BIOCHEMISTRY DEPARTMENT
AGRICULTURAL DIVISION
CIBA-GEIGY CORPORATION
GREENSBORO, N.C.

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

M11-69-11P, 11S

Report No.: ABR-79100 Issued By:

Submitted By: Issue Date October 10, 1979

A B S T R A C T

Field grown Bright tobacco was treated with ϕ-^{14}C-CGA-48988 at 3.0 lbs. ai/A broadcast followed by bedding. The ppm equivalent to ϕ-^{14}C-CGA-48988 in the leaves decreased with time. At 5 weeks bottom leaves were 12.5 ppm while at 9 weeks, 6.7 ppm. Curing the leaves increased the radioactivity about threefold, from 6.7 ppm to 21.5 ppm for cured bottom leaves. Higher levels of radioactivity were found in the bottom cured leaves than in the top cured leaves, 21.5 ppm compared to 7.3 ppm. These levels are much lower than those for greenhouse grown bright or burley tobacco (ABR-78036).

Some CGA-48988 and/or its metabolites volatilize when curing tobacco. After curing control and radioactive leaves together, controls and lower leaves contained 0.48 ppm and 21.5 ppm, respectively.

Metabolism of ϕ-^{14}C-CGA-48988 in tobacco results in the rapid formation of many metabolites. At 9 weeks, mature bottom leaves from the cured bright tobacco contained 7.5% of the total radioactivity as ϕ-^{14}C-CGA-48988 and at least 23 unknown polar metabolites. Of these, only six individually accounted for 1% or more of the total radioactivity. Seventeen polar metabolites composed a total of 10% of the total radioactivity. Two metabolites (IV and VI) composed a significant portion (14% and 9%) of the total radioactivity in these cured bottom leaves. No CGA-62826 was found in the cured tobacco.

The metabolism of ϕ-^{14}C-CGA-48988 in field tobacco is qualitatively similar to that in greenhouse grown bright or burley tobacco. Quantitative differences do occur since ϕ-^{14}C-CGA-48988 appears to be metabolized more rapidly in field tobacco than in greenhouse tobacco.

Radioactivity in the 0-3" layer of the field soil dissipated slowly from 2.0 ppm at 0 time to 1.8 ppm by 16 weeks. The residual radioactivity at 16 weeks in the 0-3" layer contained 24.1% as ϕ-^{14}C-CGA-48988 and 0.1% as the acid metabolite CGA-62826.

At 16 weeks, radioactive nonextractables in field soil accounted for 51% 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 possibly limit uptake of CGA-48988 and CGA-62826 into the tobacco at later stages of growth.

INTRODUCTION

The experimental compound CGA-48988, N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-alanine methyl ester*, is a fungicide proposed for control of blue mold and black shank on tobacco. Application is effective by preplant incorporation. A small plot of soil was treated with [U-ring-^{14}C]-N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-alanine methyl ester** by preplant incorporation followed by bedding. Tobacco slips were transplanted and grown to maturity. The objectives of this study were to: (1) determine the uptake and balance of the radioactivity in field bright (flue-cured) tobacco at various stages of growth and compare the uptake and balance to that of greenhouse grown tobacco; (2) determine the extent and nature of CGA-48988 metabolism in mature uncured and cured field grown bright tobacco; and (3) determine the extent of degradation and leaching of ϕ-^{14}C-CGA-48988 applied to field soil.

EXPERIMENTAL

Preparation of Tobacco Plot and Application of ϕ-^{14}C-CGA-48988: A plot (13' x 18') of sandy loam soil (Table I) was located at the North Carolina State University Research Farm at Reidsville, North Carolina. It was rototilled, treated ppi at 1 gal./acre Diphonate®/Tillam® and fenced off. An area of soil 5' x 10' within the fenced area was then treated at 3 lbs. ai/acre (broadcast) with ϕ-^{14}C-CGA-48988. It was applied to the soil in the following manner: 1.66 g of ϕ-^{14}C-CGA-48988 at 19.3 µCi/mg (5.6 mCi/mM) was dissolved in 25 ml of ethanol. Five ml aliquots were added to one pound of soil and the soil mixed for 3 hours. A pound of soil was then sprinkled evenly over each 2' x 5' area of the 5' x 10' plot. A total of five pounds of treated soil was thus distributed over the 5' x 10' plot. The treated soil was then incorporated to 3 inches with a hoe and bedded into two rows 35 inches apart resulting in 3-4" deep furrows on each side and between the rows. Seven bright tobacco transplants (McNair 944 variety) were placed in each row and spaced 18" apart. The soil was packed up around the plants as in normal agricultural practice and about 0.5 lb. of fertilizer (15-0-14) was sprinkled in the furrows between the rows and the furrows covered with soil. The 5' x 10' plot was then sprayed with water and soil cores immediately taken. A control (untreated) plot of tobacco was grown approximately 30 feet from the treated plot. The plants were topped and hand suckered when necessary.

*Chemical names and structures are given in Figure 1.

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

Sampling: Leaves were taken at 5 weeks and then at each priming - bottom leaves (9 weeks), middle leaves (13 weeks), top leaves (16 weeks). Soil cores were taken at these same time intervals and divided into 0-3", 3-6" and 6-9" segments.

Curing Tobacco: Tobacco leaves were yellowed at 35°C for 24-48 hours in a modified Bio-Flow hood (Germ Free Laboratories, Inc., Miami, Florida). The exit outlet and intake were sealed off and pans of water put into the sealed area and the circulator was turned on. The temperature was regulated using a heat gun and thermoregulator. The tobacco was then transferred to a 20" x 20" x 18" Blue M oven with forced air. The temperature was raised at 2°C per hour until a temperature of 68°C was attained. This temperature was held for 24 hours or until the stems were brittle at which time the oven was turned off and a pan of water placed under the tobacco to bring it to order (regain moisture).

Sample Preparation and Analysis: Uncured or cured tobacco leaves were homogenized with dry ice in a Wiley Mill (1). Samples of 100-200 mg were combusted in a Harvey oxidizer (2). Plant extractions done in accordance with AG-214 (3) gave an organic phase, aqueous phase and nonextractables. Soil samples were prepared according to AG-223 (1) and 1-2 grams of each were combusted (2). Extraction of soil was in accordance with AG-254 (4).

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) or by using a Birchover Spark Chamber. 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 (5).

RESULTS AND DISCUSSION

Radioactivity in Bright Tobacco Leaves: The levels of radioactivity equivalent to ϕ-^{14}C-CGA-48988 in tobacco leaves are shown in Table II. The radioactivity in the tobacco decreased with time. At 5 weeks, bottom leaves had 12.5 ppm equivalent to ϕ-^{14}C-CGA-48988; at 9 weeks, 6.7 ppm; and at 16 weeks, 1.7 ppm. These data suggest that radioactive metabolites of ϕ-^{14}C-CGA-48988 are being diluted by growth of the tobacco.

After curing the bottom leaves (9 weeks), the radioactivity increased about threefold to 21.3 ppm. This was due to a loss of water during the curing process. The concentrations of ϕ-^{14}C-CGA-48988 and its metabolites were higher in cured bottom leaves than in cured upper leaves, i.e., 21.5 ppm compared to 7.3 ppm. This cured field grown bright tobacco treated ppi at 3 lbs. ai/acre had much less ϕ-^{14}C-CGA-48988 and related ^{14}C-metabolites in its leaves than did greenhouse grown bright tobacco treated at 0.5 lb. ai/acre (transplant water) or burley tobacco treated ppi at 6 lbs. ai/acre (see Table IV).

Balance data (Table II) for uncured as well as cured bright tobacco show a decrease in organic radioactive solubles with leaf age, e.g., from 49.1% at 5 weeks to 31.0% by 16 weeks for uncured bright tobacco and an increase in aqueous soluble metabolites from 43.0% at 5 weeks to 61.5% by 16 weeks. These changes indicated that metabolism of CGA-48988 occurs rapidly to produce polar metabolites. Further, these changes in balance distribution occurred more rapidly in field than in greenhouse tobacco (6), and therefore indicate that metabolism of CGA-48988 is faster under field conditions.

Nonextractable radioactivity remained low until the tobacco was cured, at which time the radioactive nonextractables approach 10-20%. This increase is probably due to occlusion of ϕ-^{14}C-CGA-48988 and its metabolites during curing. Aqueous soluble polar metabolites were also higher in the middle and upper cured leaves than in the lower cured leaves, suggesting that age increases the amount of polar metabolites. Similar results were found for greenhouse grown bright and burley tobacco.

Some ϕ-^{14}C-CGA-48988 in treated tobacco is transferred to untreated tobacco during curing, if untreated tobacco leaves are cured in the same oven with the treated tobacco leaves (Table III). ϕ-^{14}C-CGA-48988 and/or its metabolites are probably volatilized out of the treated leaves at high temperatures and some transferred to the untreated leaves. The amount transferred will not only be a function of temperature but also a function of the air flow rate in the curing ovens.

Two dimensional TLC of the ^{14}C-organic solubles at 9 weeks showed 7.5% of the ^{14}C was ϕ-^{14}C-CGA-48988 (Figure 2A and Table II). Two dimensional TLC (Figure 2B and Table II) of the aqueous fraction revealed at least 23 polar metabolites in the 9-week cured bright tobacco. No CGA-62826 was found in cured tobacco. Each of the metabolites II, IV, V, VI, VII, and XVI was more than 1% of the total radioactivity in the cured leaf. There were 17 other unknown polar metabolites, each was less than 1%, and together made up no more than 10% of the total radioactivity in the cured leaf. Bright tobacco treated at 0.5 lb./A and burley tobacco treated at 6 lbs. ai/acre and grown in the greenhouse (6) have similar qualitative TLC patterns of polar metabolites as does this field grown bright tobacco (compare Figure 2B and 2C). These patterns show that the environmental conditions under which the tobacco is grown (field conditions) result in the same metabolic pathway described in previous studies (7). Some minor quantitative differences were found between the metabolism of greenhouse grown vs. field grown tobacco, which indicate that metabolism of ϕ-^{14}C-CGA-48988 is more rapid in the field and leads to early depletion of ϕ-^{14}C-CGA-48988. Compare Table I with Table IV.

Chromatographic characterization (Figure 2A and Table II) of the leaf radioactivity showed that uncured bright field tobacco (top leaves) contained 10.0% of the total radioactivity as ϕ-^{14}C-CGA-48988 while cured bright top leaves contained 3.3%. A combination of rapid metabolism of ϕ-^{14}C-CGA-48988 followed by volatilization during curing leads to low amounts of ϕ-^{14}C-CGA-48988 in top cured leaves.

Soil Balance and Characterization: Radioactivity in the field dissipated slowly (Table V). By 16 weeks, the soil was 1.8 ppm in the 0-3" layer. Leaching was observed between 5 and 16 weeks, e.g., the radioactivity in the 3-6" and 6-9" segments increased from 0.9 to 1.4 ppm and 0.3 to 0.9 ppm, respectively. Compared to greenhouse soil treated with ϕ-^{14}C-CGA-48988, radioactivity in the field dissipated more slowly and leached to a greater extent (6). This increased leaching may be due to the lower organic matter content of field soil than greenhouse soil.

The balance data for soil show that the organic radioactive solubles decreased significantly by 16 weeks while the aqueous soluble radioactivity remained low. The nonextractable radioactivity in the soil increased significantly to 51.0% by 16 weeks, suggesting that CGA-48988 and/or its metabolites become tightly bound to the soil in a short time.

Chromatographic characterization of the radioactivity in the soil showed that parent ϕ-^{14}C-CGA-48988 decreased from 49.0% at 5 weeks to 24.1% at 16 weeks in the 0-3" layer (Table V). Very little CGA-62826 was found in the 16 week soil, only 0.1% of total ^{14}C in soil sample.

Since these data show that metabolism of ϕ-^{14}C-CGA-48988 in soil leads to little CGA-62826, ϕ-^{14}C-CGA-48988 and/or its metabolites are possibly rapidly bound to the soil. The metabolism of ϕ-^{14}C-CGA-48988 in field soil and greenhouse soil is similar (6).

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

The authors would like to acknowledge Ms. 5.1.2.e Woo and Mr. 5.1.2.e Woo for their technical assistance as well as Mr. 5.1.2.e Woo who prepared the ϕ-^{14}C-CGA-48988. We would also like to acknowledge Messrs. 5.1.2.e and 5.1.2.e Woo who helped manage the plot at the North Carolina State University Research Farm in Reidsville, North Carolina.

REFERENCES

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

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

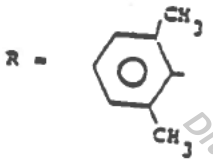
3. [redacted]., AG-214, "Biphasic Extraction of Radioactive Metabolites from Treated Biological Material."

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

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

6. [redacted], ABR-78036, "Uptake and Balance of ϕ-^{14}C-CGA-48988 and Its Metabolites in Greenhouse Bright and Burley Tobacco."

7. [redacted] and [redacted]., ABR-79008, "Identification of the Major Aglycones of ϕ-^{14}C-CGA-48988 Conjugated Metabolites in Cured Greenhouse Grown Bright Tobacco."



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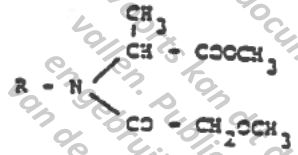
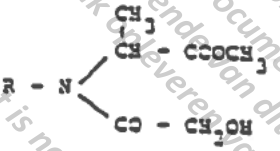
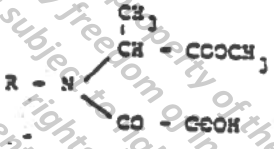
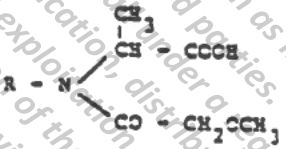
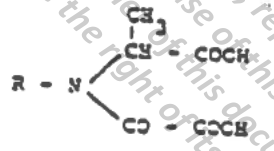
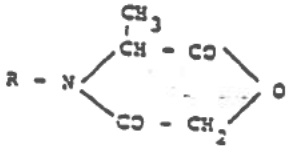
1		CGA-48988 N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-alanine methyl ester
2		CGA-67869
3		CGA-79353
4		CGA-62826
5		CGA-78532
6		CGA-68125

FIGURE 1. CHEMICAL NAMES AND STRUCTURES

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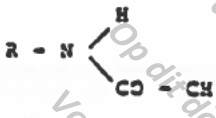
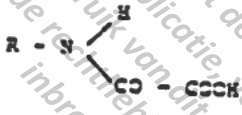
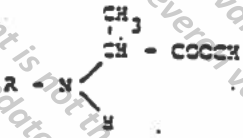
7		CGA-37734
8		CGA-68124
9		CGA-67866
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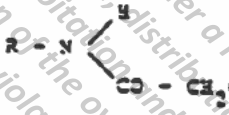
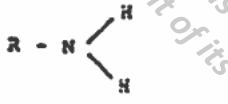
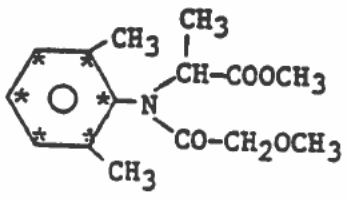
11		CGA-67868
12		CGA-72649

RADIOACTIVE COMPOUND



* = ^{14}C

ϕ-^{14}C-CGA-48988

[U-ring-^{14}C]-N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-alanine methyl ester

FIGURE 1. CHEMICAL NAMES AND STRUCTURES (Continued)

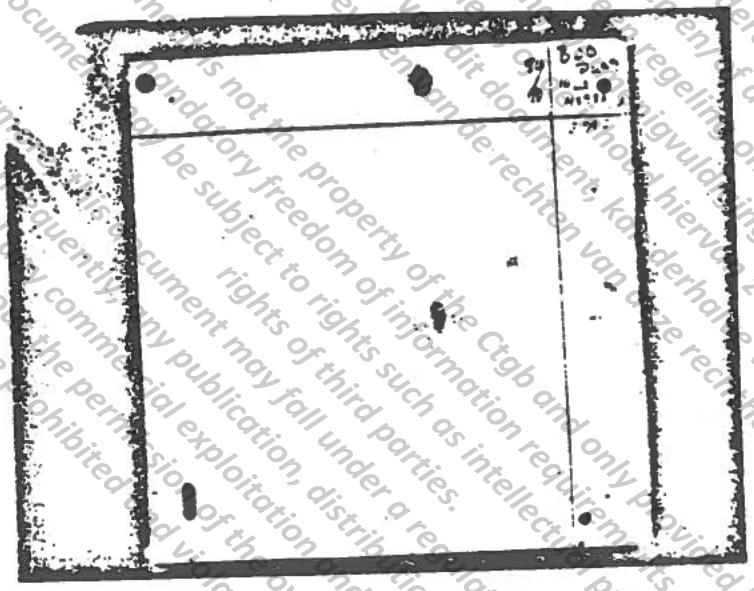
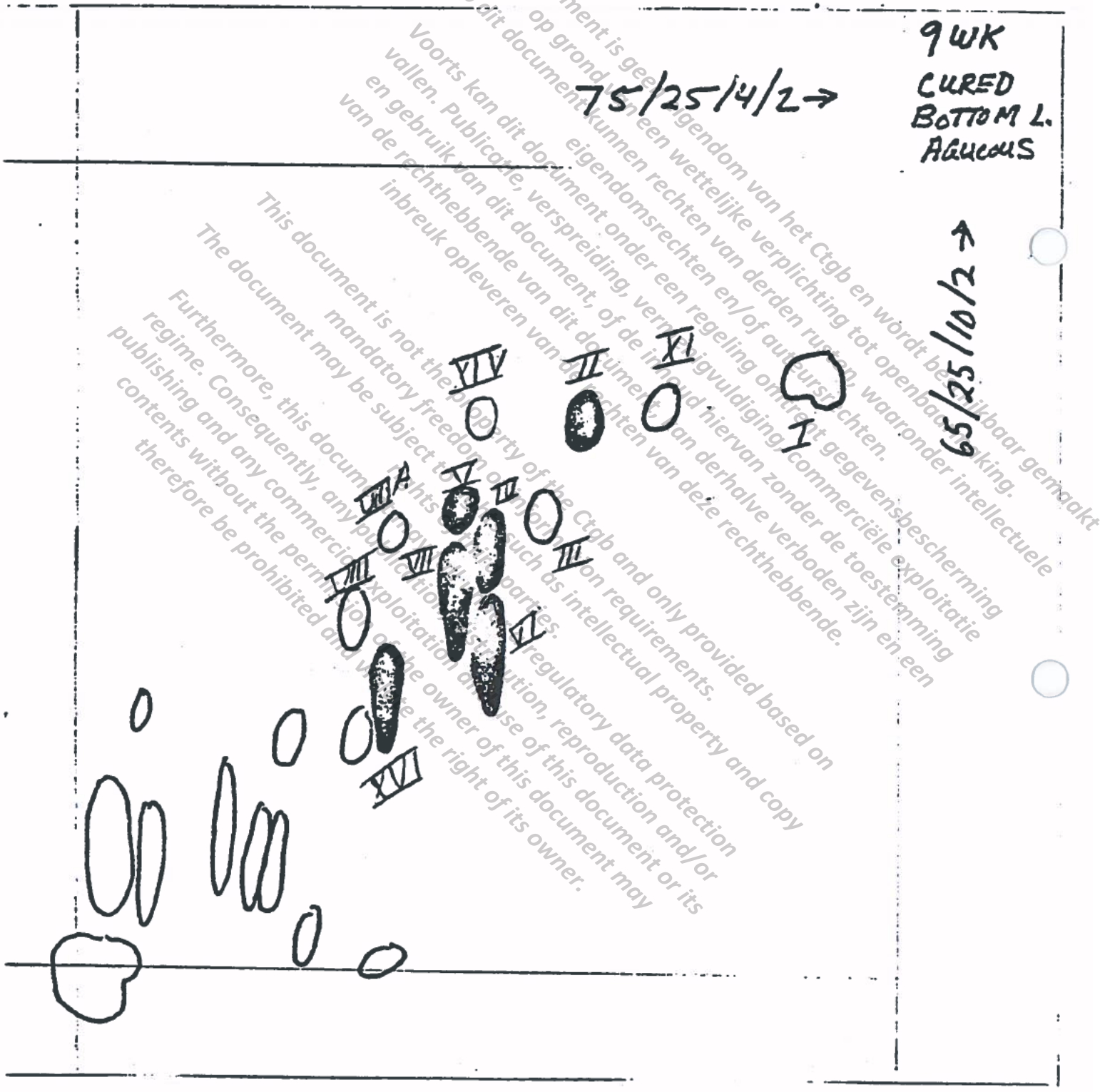


FIGURE 2A: U.V. PHOTOGRAPH OF 2D-TLC of ^{14}C-ORGANIC SOLUBLES FROM EXTRACTION OF 9 WEEK CURED BOTTOM LEAVES FROM FIELD BRIGHT TOBACCO (3 LBS. AI/ACRE). DOTTED CIRCLES ARE RADIOACTIVE ZONES.

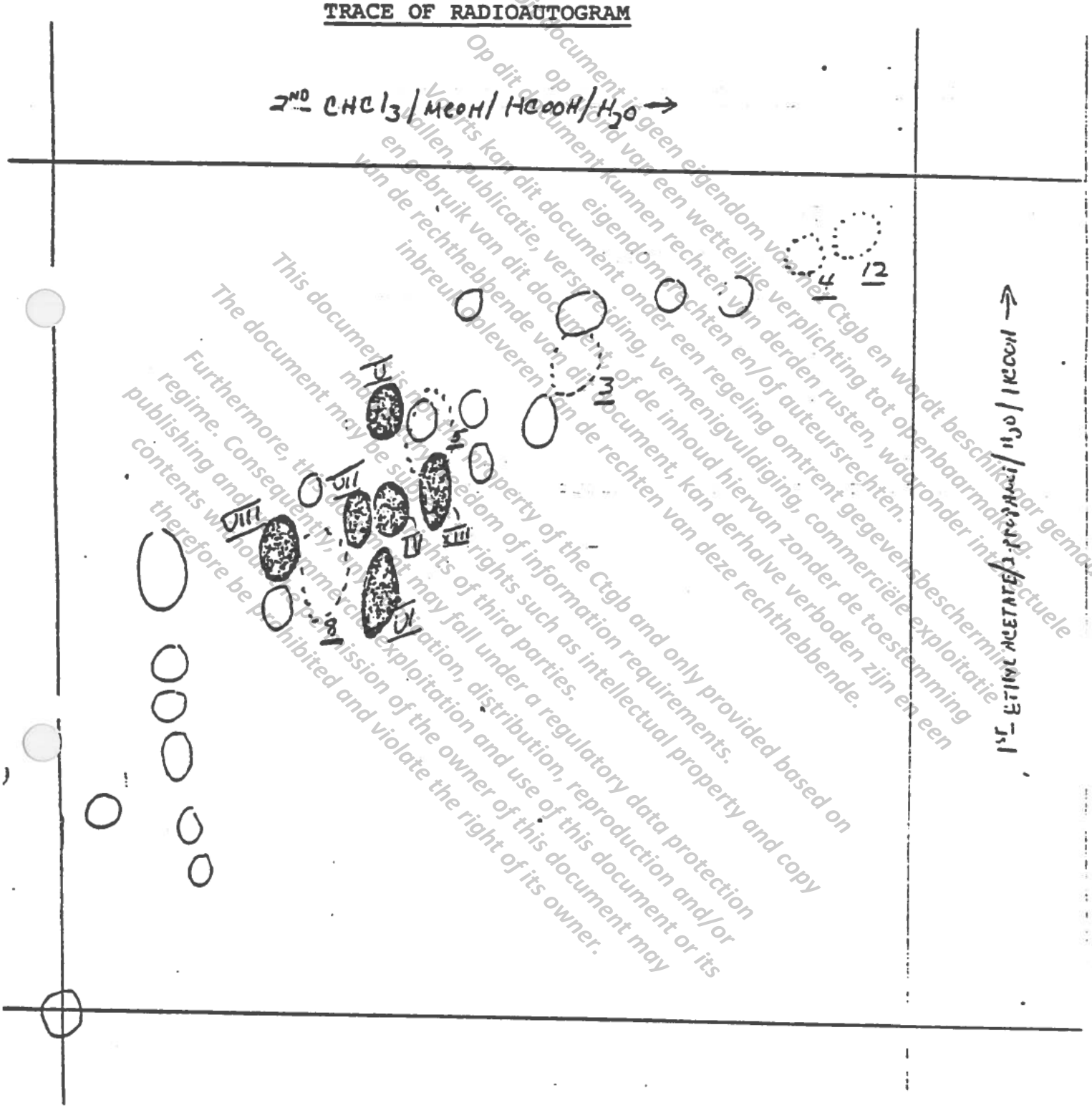
TRACE OF RADIOAUTOGRAM



Enclosed circles represent radioactive spots. Darkened areas are major (> 1%) metabolites.

FIGURE 2B: TRACE OF 2D-TLC OF ^{14}C-AQUEOUS SOLUBLES IN 9 WEEK CURED FIELD BRIGHT TOBACCO

TRACE OF RADIOAUTOGRAM



Enclosed circles represent radioactive spots. Darkened areas are major (> 3%) metabolites. Dotted circles with Arabic numbers are standards (See FIGURE 1 for code #'s).

FIGURE 2C: TRACE OF 2D-TLC of ^{14}C-AQUEOUS SOLUBLES IN 16-WEEK CURED BRIGHT TOBACCO (0.5 LB. AI/A) (FROM ABR-78036)

TABLE I: CHARACTERISTICS OF REIDSVILLE FARM SOIL*

Texture	Sandy loam
pH	5.7
% organic matter	0.9
% sand	76.0
% silt	14.0
% clay	10.0

*Data from plot near radioactive plot.

Table II: BALANCE AND CHARACTERIZATION OF ϕ-^{14}C-CGA-48988 IN FIELD BRIGHT TOBACCO

Rate = 3 lbs. ai/acre (ppi)

	Bottom Leaves			Middle Leaves		Upper Leaves	
THI* (weeks)	5 UC**	9 UC	9 C***	13 UC	13 C	16 UC	16 C
PPM	12.5	6.7	21.5	4.0	10.3	1.7	7.3
Balance	Percent of Total ^{14}C in Plant Sample						
Organic	49.1	38.4	14.9	42.0	13.3	31.0	13.1
Aqueous	43.0	49.1	50.3	47.0	80.0	67.5	71.2
Nonextractable	5.0	3.9	20.3	2.5	11.5	4.5	10.4
TLC Characterization							
CGA-48988	-	-	7.5	-	4.3	10.0	3.3
Major unknown polar Met. each >1.1% in field cured tobacco:							
II	-	-	1.9	-	-	-	-
IV	-	-	13.7	-	-	-	-
V	-	-	1.1	-	-	-	-
VI	-	-	9.2	-	-	-	-
VII	-	-	4.1	-	-	-	-
XVI	-	-	2.0	-	-	-	-
17 Minor unknown met. each from 0.06 to 1.0% in cured tobacco.	-	-	10.0	-	-	-	-
unrecovered ^{14}C	-	-	8.3	-	-	-	-

*THI = Treatment to harvest interval.
**UC = Uncured
***C = Cured

TABLE III: TRANSFER OF ϕ-^{14}C-CGA-48988 AND/OR ^{14}C METABOLITES FROM TREATED TOBACCO TO CONTROL TOBACCO (LOWER LEAVES)

Leaf and Conditions	ppm*
Treated cured bottom leaves**	21.5
Nontreated bottom leaves cured with treated leaves**	0.48
Nontreated bottom leaves not cured with treated leaves.	<0.01

*ppm equivalent to ϕ-^{14}C-CGA-48988.

**Treated and nontreated leaves were cured together in the same oven.

TABLE IV. BALANCE AND CHARACTERIZATION OF ϕ-^{14}C-CGA-48988 IN GREENHOUSE BRIGHT TOBACCO (FROM ABR-78036)

Rate = 0.50 lb. ai/A (Transplant Water Treatment)

	Lower Leaves				Middle Leaves		Upper Leaves	
THI* (weeks)	3 UC**	6 UC	12 UC	12 C***	16 UC	16 C	19 UC	19 C
PPM	73.9	32.6	14.1	147.7	—	74.0	—	93.7
Balance	Percent of Total ^{14}C in Plant Sample							
Organic	73.4	73.5	56.2	31.3	—	24.2	—	34.0
Aqueous	25.1	29.6	46.8	49.6	—	60.7	—	64.6
Nonextractable	1.3	2.0	2.4	9.9	—	12.2	—	6.5
TLC Characterization								
Organic CGA-48988	64.7	58.2	34.7 (4.9 ppm)	26.9 (40 ppm)	—	10.9	—	—
7 Unknown Met.	—	—	—	—	—	9.9	—	—
Aqueous CGA-62826	1.0	0.4	1.5	<0.3	—	—	—	—
6 Major Unknown Polar Met., each >3% in Cured Tobacco								
IV	1.7	2.7	4.7	6.5				
V	1.0	0.8	2.5	3.2				
VI	5.7	7.7	13.5	6.9				
VII	1.8	2.2	3.3	2.8				
VIII	0.8	3.2	3.2	3.0				
XIII	—	—	2.9	3.2				
19 Minor Unknown Polar Met., each from 0.3 to 3.0% in Cured Tobacco	10.2	7.3	12.1	19.6				

*THI = treatment to harvest interval
**UC = uncured
***C = cured

TABLE V: BALANCE AND CHARACTERIZATION OF ϕ-^{14}C-CGA-48988 IN TREATED FIELD SOIL - BRIGHT TOBACCO

THI* (weeks)	0			5			16		
Depth	0-3	3-6	6-9	0-3	3-6	6-9	0-3	3-6	6-9
PPM	3.0	0.07	0.04	2.0	0.9	0.3	1.8	1.4	0.9
Balance	Percent of Total ^{14}C in Soil Sample								
Organic	104.1	-	-	51.5	-	-	24.7	-	-
Aqueous	1.3	-	-	6.1	-	-	17.8	-	-
Nonextractable	6.3	-	-	27.1	-	-	51.0	-	-
TLC Characterization (organic)									
CGA-48988	-	-	-	49.0	-	-	24.1	-	-
CGA-62826	-	-	-	0.7	-	-	0.1	-	-
Unknowns	-	-	-	2.4	-	-	0.7	-	-

*THI = treatment to harvest interval.