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METABOLISM OF CGA 48 988 IN GRAPEVINE

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Contents

A B S T R A C T

The fate of CGA 48 988, i.e. D,L-N-(2,6-dimethylphenyl)-N-(2'-methoxyacetyl)-alanine methylester, the active ingredient of the fungicide RIDOMIL ®, was studied in field grown grapevine after regular application of phenylring-^{14}C-labelled pesticide.

At the time of harvest 22.4 % of the radioactivity in the leaves and 64.1 % of that in the grapes were in form of the unchanged fungicide. In the grapes the total radioactivity corresponded to 3.06 ppm CGA 48 988 equivalents, whereby the portion of unchanged CGA 48 988 was 1.96 ppm.

After pressing the grapes 1.04 ppm of the total radioactivity or 0.45 ppm of CGA 48 988 were found in the juice.

Degradation of CGA 48 988 in grapevine proceeds primarily via oxydation probably at one of the ring-methyl groups yielding a benzylic alcohol derivative, which is subsequently conjugated with endogenous plant material, most probably sugars.

1. INTRODUCTION

CGA 48 988, i.e. D,L-N-(2,6-dimethylphenyl)-N-(2'-methoxy-acetyl)-alanine methylester, is the active ingredient of the fungicide RIDOMIL® which effectively controls Late Blight of potatoes and Downy Mildews on various crops and seedling diseases caused by Oomycetes.

This use makes an understanding of its fate in plants necessary. In this report the result of studies on the fate of the fungicide and the nature of its metabolites in grapevine is presented.

2. RESULTS AND DISCUSSION

Grapevine, variety Riesling & Sylvaner located in a vineyard in Sisseln, near Basle, were sprayed seven times at 14 day intervals with ^{14}C-labelled fungicide. The spraying calendar as well as the mode and rate of application were as recommended for practical use (for details see 3.2.1).

Fifty two days after the last application the ripe grapes were harvested and processed to juice and press-cake. The radioactivity in these fractions as well as that of the leaves were analyzed as given under 3.2.2

2.1 Recovery and distribution of radioactivity

The concentrations of radioactive CGA 48 988 equivalents in various plant parts are given in Table II.

As part of the plant material (about 35 % of the leaves, 60 % of the grapes) was lost during a hailstorm shortly before the maturity, exact establishment of the radioactivity balance was not possible. However, the concentration of the radioactivity in the damaged and remaining plant material was measured. The results strongly indicate that only less than 5 % of the radioactivity totally applied was present in the plants shortly before the harvest. The rest of the radioactivity was probably lost partly due to drift during the application and partly due to volatilization from the leaf surface. In fact, as shown by additional short-term experiments, more than 80 % of the radioactivity applied to the leaf surface as CGA 48 988 was volatilized within 7 hours after the application.

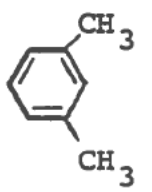
The radioactivity in young shoots grown within two weeks after the last application amounted to 1.2 - 1.6 ppm CGA 48 988 equivalents. This indicates that the acropetal translocation of CGA 48 988 and its metabolites took place to a small extent.

As shown by additional experiments this translocation proceeded primarily after penetration of the radioactivity through the outer stem layers with the water stream in the xylem. Basipetal translocation took place, if at all, only to a very limited extent.

2.2 Characterization of the radioactivity

The radioactivity in the grape juice as well as that in the extracts of the presscake and leaves was fractionated as described under 3.2.2. The portion of unchanged CGA 48 988 and the number of its metabolites were determined by TLC. The results are presented in Table III.

The zone consisting of CGA 48 988 was identified by comparison with reference material on TLC and by GLC/MS. The following fragments were obtained:

m/e	279	M
	264	$M - CH_3$
	249	$M - OCH_2$
	234	$M - CH_2OCH_3$
	220	$M - COOCH_3$
	206	$M - C(=O)-CH_2OCH_3$
	192	$M - CH(CH_3)-COOCH_3$
	160	$192 - CH_3OH$
	148	$220 - C(=O)-CH_2OCH_3$
	105	

The unchanged fungicide, which was primarily found in the hexane phase amounted to 22.4 % and 64.1 % of the total radioactivity in the leaves and grapes, respectively. Nearly 90 % of CGA 48 988 present in the grapes was found after pressing in the presscake, indicating that most of CGA 48 988 was connected with the cell matrix possibly still being adsorbed to the skin of the fruits.

The non-extractable radioactivity was low amounting to 9.4 and 4.2 % of the total radioactivity in grapes and leaves, respectively.

Almost 75 % of the radioactivity in the leaves and 27 % of that in the grapes were in form of degradation products, which behaved qualitatively identical in both plant parts. The methylene chloride soluble metabolite fraction consisted of one major (designated as zone III) and three minor metabolites (zone I, II and IV). TLC of the water soluble radioactivity in solvent systems 43 and 16A revealed the presence of one major and at least three minor compounds (see also Appendix, TLC 3).
The radioactivity of the ethyl acetate phase consisted of metabolites which were already found in the methylene chloride as well as in the water phase.

The water soluble radioactivity of the leaves was purified according to Scheme 2. After incubation with cellulase according to 3.3.8 all of the radioactivity became methylene chloride soluble indicating the presence of conjugated metabolites only. TLC in solvent system 60 showed, that this radioactivity consisted of the same metabolites (zones I through IV)

which were already found in the original methylene chloride phase (see Appendix, TLC 1 and 2). These fractions were quantitated and the results are summarized in Table IV.

For the isolation of sufficient amounts of the major degradation product (zone III) for GLC/MS, the original methylene chloride phase was fractionated on a preparative silica gel column according to 3.3.5. The metabolite corresponding to zone III was further purified by HPLC as described under 3.3.4.

In order to get a volatile and thermally stable product for GLC, zone III was silylated according to 3.3.9. Separation and fragmentation by coupled capillary GLC and MS in the EI mode revealed the following fragments:

m/e 367 M^+ (1 x TMS)

352 $M^+ - CH_3$ "

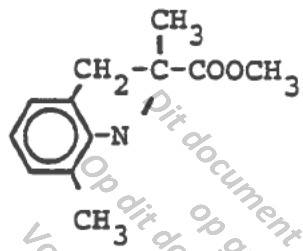
336 $M^+ - OCH_3$ "

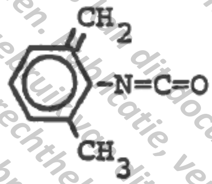
322 $M^+ - CH_2OCH_3$ "

308 $M^+ - COOCH_3$ "

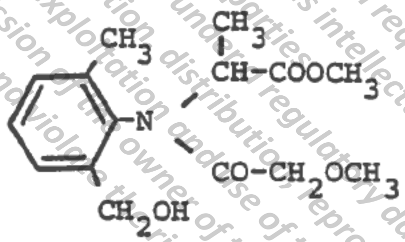
294 $M^+ - C(=O){-}CH_2OCH_3$ "

280 $M^+ - CH(CH_3){-}COOCH_3$ "

232 

146 

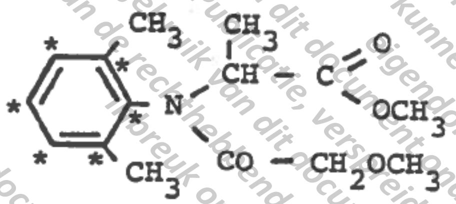
The fragmentation pattern indicates the presence of a metabolite formed after oxydation of the parent compound but still having the aliphatic N-substituents unchanged. Most probably, oxydation had taken place at the 2-(6)-methyl substituent resulting in the following structure:



In conclusion, the results indicate that CGA 48 988 is degradated in grapevine primarily by oxydation probably at one of the ring-methyl groups yielding a benzylic alcohol derivative, which is subsequently conjugated with endogenous plant material, most probably sugars.

3. MATERIALS AND METHODS

3.1 Chemicals



Uniformly [^{14}C]-ring labelled D,L-N-(2,6-dimethylphenyl)-N-(2'-methoxyacetyl)-alanine methylester, specific radioactivity: 20.0 µCi per mg.

For this experiment ^{14}C-CGA 48 988 was diluted with unlabelled CGA 48 988 to obtain a specific radioactivity of 0.5 µCi per mg.

3.2 Experimental conditions

3.2.1 Plants and their treatment

Two grapevines, variety Riesling & Sylvaner, cultivated in a vineyard at the CIBA-GEIGY research farm in Sisseln near Basle were sprayed seven times until run-off with a spray mixture of ^{14}C-CGA 48 988 formu-

lated as WP 50 (A-5514-A) at a concentration of 50 g a.i. / 100 l in water.

The spraying calendar followed is given below:

No.	Date	ml sprayed per plant
1	June 7, 1977	150
2	June 21	175
3	July 5	200
4	July 19	250
5	August 2	300
6	August 16	350
7	August 30	400

3.2.2 <u>Harvest and analysis</u>

Shortly before the maturity a great part of the leaves and grapes were damaged during a hailstorm in spite of a shed over the vine plants. The remaining plant material including ripe grapes were harvested on October 21, 52 days after the last application. The following amounts of biological materials were obtained:

	0.5 µCi/mg
- ripe grapes	2.0 kg
- leaves	1.9 kg
- green and wooden stems, cut above the 8th eye	2.6 kg

The ripe grapes were processed and analyzed as given in Scheme 1.

The grape juice had a density of 1.07 corresponding to 70^{o} Oechsle indicating a moderate sugar content.

The leaves were extracted and analyzed according to Scheme 2.

3.3 Analytical methods

3.3.1 Measurement of radioactivity

All measurements were performed with a Packard Tri-Carb Liquid Scintillation spectrometer Mod. 3320 or Mod. 3375 (Packard Instr. Comp., Downers Grove, Ill. USA).

Three scintillation mixtures were used:

Mixture A: (for organic and aqueous solutions;
0.05 - 0.2 ml extract
+ 15 ml mixture)

Toluene	500 ml
Dioxane	500 ml
Methanol	300 ml
Naphthalene	104 g
Butyl-PBD [1]	10 g

Mixture B: (for $^{14}CO_2$, 10 ml absorption solution
+ 10 ml mixture)

Toluene	1000 ml
Butyl-PBD	8 g
PBBO [2]	0.5 g

Mixture C: (for silicagel scraped off thin-layer plates; 5 ml water + 10 ml mixture)

Instagel solution (Packard Instr. Comp.)

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1) Butyl-PBD (CIBA-GEIGY): 2-(4'-t-butylphenyl)-5-(4"-biphenylyl)-1,3,4-oxadiazole

2) PBBO (CIBA-GEIGY): 2-(4'-biphenylyl)-6-phenyl benzoxazole

Quenching was corrected for by the internal standard method (scintillation mixtures B and C) or by the channels ratio- (Mod. 3320) or the AES channels ratio method (Mod. 3375; scintillation mixture A).

Radioactive zones on thin layer plates were localized with a Radiochromatogram Scanner Berthold Model II (Laboratory Dr. Berthold, Wildbad, Western Germany) or a LKB Model 2105 Radiochromatogram Camera (LKB, Bromma, Sweden).

Non-extractable radioactivity was determined by combustion of the dry residue (approximately 100 mg) in an oxygen stream at 900 °C. $^{14}CO_2$ liberated was absorbed in three traps each containing 10 ml of ethanolamine/methanol (12:88). Radioactivity was measured after addition of 10 ml scintillation mixture B.

3.3.2 Thin layer chromatography (TLC)

Support: Silica gel with fluorescent indicator (MERCK, Darmstadt, Germany).

Layer thickness: 0.25 mm for analytical, 0.5 and 2 mm for preparative separations.

Non-labelled reference substances were located under UV-light (254 nm).

The following solvent systems were used:

60.	ethyl acetate	
103 A.	ethyl acetate / acetic acid	9:1
97 A.	methylene chloride / methanol / acetic acid	95:4:1
43.	chloroform / ethanol	1:1
16 A.	ethyl acetate / ethanol / acetic acid	44:44:11

The following Rf-values were obtained:

Compound	Solvent system		
	60	103 A	97 A
CGA 48 988	0.45	0.65	0.51
CGA 37 734	0.23	0.51	
CGA 72 649	0.60	0.75	
CGA 62 826	0.03	0.44	
CGA 67 866	0.64	0.89	
CGA 67 867	0.15	0.73	0.22
CGA 67 868	0.40	0.67	0.59
CGA 67 869	0.46	0.70	0.45
CGA 68 124	0.00	0.18	
CGA 68 125	0.48	0.55	0.67
CGA 78 532	0.00	0.11	
CGA 79 353	0.00	0.28	

- The absolute Rf-values may vary to some extent. However, the relative rates of migration remain unchanged.

3.3.3 Gas chromatography (GLC)

The radioactive substances were co-chromatographed with reference material using a Hewlett Packard 5750 Gas chromatograph (Hewlett Packard, Anal. Instr., Avondale, Penn., USA). The apparatus was equipped with both a flame ionisation (FID) and a ^{14}C-Perkin-Elmer RGC 170 (Perkin Elmer Corp., Norwall Connecticut, USA) radioactivity monitor (RAM). The RAM signal was synchroneously registered with the FID signal by a two channel recorder (W + W, Münchenstein, Switzerland).

Details of the conditions:

Column

Material	:	Stainless steel Length: 1.8 m ID: 2 mm
Support	:	Chromosorb GAW DMCS Mesh 80/100
Liquid phase	:	UCCW 982 10 %

Temperatures

Injector	:	280°C

Detector	:	280°C
Column oven	:	100°C - 250°C
Program rate	:	10°C/min
Flow rate of carrier gas (He)	:	40 ml/min Pressure: 7 kp/cm^2

Compound	Retention time (min)
CGA 48 988	13.3
CGA 37 734	10.9
CGA 72 649	4.9
CGA 62 826 *	13.3
CGA 67 866	8.9
CGA 67 867 *	8.9
CGA 67 868	10.2
CGA 67 869	12.9
CGA 68 124 *	10.7
CGA 68 125	12.9
CGA 78 532 *	13.6
CGA 79 353 *	13.6

* After methylation with diazomethane

3.3.4 High performance liquid chromatography (HPLC)

HPLC of the radioactive substances was carried out on a Spectra-Physics Model 3500 Liquid chromatograph (Spectra-Physics, Santa Clara, California 95051, USA). The apparatus was equipped with both a UV detector (Model 770, Spectra-Physics) and a Berthold BF 5025 Flow Cell (Laboratory Dr. Berthold, Wildbad, Germany) radioactivity detector (RAM). The RAM signal was synchroneously registered with the UV signal by a two channel recorder (W + W, Münchenstein, Switzerland).

Metabolite zone III was successively purified on three different columns under the following conditions:

Column	Lichrosorb Si 60, 10 µm 50 cm x 8 mm i.d.	Spherisorb ODS, 5 µm 25 cm x 3 mm i.d.	XAD-4, 10 µm 25 cm x 1.3 cm i.d.
Mobile phase	from 0 % to 100 % ethylacetate in isopropylether	from 0 % to 100 % MeOH in water	from 0 % to 100 % MeOH in water
Delay time	10 min	10 min	30 min
Sweep time	20 min	30 min	120 min
Flow rate	1.6 ml / min	0.8 ml / min	0.4 ml / min

3.3.5 Silica gel chromatography

Stationary Phase : Silica gel Woelm [1)]
activity grade I
100 - 200 µm particle size

Column : 15 x 600 mm (i.d. x h)

Eluent : For CH_2Cl_2-soluble radioactivity

1. ethyl acetate (350 ml)
2. $CHCl_3$ / ethanol 1:1 (100 ml)

For Water-soluble radioactivity

$CHCl_3$ / ethanol 1:1

Flow rate : 60 ml/hour

The radioactivity of the eluent was continuously monitored using a Berthold BF 5025 Flow Cell (Laboratory Dr. Berthold, Wildbad, Germany) equipped with a glass scintillator-cell.
The eluate was collected in 10 ml fractions.

1) 5.1.2.e Woo, 344 Eschwege, Germany

3.3.6 Amberlite XAD-2 chromatography

A glass column with an internal diameter of 2 cm was filled with a slurry of Amberlite XAD-2 300 - 1000 µ (Serva Feinbiochemica, Heidelberg, Germany) in water to a height of 20 cm.

The water phase of the leaves was concentrated to 20 ml and carefully added on the top of the column. The ethyl acetate phase of the leaves was evaporated to dryness, redissolved in 20 ml water and then applied onto the column. After application of the sample the column was washed with 200 ml water to remove the bulk of contaminating sugars and other plant constituents. The radioactivity was then eluted with 150 ml methanol/water 8:2. Flow rate of the effluent was 180 ml/hour.

3.3.7 Sephadex gel filtration

Filtration experiments were carried out using Sephadex G 15 and Sephadex LH 20 gel (Pharmacia, Uppsala, Sweden). The dry Sephadex was allowed to swell in water and packed in a column with an internal diameter of 2.5 cm to a height of 55 cm. Deionized water at a flow rate of 60 ml/hour was used as the eluent. Fractions of 10 ml were collected.

Before reaching the fraction collector the radioactivity of the effluent was continuously measured using a Berthold BF 5025 Flow Cell (Laboratory Dr. Berthold, Wildbad, Western Germany) equipped with

an anthracene cell.

3.3.8 Enzymatic cleavage

An aliquot of the purified water soluble radioactivity was placed in a 25 ml Erlenmeyer flask. The solvent was evaporated under a gentle stream of air at 40°C. The residue was dissolved in 10 ml of Mc Ilvaine-buffer pH 5.0 which was made from 0.1 M citric acid and 0.2 M disodium phosphate 1:1. Then 5 mg of cellulase, practical grade type II (from Aspergillus niger, 1.6 Unit/mg, Sigma Chemical Company, St. Louis, Mo., USA) were added and the flask was shaken at 37°C for five hours.

The hydrolysate was extracted with methylene chloride.

3.3.9 Silylation

50 µg of the dry metabolite fraction was dissolved in 50 µl BSTFA (Pierce Chemical Company, Rockford, Ill., U.S.A.). The mixture was heated for 15 minutes at 50°C and afterwards directly subjected to gas-chromatography.

3.3.10 Gas chromatography - Mass spectrometry (GLC/MS)

The identity of the radioactivity corresponding to CGA 48 988 in the hexane and methylene chloride

phases of the grapes and leaves was confirmed by comparison with authentic CGA 48 988 using coupled GLC and MS in the EI mode.

Metabolite "zone III" was identified by electron-impact mass spectrometry using a Finnigan, Model 10 - 15 C mass spectrometer (Finnigan Instruments Corp., Sunnyvale, Calif., USA) coupled with a glass capillary gas chromatograph (Model Carlo Erba Fractovap, Carlo Erba, Milano, Italy).

The experimental conditions were:

Column

20 m SE-54, 0.3 mm ID

Carrier gas

Helium, pressure 1.7 bar

Temperatures

Injector : $180^{o}C$

Column oven : $100^{o} \longrightarrow 200^{o}C$, $3^{o}C/min$

4. ACKNOWLEDGEMENT

The skilled technical assistance of Miss [redacted] and Mr. [redacted] is gratefully acknowledged.

Labelled CGA 48 988 was synthesized by Dr. [redacted] Agricultural Division, CIBA-GEIGY Corporation, Greensboro, N.C., USA.

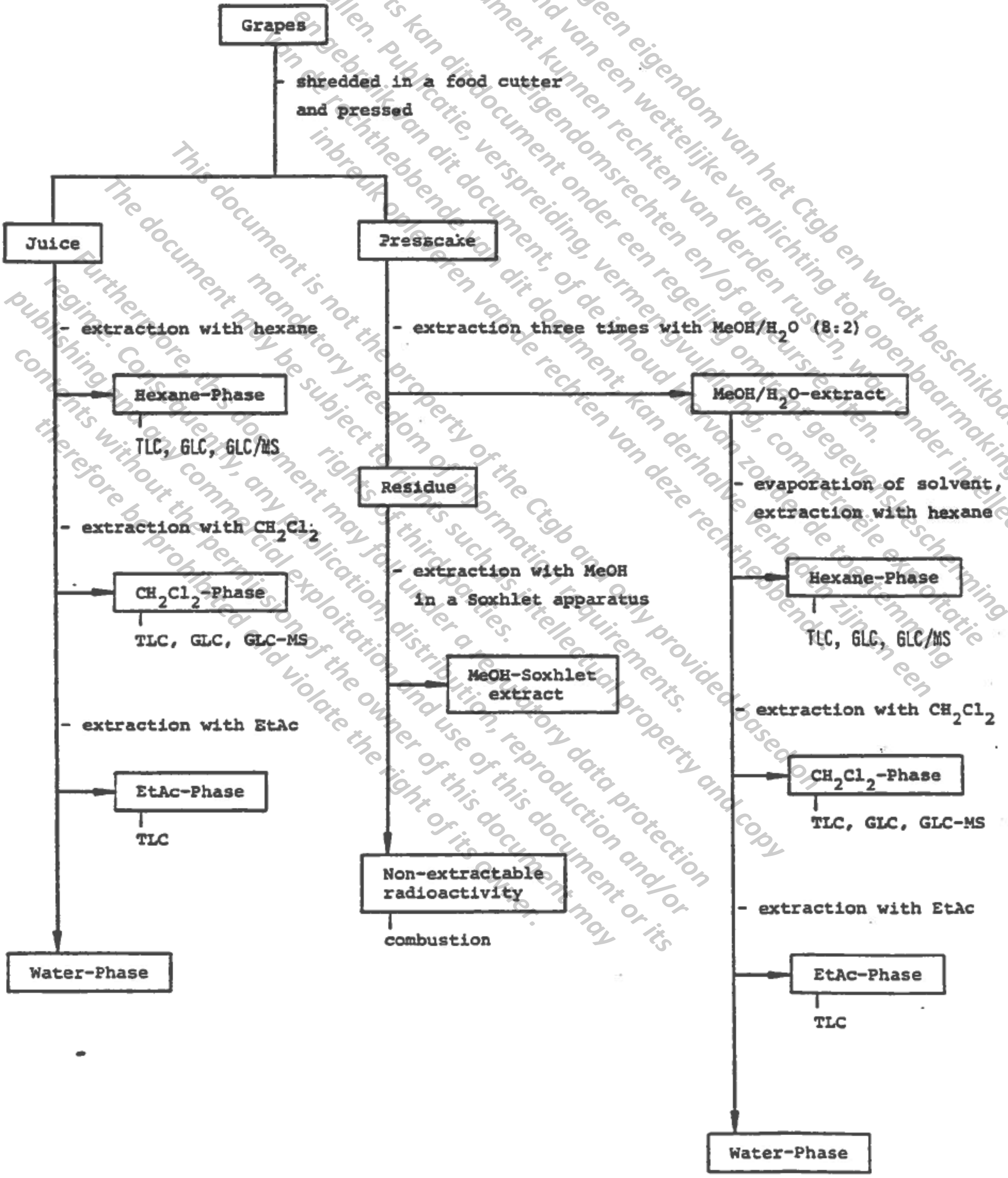
Non-labelled reference substances were synthesized by Mr. [redacted], Biochemistry, Agrochemicals Division, CIBA-GEIGY Limited, Basle, Switzerland.

Furthermore, we thank Dr. [redacted], Agrochemical Research and Development Dept., and Mr. [redacted] Analytical Department, CIBA-GEIGY Limited, Basle, for performance and interpretation of the GLC/MS analysis.

[redacted]

5. SCHEMES AND TABLES

Scheme 1 Analysis of the grapes



Scheme 2 Analysis of the leaves

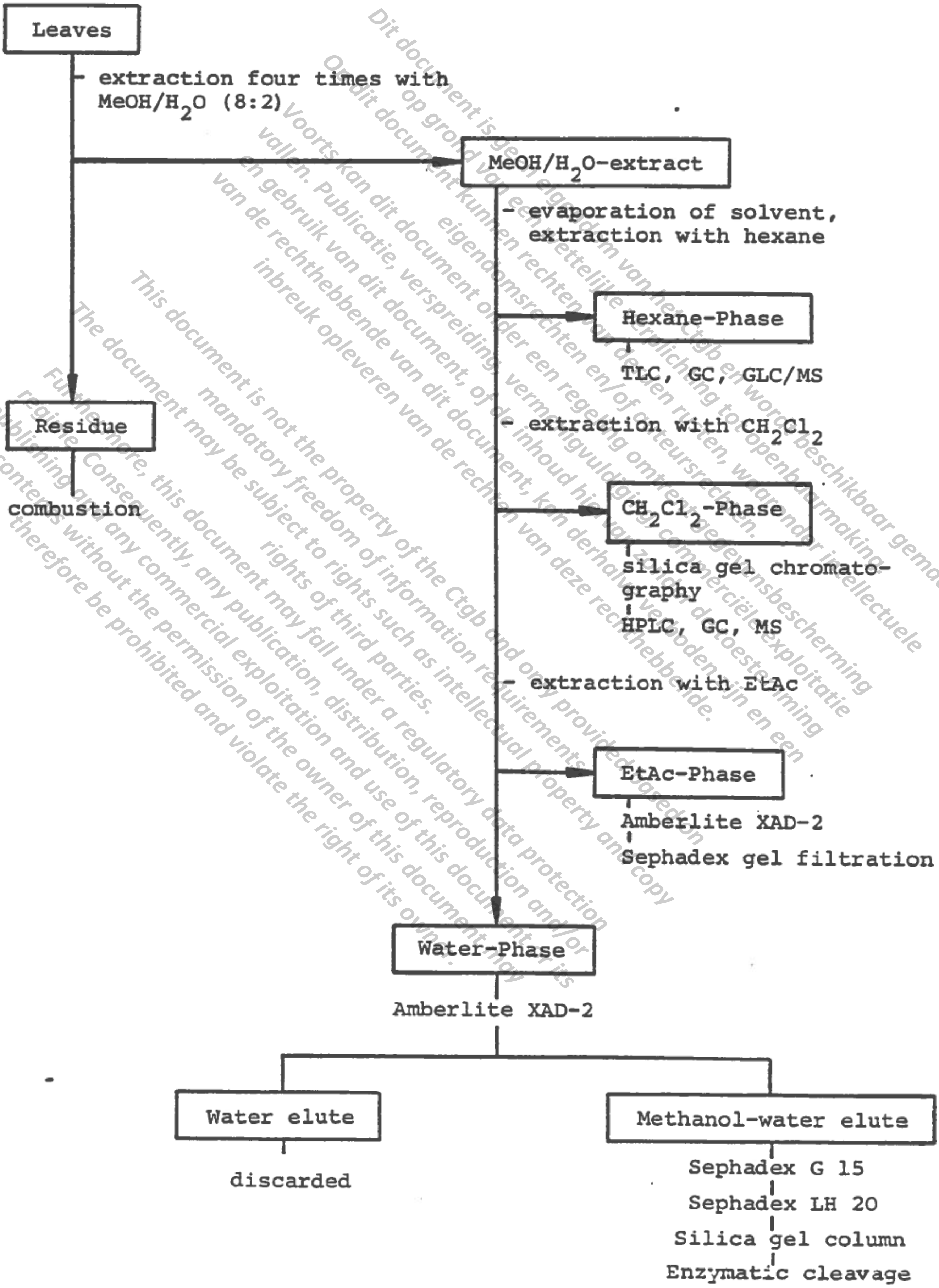


Table I Structure and code numbers of reference materials

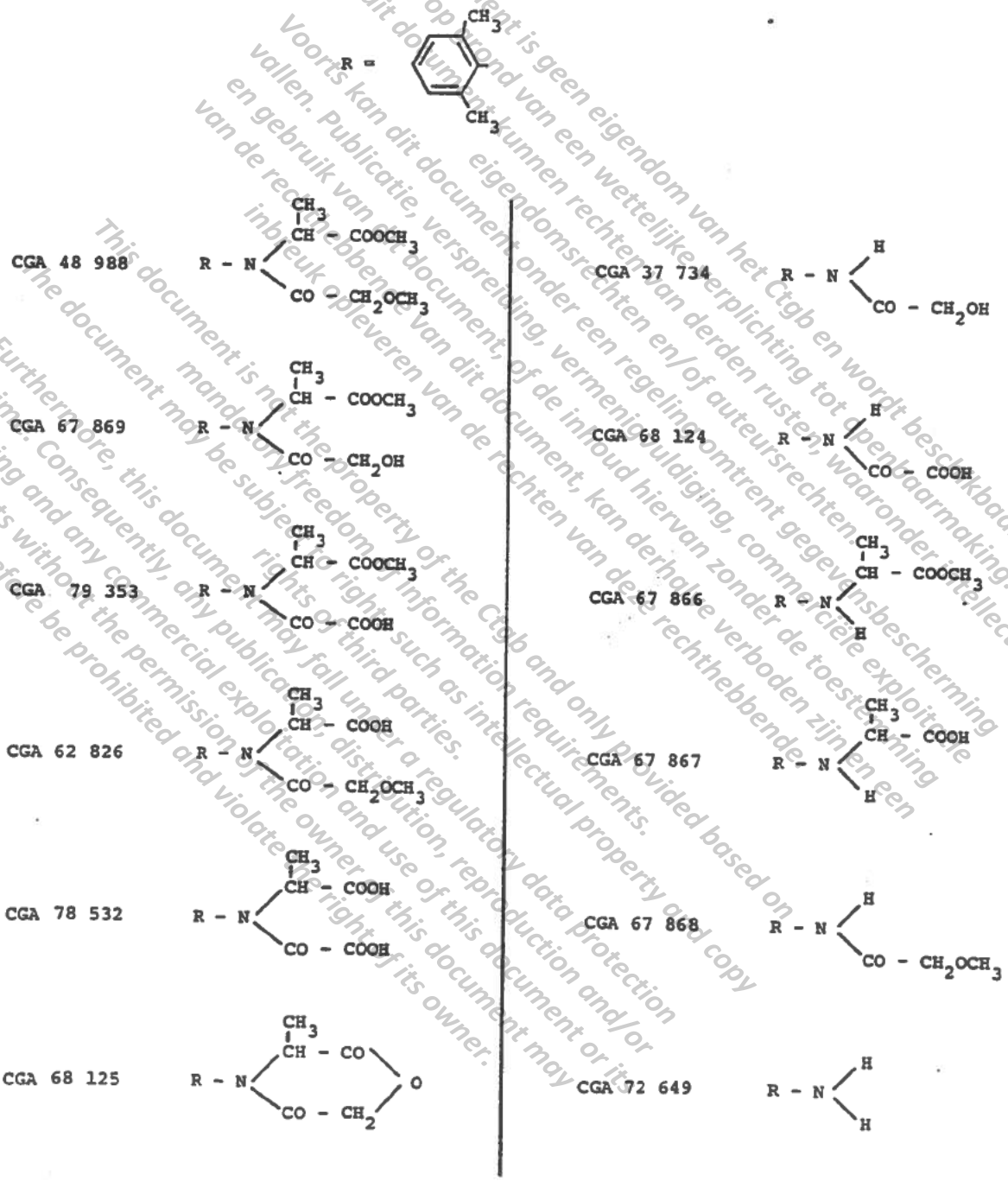


Table II Content of radioactivity in grapevine at maturity (ppm CGA 48 988 equivalents)

Grapes	Juice	1.04
	Presscake	7.31
	Total	3.06
Leaves		30.13

Table III Degradation of CGA 48 988 in grapevine (in % of the radioactivity found in grapes and leaves, respectively)

	CGA 48 988[1)]	Degradation products		
		CH_2Cl_2 and EtAc soluble	water-soluble	non-extractable
Grapes				
Juice	7.8	7.0	2.7	-
Presscake	56.3	9.8	7.0	9.4
Total	64.1	16.8	9.7	9.4
Leaves	22.4	34.2	39.1	4.2

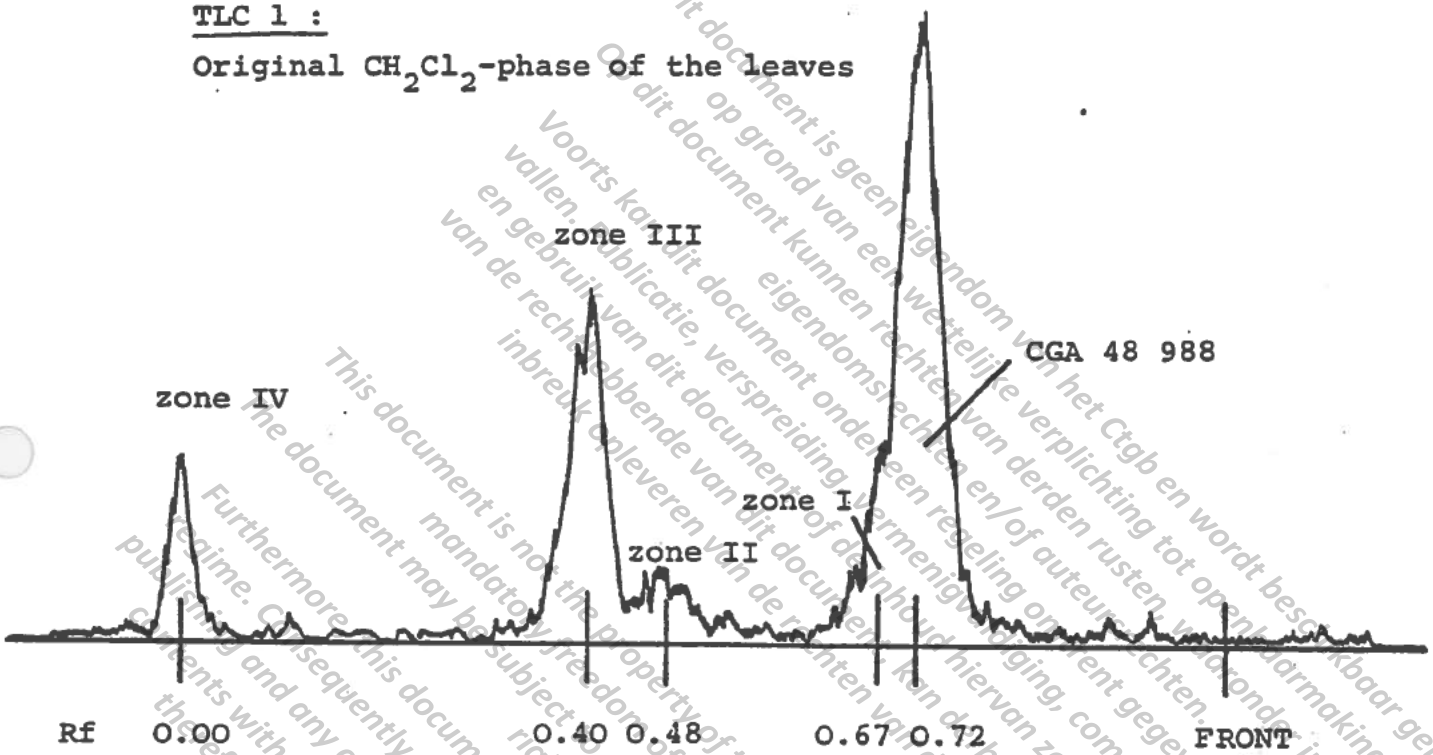
1) corresponds to 0.45, 4.98, 1.96 and 6.75 ppm in juice, presscake, whole grapes and leaves, respectively

Table IV Pattern of metabolites in grapevine at harvest time (in % of the radioactivity found in grapes and leaves, respectively)

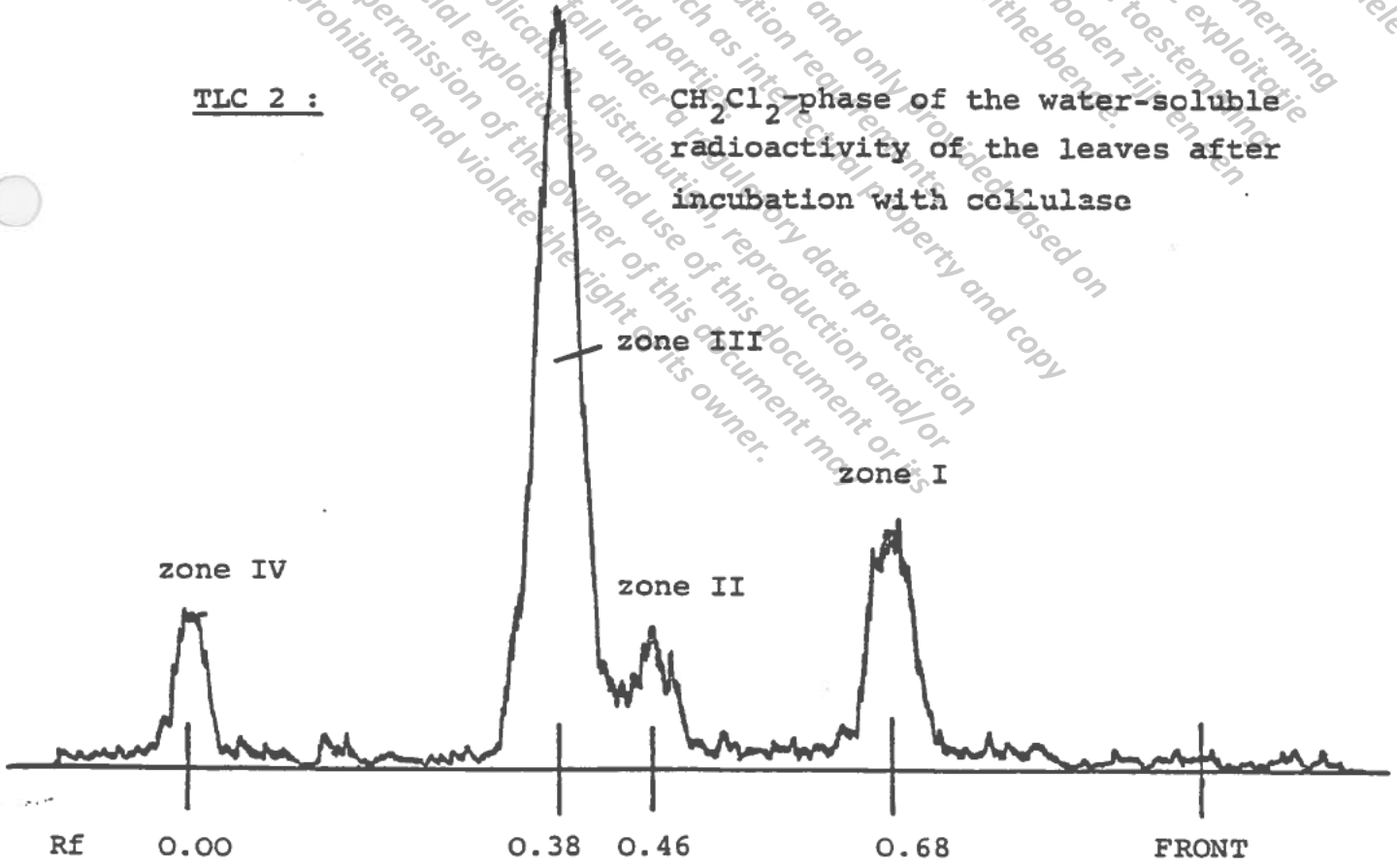
	CGA 48 988	Zone III		Zones I, II and IV		Non-extractable
		free	conjugated	free	conjugated	
Grapes						
Juice	7.8	3.5	2.2	3.5	0.5	-
Presscake	56.3	6.2	5.8	3.6	1.2	9.4
Total	64.1	9.7	8.0	7.1	1.7	9.4
Leaves	22.4	18.7	29.4	15.5	9.7	4.2

6. APPENDIX

TLC 1 :
Original CH_2Cl_2-phase of the leaves



TLC 2 :
CH_2Cl_2-phase of the water-soluble radioactivity of the leaves after incubation with cellulase



Solvent: Ethyl acetate

TLC 3 : Water-soluble radioactivity of the leaves
solvent system: 16 A