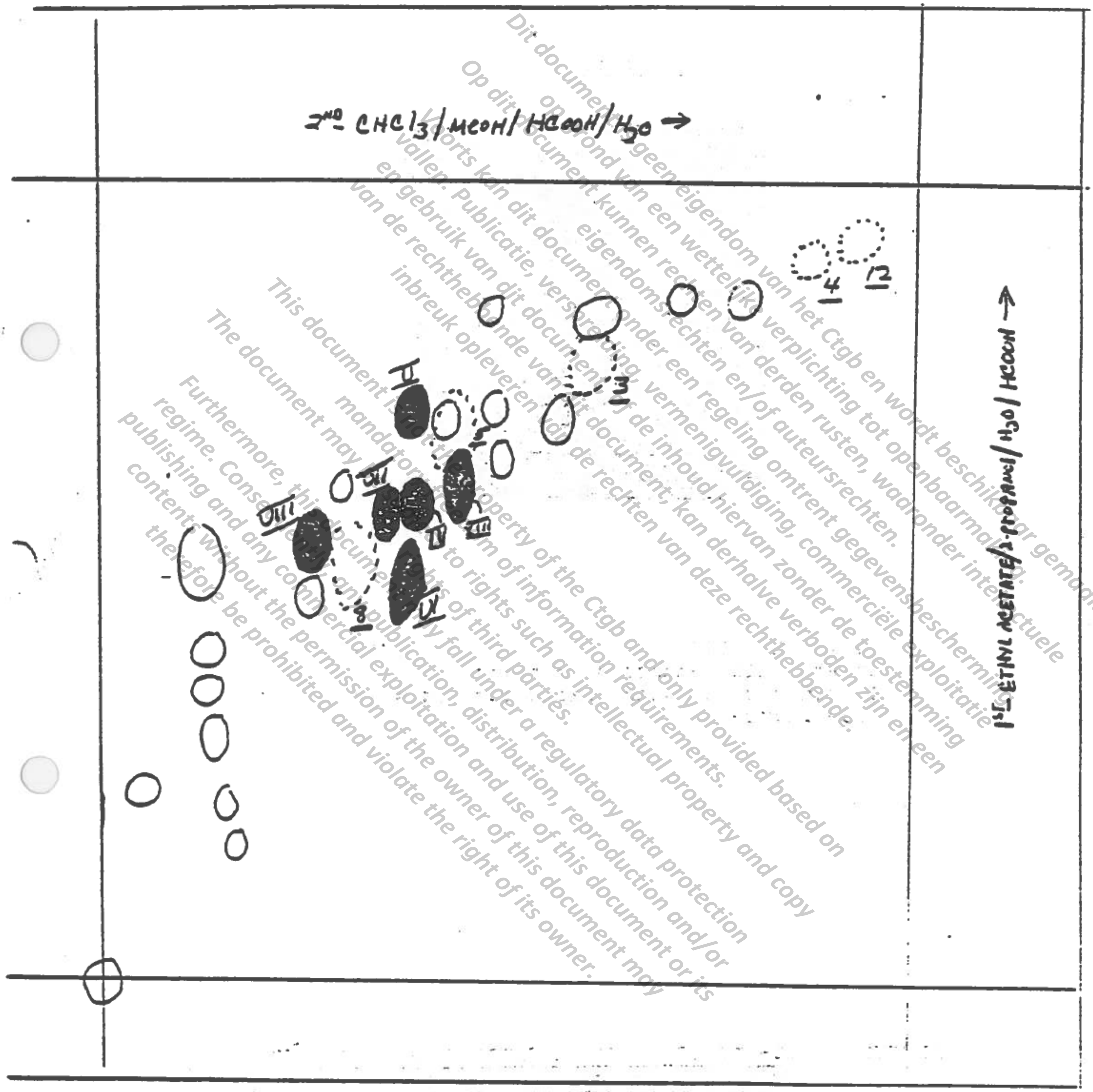
Radioautogram



#1 = CGA-48988 = C
Other capital letters = unknowns
Dotted circles with Arabic numbers = standards
(See Figure 1).

FIGURE 2A. 2D-TLC OF ORGANIC ^{14}C-SOLUBLES IN 12 WEEK CURED BRIGHT TOBACCO (0.5 LB. AI/A)

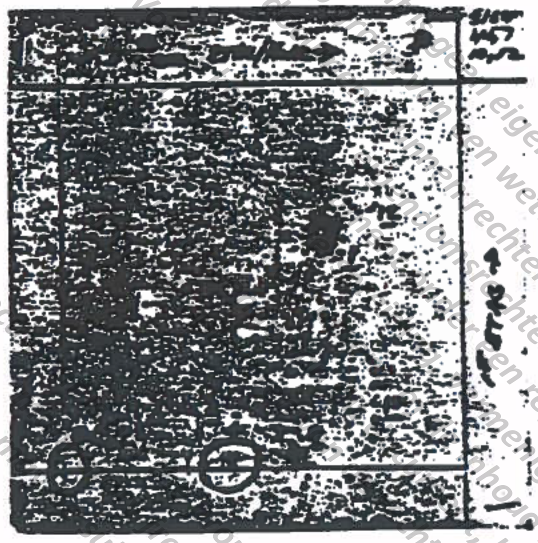
Trace of Radioautogram



Enclosed circles represent radioactive spots. Darkened areas are major (>3%) metabolites. Dotted circles with Arabic numbers are standards (See Figure 1).

FIGURE 2B. TRACE OF 2D-TLC OF ^{14}C-AQUEOUS SOLUBLES IN 12-WEEK CURED BRIGHT TOBACCO (0.5 LB. AI/A)

Radioautogram



#1 = CGA-48988 #4 = CGA-62826
Other small letters = unknowns
Dotted circles with Arabic numbers = standards
(See Figure 1).

FIGURE 3. 2D-TLC OF METHANOL EXTRACT OF ϕ-^{14}C-CGA-48988 IN BURLEY TOBACCO SOIL (12 WEEK)

TABLE I. BALANCE AND CHARACTERIZATION OF ϕ-^{14}C-CGA-48988 IN GREENHOUSE BRIGHT TOBACCO - TRANSPLANT WATER TREATMENT

Rate (lb. ai/A)	0.25								0.50							
			Lower Leaves		Middle Leaves		Upper Leaves				Lower Leaves		Middle Leaves		Upper Leaves	
THI[1] (weeks)	3	6	12UC[2]	12C[3]	18UC	18C	20UC	20C	3	6	12UC	12C	16UC	16C	19UC	19C
PPM	35.3	15.2	7.8	69.3	--	69.3	--	36.6	73.9	32.6	14.1	147.7	--	74.0	--	93.7
Balance	Percent of Total ^{14}C in Plant Sample								Percent of Total ^{14}C in Plant Sample							
Organic	67.1	65.8	53.4	47.8	--	18.9	--	25.6	73.4	73.5	56.2	31.3	--	24.2	--	34.0
Aqueous	27.2	20.7	40.5	44.8	--	76.4	--	73.4	25.1	29.6	46.8	49.6	--	60.7	--	64.6
Nonextractable	1.7	1.6	2.7	5.9	--	9.6	--	5.2	1.3	2.0	2.4	9.9	--	12.2	--	6.5
TLC Characterization																
Organic CGA-48988	--	--	38.4	34.5	--	--	--	--	64.7	58.2	34.7	26.9	--	10.9	--	--
7 Unknown Met.	--	--	--	12.5	--	--	--	--	--	--	--	--	--	9.9	--	--
Aqueous CGA-62826	--	--	0.9	<0.3	--	--	--	--	1.0	0.4	1.5	<0.3	--	--	--	--
6 Major Unknown Polar Met. each >3% in Cured Tobacco																
IV			5.4	4.6					1.7	2.7	4.7	6.5				
V			3.3	3.5					1.0	0.8	2.5	3.2				
VI			11.0	6.1					5.7	7.7	13.5	6.9				
VII			2.6	1.9					1.8	2.2	3.3	2.8				
VIII			2.9	2.4					0.8	3.2	3.2	3.0				
XIII			1.8	3.3					--	--	2.9	3.2				
19 Minor Unknown Polar Met., each from 0.3 to 3.0% in Cured Tobacco			11.2	16.4					10.2	7.3	12.1	19.6				

1. THI = treatment to harvest interval
2. UC = uncured
3. C = cured

TABLE II. BALANCE AND CHARACTERIZATION OF ϕ-^{14}C-CGA-48988 IN GREENHOUSE BURLEY TOBACCO

Rate = 6 lb. ai/A (ppi)

			Bottom Leaves		Middle Leaves		Top Leaves	
THI	3	6	12UC	12C	13UC	13C	16UC	16C
PPM	23.4	31.3	15.0	161.8	--	110.7	--	80.2
			Percent of Total ^{14}C in Plant Sample					
Balance								
Organic	73.5	53.9	49.4	43.6				41.3
Aqueous	19.0	37.1	44.6	44.7				47.9
Nonextractable	1.6	2.1	2.1	7.8				9.4
TLC Characterization								
Organic CGA-48988	61.2	38.9	32.9	28.1				
Aqueous CGA-62826	1.1	0.8	0.7	--				
4 Major Unknown Polar Met. each >3% in Cured Tobacco								
IV	0.7	3.6	5.4	5.4				
V	--	1.2	2.2	3.0				
VI	4.6	10.3	12.0	7.5				
VII	2.0	3.0	4.7	3.1				
21 Minor Unknown Polar Met. each from 0.3 to 3.0% in Cured Tobacco	5.5	11.9	16.8	17.3				

TABLE III. BALANCE AND CHARACTERIZATION OF ϕ-^{14}C-CGA-48988 IN TREATED GREENHOUSE SOIL - BURLEY TOBACCO

THI* (weeks)	0			3			12		
Depth (inches)	0-3	3-6	6-11	0-3	3-6	6-11	0-3	3-6	6-11
PPM	6.53	1.57	--	4.94	1.65	0.20	2.88	2.70	0.41
Balance	Percent of Total ^{14}C in Soil Sample								
Organic	84.4			77.6			31.8	31.4	
Aqueous	1.5			2.3			3.9	4.6	
Nonextractable	15.4			28.6			42.5	46.7	
TLC Characterization									
CGA-48988 (c)	80.1			65.4			28.8	31.7	
CGA-62826 (4)	<0.3			1.1			2.8	6.0	
Unknowns									
(1)	--			--			0.5	0.5	
(o)	--			--			1.3	1.7	

*THI = Treatment to harvest interval.

TABLE IV. ^{14}C-EQUIVALENT TO CGA-48988 IN GREENHOUSE SOIL - BRIGHT TOBACCO

Rate (lb. ai/A)	0.25									0.5								
THI* (weeks)	0			6			12			0			6			12		
Depth (inches)	0-3	3-6	6-11	0-3	3-6	6-11	0-3	3-6	6-11	0-3	3-6	6-11	0-3	3-6	6-11	0-3	3-6	6-11
PPM	13.7	0.7	0.3	2.2	0.7	0.2	1.1	0.6	0.2	17.4	3.6	0.8	3.8	2.2	0.6	2.3	1.3	0.4

*THI = Treatment to harvest interval.

TABLE V. CHARACTERISTICS OF GEORGIA SANDY LOAM SOIL

Texture	Loamy sand
pH	5.0
% organic matter	2.3
% sand	87.2
% silt	9.6
% clay	3.2