European Registration Dossier
Dossier File N°: 6.1 /04
Ciba File N°: 48988/2020

CONFIDENTIAL

BIOCHEMISTRY
DEPARTMENT R & D PLANT PROTECTION
AGRICULTURAL DIVISION
CIBA-GEIGY LIMITED
BASLE, SWITZERLAND

Dit document is eigendom van het Ctgb en wordt beschikbaar gemaakt voor openbaarmaking. Ongeoorloofd gebruik van een eigendom van een wettelijke verplichting tot openbaarmaking. Voor dit document kunnen rechten van derden rusten, waaronder intellectuele eigendomsrechten en/of auteursrechten. vallen. Publicatie, verspreiding, vermenigvuldiging, commerciële exploitatie en gebruik van dit document onder een regeling omtrent gegevensbescherming van de rechthebbende van dit document, of de inhoud hiervan zonder de toestemming inbreuk opleveren van deze rechten van deze rechthebbende. kan derhalve verboden zijn en een

This document is not the property of the Ctgb and may be subject to rights of third parties such as intellectual property and copy
The document may be subject to rights of information requirements. provided based on
mandatory freedom of information regulations
Furthermore, this document may fall under a regulatory data protection
regime. Consequently, any publication, distribution, reproduction and/or
publishing and any commercial exploitation and use of this document or its
contents without the permission of the owner of this document may
therefore be prohibited and violate the right of its owner.

PROJECT REPORT 38/80

IDENTIFICATION OF DEGRADATION PRODUCTS OF CGA 48 988 (RIDOMIL®) IN LETTUCE

(Addendum to Project Report 38/79)

5.1.2.e/Woo

AG 2.52 / sch
Basle, Switzerland, October 5, 1980

Content

Dit document is geen eigendom van het Ctgb en wordt beschikbaar gemaakt
Op dit document kunnen rechten van derden rusten, waaronder intellectuele
Voorts kan dit document ...
This document is not the property of the Ctgb ...

ABSTRACT

In further studies on the metabolism of CGA 48 988 in lettuce the following metabolite structures have been identified:

- two atropisomeric forms of N-(2-hydroxymethyl-6-methylphenyl)-N-(methoxyacetyl)-alanine methylester (22.1 % of the total radioactivity)

- N-(2,6-dimethyl-3-hydroxyphenyl)-N-(methoxyacetyl)-alanine methylester (6.2 %)

- N-(2,6-dimethylphenyl)-N-(hydroxyacetyl)-alanine methylester (8.9 %)

- 2,6-dimethyl-N-hydroxyacetyl-aniline (2.9 %)

- N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-alanine (6.0 %)

- N-(2,6-dimethylphenyl)-N-(hydroxyacetyl)-alanine (10.1 %)

- N-(2-carboxy-6-methylphenyl)-N-(methoxyacetyl)-alanine methylester (1.2 %)

All metabolites thus formed - with the exception of the benzoic acid derivative - were found partially conjugated with glucose.

Consequently, the degradation of CGA 48 988 in lettuce proceeds via

- oxydation of the phenyl ring
- oxydation of a ring methyl group
- cleavage of the methylester and methylether bonds
- N-dealkylation

The known structures account for 76 % of the radioactivity found in the aerial parts of lettuce.

Dit document is een eigendom van het Ctgb en wordt beschikbaar gemaakt op grond van een wettelijke verplichting tot openbaarmaking.
Op dit document kunnen rechten van derden rusten, waaronder intellectuele eigendomsrechten en/of auteursrechten.
Voorts kan dit document onder een regeling omtrent gegevensbescherming vallen. Openbaarmaking, vermenigvuldiging, commerciële exploitatie of gebruik van dit document, of de inhoud hiervan zonder de toestemming van de rechthebbende van dit document, kan derhalve verboden zijn en een inbreuk opleveren van de rechten van deze rechthebbende.
This document is not the property of the Ctgb and only provided based on mandatory freedom of information requirements.
The document may be subject to rights such as intellectual property and copy rights of third parties.
Furthermore, this document may fall under a regulatory data protection regime. Consequently, any publication, distribution, reproduction and/or publishing and any commercial exploitation and use of this document or its contents without the permission of the owner of this document may therefore be prohibited and violate the right of its owner.

1. INTRODUCTION

In a previous report [1] the results of a study on the fate of the fungicide CGA 48 988 (RIDOMIL®) in lettuce have been presented. The metabolites found in the plant, however, were only tentatively characterized. Therefore, additional efforts were undertaken to elucidate the genuine character of these metabolites and to determine their relative significance. The results are presented in this report.

2. MATERIAL AND METHODS

If not stated otherwise the same materials and methods were used as described in earlier experiments [1].

2.1 Thin Layer Chromatography (TLC)

TLC was performed on precoated plates of silica gel 60 F_{254}, 0.25 mm thick (E. Merck, Darmstadt, G.F.R.).

The following solvent systems were used:

ss 60	ethyl acetate
ss 79	chloroform/methanol/formic acid/water 75:20:4:2

ss 103 A ethyl acetate/acetic acid 9:1
ss 106 ethyl acetate with 1 % NH_4OH
ss 107 ethyl acetate/iso-propanol/water/acetic acid
65:25:10:2

The following Rf-values were obtained:

Compound	Solvent system 60	106	103 A	79	107
CGA 48 988	0.70	0.76	0.85	-	0.92
CGA 67 869	0.70	0.0-0.2	0.85	-	0.96
CGA 100 255	0.67	0.54	0.85	-	0.93
CGA 83 048	0.56	0.35	-	-	-
CGA 62 826	0.00	0.00	0.60	0.71	0.86
CGA 107 955	0.00	0.00	0.63	0.47	0.93
CGA 37 734	0.50	-	0.71	-	0.92
CGA 94 689 A	0.48	0.37	0.63	-	0.86
CGA 94 689 B	0.40	0.32	0.60	-	0.82
CGA 108 905	0.00	0.00	0.47	0.56	0.86
CGA 109 097	0.64	-	-	-	0.91
CGA 114 523	0.00	0.00	0.05	0.38	0.40

The Rf-values may vary to some extend. However, their relative rates of migration remain unchanged.

2.2 Gas Chromatography (GC)

GC-Analysis was performed on a Hewlett Packard Gas Chromatograph Model 5750 (Hewlett Packard, Analytical

Instruments, Avondale, Pa., USA) equipped with a RGC-170 radioactivity monitor (RAM, Perkin Elmer, Norwalk, Conn., USA).

Details of experimental conditions:

	Column A	Column B
Liquid phase:	SE 30 3 %	SP 1000 3 %
Support:	Gas-chrom Q mesh 80/100	Gas-chrom Q mesh 80/100
Material:	Glas, 1.8 m x 2 mm ID	Glas, 1.0 m x 2 mm ID

Temperatures:

	Column A	Column B
Injector:	250 °C	250 °C
Detector:	280 °C	280 °C
Column oven:	100 °C - 250 °C	120 °C - 240 °C
Program rate:	10 °C/min	5 °C/min
Flow rate of carrier gas (He):	40 ml/min	40 ml/min

The following retention times (min) were obtained:

	Column A	Column B
CGA 48 988	9.8	18.0
CGA 67 869*	9.4	22.0

Dit document is geen eigendom van het Ctgb en wordt beschikbaar gemaakt op grond van een wettelijke verplichting tot openbaarmaking. Op dit document kunnen rechten van derden rusten, waaronder intellectuele eigendomsrechten en/of auteursrechten. Voorts kan dit document onder een regeling op het gebied van gegevensbescherming vallen. Publicatie, verspreiding, vermenigvuldiging, commerciële exploitatie en gebruik van dit document of de inhoud hiervan zonder de toestemming van de rechthebbende van dit document is derhalve verboden en kan een inbreuk opleveren van de rechten van deze rechthebbende.
This document is not the property of the Ctgb and only provided based on mandatory freedom of information requirements. The document may be subject to rights such as intellectual property and copy rights of third parties. Furthermore, this document may fall under a regulatory data protection regime. Consequently, any publication, distribution, reproduction and/or publishing and any commercial exploitation and use of this document or its contents without the permission of the owner of this document may therefore be prohibited and violate the right of its owner.

	Column A
CGA 68 125	9.4
CGA 100 255	12.2
CGA 83 048	12.6
CGA 109 097	11.5
CGA 94 689	10.8
CGA 37 734	7.1

* cyclises via GLC to CGA 68 125

2.3 High Pressure Liquid Chromatography (HPLC)

HPLC was run on an Altex system Model 312 (Altex Scientific Inc., Berkeley, Ca., USA), equipped with a Kontron UV detector Model LCD 725 (Kontron Ltd., Zurich, Switzerland) and a Berthold flow cell radioactivity detector Model BF 5025 (Laboratory Dr. Berthold, Wildbad, G.F.R.).

The following general conditions were used:

LiChrosorb Si 60

Column: stainless steel, 250 x 4.6 mm i.d. filled with Si 60, 10 µm particle size (E. Merck, Darmstadt, G.F.R.). Separation of the metabolite fractions 4 through 8 was achieved using a linear gradient of ethanol in hexane (20 % → 40 %). Each individual fraction was further purified on the same column using a linear gradient of acetonitrile in methylene chloride (5 % → 35 %).

Dit document is een eigendom van het Ctgb en wordt beschikbaar gemaakt op grond van een wettelijke verplichting tot openbaarmaking. Op dit document kunnen rechten van derden rusten, waaronder intellectuele eigendomsrechten en/of auteursrechten. Voorts kan dit document onder een regeling omtrent gegevensbescherming vallen. Publicatie, verspreiding, vermenigvuldiging, commerciële exploitatie en gebruik van dit document, of de inhoud hiervan, zonder toestemming van de rechthebbende van dit document zijn verboden en kan een inbreuk opleveren van de rechten van de rechthebbende.
The document is not the property of the Ctgb ... regulatory data protection regime. Consequently, any publication, distribution, reproduction and/or publishing and any commercial exploitation and use of this document or its contents without the permission of the owner of the rights of this document may therefore be prohibited and violate the rights of its owner.

<u>LiChrosorb RP-18</u>

Column: stainless steel, 250 x 4.6 mm i.d. filled with RP-18, 5 µm particle size material ([redacted], Darmstadt, G.F.R.). The reverse phase HPLC was used for the separation of the metabolite fractions 1 through 3 and for the water soluble radioactivity (fractions I through V) using a linear gradient of acetonitrile in water (0 % → 50 %). Each individual fraction was further purified on this column using a linear gradient of isopropanol/acetonitrile 3:1 in water (0 % → 45 %).

<u>LiChrosorb Diol</u>

Column: stainless steel, 250 x 4.6 mm i.d. filled with Diol, 10 µm particle size material [redacted], Darmstadt, G.F.R.). The column was used for the purification of the methylated and/or acetylated derivatives of the metabolite fractions 1 through 3 and of the conjugate fractions I through V using a linear gradient of chloroform/ethanol 95:5 in hexane (0 % → 50 %).

2.4 <u>Liquid Chromatography (LC)</u>

Column: glas, 20 mm i.d. filled to a height of 150 mm with silica gel ([redacted] G.F.R.) activity grade I, 100 - 200 µm particle size

Eluent:	1. ethyl acetate	(400 ml)
	2. ethanol with 2 % acetic acid	(150 ml)

2.5 Chemical reactions

Enzymatic cleavage of the glucose conjugates was performed in citrate-phosphate buffer at pH 5.0 (0.1 M citric acid and 0.2 M disodium phosphate 1:1) with cellulase 5.1.2.e Woo, Darmstadt, G.F.R.) (2000 Unit/g) by shaking the mixture for 5 hours at 38 °C.

Acetylation was performed with Ac_2O/pyridine (2:8) overnight at room temperature.

Methylation reactions were carried out in an ethereal solution of diazomethane for 2 hours at room temperature. The metabolites were first dissolved in ethylacetate.

2.6 Spectroscopic methods

Mass spectra of the glucose conjugates were obtained using the solid probe inlet of a Finnigan instrument Model 4000 (Finnigan, Sunnyvale, Calif. USA).

For GLC/MS the Model 4000 instrument was coupled with a Finnigan Gas Chromatograph Model 9610. All MS-spectra were run in the EI mode.

3. RESULTS AND DISCUSSION

As shown in Figure 1 nearly 77 % of the radioactivity present in the green parts were extractable with methanol/water 8:2. This radioactivity was further characterized as hexane, methylene chloride and water soluble metabolites.

3.1 Identification of the organo-soluble metabolites

As shown by TLC, GC and MS analysis, the hexane phase contained the unchanged fungicide exclusively (18.6 % of the total radioactivity).

Two-dimensional TLC of the methylene chloride phase in solvent systems 60/103A revealed the presence of at least 8 different polar metabolites (Figure 4, metabolite Zone 1 through 8). The methylene chloride soluble radioactivity was further fractionated and analysed according to Figure 2.

Metabolite identification

Fraction 1 (3.0 % of the total radioactivity) behaves electrophoretically as a neutral compound. It was analysed by MS (direct probe inlet, EI) after derivatization with Ac_2O/pyridine.

The spectrum shows the following main features: highest fragment at m/e 595 ($M^+ - CH_2O$), major fragments at m/e 294 (benzyl alcohol derivative) and

m/e 331 (tetraacetyl glucose oxonium ion).
These features are characteristic for the glucoside conjugate of CGA 94 689.

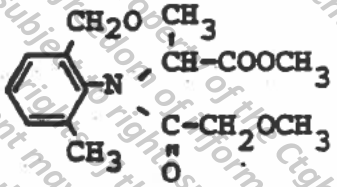
Fragmentation:

m/e 595 $M^+ - CH_2O$

580 $M^+ - CH_2OCH_3$

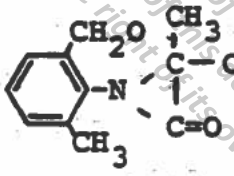
538 $M^+ - CH_3\text{-}\dot{C}H\text{-}COOCH_3$

331 tetraacetyl glucose oxonium ion

294 

264 $294 - CH_2O$

262 $294 - CH_3OH$

248 

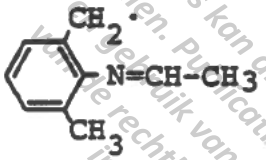
234 $294 - HCOOCH_3$

218 $294 - O{=}C{=}CH\text{-}OCH_3$

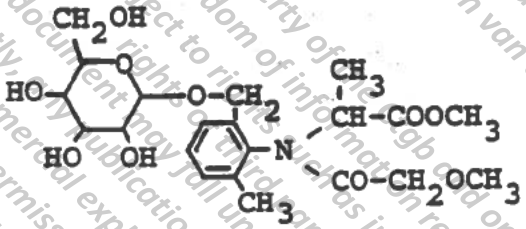
Het document is geen eigendom van het Ctgb en wordt beschikbaar gemaakt op grond van een wettelijke verplichting tot openbaarmaking. Op dit document kunnen rechten van derden rusten, waaronder intellectuele eigendomsrechten en/of auteursrechten. Voorts kan dit document onder een regeling omtrent gegevensbescherming valen. Publicatie, verspreiding, vermenigvuldiging, commerciële exploitatie en gebruik van dit document, of de inhoud hiervan zonder de toestemming van de rechthebbende van dit document, kan derhalve verboden zijn en een inbreuk opleveren op de rechten van deze rechthebbende.
This document is not the property of the Ctgb and only provided based on mandatory freedom of information requirements. The document may be subject to rights such as intellectual property and copyright of third parties.
The document may full under a regulatory data protection regime. Furthermore, this document may be subject to rights such as intellectual property and copyright of third parties. Consequently, any publication, distribution, reproduction and/or commercial exploitation and use of this document or its contents without the permission of the owner of its document may therefore be prohibited and violate the rights of its owner.

207 294 - CH_3-CH-$COOCH_3$

190 294 - $COOCH_3$ + CH_2OCH_3

146 

The corresponding derivative of the reference compound CGA 114 523 shows identical spectra and confirms the structure of fraction 1 as the O-glucoside derivative of CGA 94 689



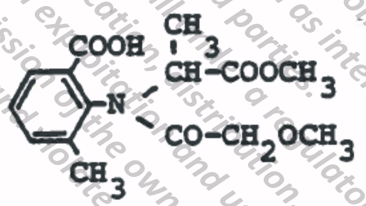
Fraction 2 (1.2 % of the total radioactivity) behaves electrophoretically like an acid. It co-chromatographed on TLC with the reference compound CGA 108 905 and on GC, after methylation, with the reference compound CGA 109 097 (methylester of CGA 108 905). GC/MS of the methylated metabolite fraction in the EI mode shows a molecular ion at m/e 323 and a base peak at m/e 160, characteristic for the benzoyl acid moiety.

Dit document is geen eigendom van het Comité en wordt beschikbaar gemaakt op grond van een wettelijke verplichting tot openbaarmaking. Op dit document kunnen rechten van derden rusten, waaronder intellectuele eigendomsrechten en/of auteursrechten. Voorts kan dit document onder een regeling omtrent gegevensbescherming vallen. Publicatie, verspreiding of enige openbaarmaking, commerciële exploitatie of gebruik van dit document of de inhoud hiervan zonder de toestemming van de rechthebbende(n) is niet toegestaan. Door inbreuk op eventuele rechten van derden, kan derhalve verboden zijn en een This document is not the property of [illegible] and only provided based on mandatory freedom of information requirements. The document may be subject to rights of third parties, such as intellectual property and copyright. Furthermore, this document may fall under a regulatory regime. Consequently, any publication, distribution, reproduction, commercial exploitation and use of this document and/or its contents without the permission of the owner of this document or its author may therefore be prohibited and violate the rights of the owner or author.

Fragmentation:

m/e	323	M^+
	264	M^+ - $COOCH_3$
	234	264 - CH_2=O
	204	264 - $HCOOCH_3$
	192	264 - O=C=$CHOCH_3$
	174	204 - CH_2=O
	160	192 - CH_3OH
	132	174 - CH_2=C=O

An identical fragmentation pattern was found for the synthetic reference compound CGA 109 097. Therefore, fraction 2 has the structure:

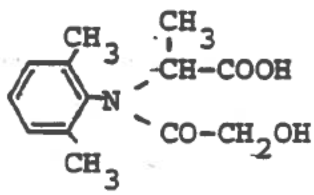


<u>Fraction 3</u> (8.5 % of the total radioactivity) was separated on a reverse phase HPLC column in two metabolite fractions, designated as fractions 3a and 3b.

Fraction 3a (3.9 %) behaves electrophoretically like an acid. It co-chromatographed on TLC in a two-dimensional solvent system (103A/79) with CGA 107 955. MS-analysis of the metabolite was achieved by GC/MS of the methyl/acetyl derivative in the EI mode. The spectrum shows the following main features: a molecular ion at m/e 307 and a base peak at m/e 148 (characteristic for the intact 2,6-dimethylphenyl moiety)

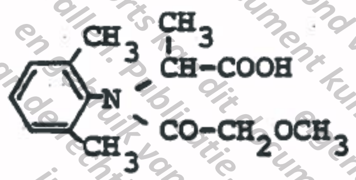
m/e 307	M^+
276	$M^+ - CH_2OH$
248	$M^+ - COOCH_3$
234	$M^+ - CH_2O\text{-}COCH_3$
206	$M^+ - CO\text{-}CH_2O\text{-}COCH_3$
186	
174	$206 - CH_3OH$
158	
148	$206 - CO{=}CHOH$
146	$174 - CO$

The corresponding derivative of the reference compound CGA 107 955 shows identical mass spectra and confirms the structure of fraction 3a as the acid-alcohol derivative



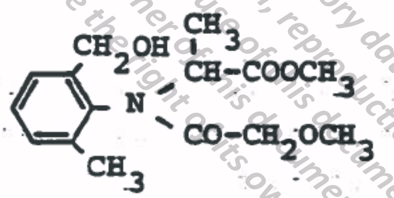
Dit document is geen eigendom van het Ctgb en wordt beschikbaar gemaakt op grond van de wettelijke verplichting tot openbaarmaking. Op dit document kunnen intellectuele eigendomsrechten van derden rusten, waaronder intellectuele eigendomsrechten en/of auteursrechten. Voorts kan dit document onder een regeling omtrent gegevensbescherming vallen. Publicatie, verspreiding, vermenigvuldiging, commerciële exploitatie en/of gebruik van dit document, of de inhoud hiervan zonder de toestemming van de rechthebbende van dit document, kan derhalve verboden zijn en een inbreuk opleveren van de rechten van deze rechthebbende.
This document is not the property of the Ctgb and only provided based on mandatory freedom of information requirements. The document may be subject to rights such as intellectual property and copy rights of third parties. Furthermore, this document may fall under a regulatory data protection regime. Consequently, any publication, distribution, reproduction and/or publishing and any commercial exploitation and use of this document or its contents without the permission of the owner of the rights of this document may therefore be prohibited and violate the rights of its owner.

Fraction 3b (4.6 %) co-chromatographed in the two-dimensional system 103 A/79 with CGA 62 826, i.e. N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-alanine:



To confirm its structure the metabolite was converted to its methylester and submitted to TLC, GC and GC/MS. In all systems the methylester derivative behaved identically to the parent compound CGA 48 988.

Fractions 4 and 5 (4.5 % and 0.6 % of the total radioactivity) co-chromatographed on TLC with the two atropisomeric forms of CGA 94 689, i.e. N-(2-methyl-6-hydroxymethylphenyl)-N-(methoxyacetyl)-alanine methylester:



The identity of the metabolite fractions with the synthetic reference compound CGA 94 689 was confirmed by GC/MS (EI). The mass spectra obtained shows a

molecular ion at m/e 263 and a base peak at m/e 146 (characteristic for the hydroxy benzylic moiety). The M^+ 263 fragment can be explained by an intramolecular rearrangement during GC separation involving cleavage of methanol and formation of a lactone:

M = 263

For both, the metabolites and the reference compound, the following fragments were obtained:

m/e 263	M^+
233	
232	$M^+ - CH_2O/CH_2OH/CH_3OH$
231	
219	$M^+ - CO_2$
174	$219 - CH_2OCH_3$
160	$174 - CH_3$
<u>146</u>	$174 - CO$

Fraction 6 (1.2 % of the total radioactivity) behaved on TLC and GC like CGA 37 734. The mass spectrum of the metabolite (GC/MS, EI mode) shows a molecular ion at m/e 179 and a base peak at m/e 148.

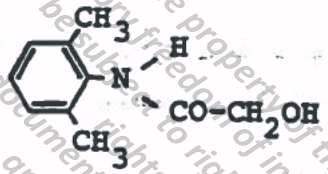
Dit document is geen eigendom van het Ctgb en wordt beschikbaar gemaakt op grond van een wettelijke verplichting tot openbaarmaking. Op dit document kunnen rechten van derden rusten, waaronder intellectuele eigendomsrechten en/of auteursrechten. Voorts kan dit document onder een regeling omtrent gegevensbescherming vallen. Publicatie, verspreiding, vermenigvuldiging, commerciële exploitatie en gebruik van dit document, of de inhoud hiervan zonder de toestemming van de rechthebbende van dit document zijn derhalve verboden en een inbreuk opleveren van de rechten van deze rechthebbende.
This document is not the property of the Ctgb and only provided based on mandatory freedom of information requirements. The document may be subject to rights such as intellectual property and copy rights of third parties. Furthermore, this document may fall under a regulatory data protection regime. Consequently, any publication, distribution, reproduction and/or publishing and any commercial exploitation and use of this document or its contents without the permission of the owner of this document may therefore be prohibited and violate the rights of its owner.

The following fragments were obtained:

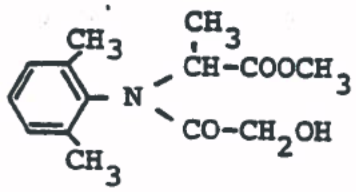
m/e	179	M^+
	<u>148</u>	$M - CH_2OH$
	121	M - CO=CH-OH
	120	$M - CO\text{-}CH_2OH$

An identical fragmentation pattern is found for the synthetic reference compound CGA 37 734.

Therefore, fraction 6 has the structure



Fraction 7 (25.8 % of the total radioactivity) consisted of three different polar fractions as demonstrated by TLC in solvent system 106. This mixture was subjected to HPLC on silica gel. Using a linear gradient of ethanol in hexane two peaks were eluted, one of which (7b) as a radiochemically pure compound, the other (7a), as a composite fraction containing two metabolites. The two metabolites present in fraction 7a were identified by GC/MS (after separation on a SP-1000 column) as the parent compound CGA 48 988 (18.6 %) and CGA 67 869 (4.5 %), i.e.



Dit document is geen eigendom van het Ctgb en wordt beschikbaar gemaakt op grond van een wettelijke verplichting tot openbaarmaking. Op dit document kunnen rechten van derden rusten, waaronder intellectuele eigendomsrechten en/of auteursrechten. Voorts kan het document onder een regeling omtrent gegevensbescherming vallen. Publicatie, verspreiding, vermenigvuldiging, commerciële exploitatie en gebruik van dit document, of de inhoud hiervan, kan derhalve verboden zijn zonder de toestemming van de rechthebbende en een inbreuk opleveren van de rechten van deze rechthebbende.
This document is not the property of the Ctgb and only provided on mandatory freedom of information requirements. The document may be subject to rights such as intellectual property rights of third parties. Furthermore, this document may fall under a regulatory data protection regime. Consequently, any publication, distribution, reproduction and/or publishing and any commercial exploitation and use of this document or its contents without the permission of the owner of this document may therefore be prohibited and violate the rights of its owner.

The mass spectra of CGA 67 869 shows a molecular ion at m/e 233 and a base peak at m/e 132.
The loss of 32 amu indicates a ring closure reaction during the GC separation, which leads to the formation of the lactone CGA 68 125.

Fragmentation:

m/e	233	M^+
	218	$M^+ - CH_3$
	175	$M^+ - CO_2CH_2$
	174	$M^+ - CO_2CH_3$
	160	$174 - CH_3$
	147	$M^+ - CO_2/COCH_2$
	<u>132</u>	$147 - CH_3$

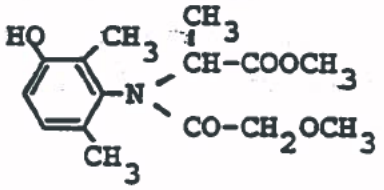
<u>Fraction 7b</u> (2.7 %) behaved on GC and TLC (solvent system 106) like CGA 100 255, the 3-hydroxyphenyl derivative of CGA 48 988. The identity of the metabolite fraction with the synthetic reference compound CGA 100 255 was confirmed by GC/MS (EI).

As shown below the MS-fragmentation pattern confirms the identity of the two compounds. One notices that the fragmentation pattern of the 4-hydroxy isomer CGA 83 048, which behaves differently on TLC and GC, is identical. Solely, the relative peak intensities are different, especially for the fragments 148, 158, 162, 176, 190 and 250. Base peak at m/e 130 is characteristic for both phenolic structures.

The following table compares the fragmentation pattern and relative peak intensities of the two hydroxy isomers and metabolite fraction 7b.

		relative intensities		
m/e		fraction 7b	CGA 100 255	CGA 83 048
295	M^+	8	6	6
250	$M^+ - \dot{C}H_2OCH_3$	39	27	4
236	$M^+ - \dot{C}OOCH_3$	17	14	7
222	$M^+ - \dot{C}O\text{-}CH_2OCH_3$	60	48	25
208	$M^+ - CH_3\text{-}\dot{C}H\text{-}COOCH_3$	24	21	14
190	$236 - \dot{C}H_2OCH_3$	45	38	9
176	$208 - CH_3OH$	72	65	7
164	$236 - O{=}C{=}CHOCH_3$	33	32	14
162	190 - CO	86	83	43
158		25	30	82
148	$163 - CH_3$	88	86	54
130	base peak	100	100	100

Therefore, fraction 7b has the structure



Dit document is gepubliceerd onder eigendomsrechten van het Ctgb en wordt beschikbaar gemaakt op grond van een wettelijke verplichting tot openbaarmaking. Op dit document kunnen rechten van derden rusten, waaronder intellectuele eigendomsrechten en/of auteursrechten. Voorts kan dit document onder een regeling omtrent gegevensbescherming vallen. Publicatie, verspreiding, vermenigvuldiging, commerciële exploitatie en gebruik van dit document, of de inhoud hiervan zonder de toestemming van de rechthebbende van dit document, kan derhalve verboden zijn en een inbreuk opleveren van de rechten van deze rechthebbende.
This document is notthe property of the Ctgb and only provided based on the document may be subject to rights such as intellectual property and copy-right. Furthermore, this document may fall under a regulatory data protection regime. Consequently any publication, distribution, reproduction and/or commercial exploitation and use of this document or its contents without the permission of the owner of this document may therefore be prohibited and violate the right of its owner.

Fraction 8 (0.4 % of the total radioactivity) did not migrate with any of the reference substances. Due to the small amount available it was not further characterized.

3.2 Identification of the water soluble radioactivity

Two-dimensional TLC of the water soluble radioactivity in solvent systems 107/79 revealed the presence of at least 5 different polar metabolites (Figure 4, Fractions I through V).

The water soluble radioactivity was further fractionated and analysed according to Figure 3.

Metabolite identification

Fraction I (6.2 % of the total radioactivity) was found to be an acidic compound, migrating by HVE at pH 7 and 4 but not at pH 2. Its nature as a conjugate was evidenced by the release of the hydroxy-acid derivative CGA 107 955 as pesticide moiety upon cellulase cleavage.

MS-analysis of the conjugate was achieved by direct probe inlet of the acetyl/methyl derivative in the EI mode.

The spectrum shows the following main features: a molecular ion at m/e 595 and major fragments at m/e 331 (tetraacetyl glucose oxonium ion) and m/e 294 (aglycone).

m/e 595 M^+

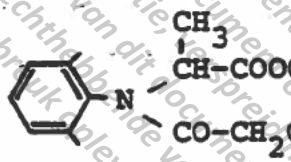
564 $M^+ - OCH_3$

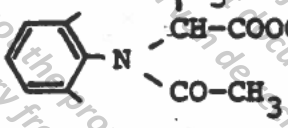
536 $M^+ - \cdot COOCH_3$

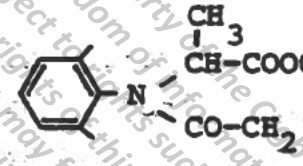
535 $M^+ - HCOOCH_3/CH_3COOH$

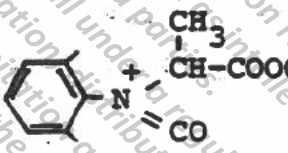
475 535 - $HCOOCH_3/CH_3COOH$

331 tetraacetyl glucose oxonium ion

294 

249 

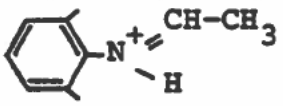
248 

234 

206 249 - $\cdot COCH_3$

190 249 - $\cdot COOCH_3$

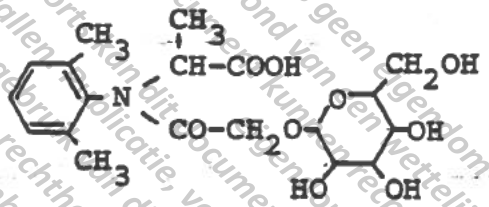
160 248 - $CH_3-CH_2-COOCH_3$

148 

Dit document is geen eigendom van het Ctgb en wordt beschikbaar gemaakt onder voorbehoud van eventuele wettelijke verplichting tot openbaarmaking. Op dit document kunnen rechten van derden rusten, waaronder intellectuele eigendomsrechten en/of auteursrechten. Voortzetting van de verspreiding, vermenigvuldiging, commerciële exploitatie en gebruik van dit document, of de inhoud hiervan zonder de toestemming van de rechthebbende(n) van dit document, kan derhalve verboden zijn en een inbreuk opleveren op de rechten van deze rechthebbende.

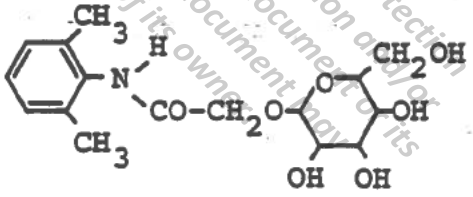
This document is not the property of the Ctgb and only provided based on mandatory freedom of information requirements. The document may be subject to intellectual property and copy rights of third parties such as regulatory data protection. Furthermore, this document may fall under various regulatory data protection regime. Consequently, any publication, distribution, reproduction and/or publishing and any commercial exploitation and use of this document or its contents without the permission of the owner of this document may therefore be prohibited and violate the right of ownership.

This MS-fragmentation pattern is characteristic for the O-tetraacetylglucoside of CGA 67 869. Therefore, the following structure is proposed for fraction I



Fraction II (1.7 % of the total radioactivity) behaves electrophoretically as a neutral compound. Enzymatic cleavage by cellulase liberated a pesticide moiety, which was found to be identical with CGA 37 734. It was analysed by MS (direct probe inlet, EI) after derivatization with Ac_2O/pyridine.

The spectrum shows a molecular ion at m/e 509 and major fragments at m/e 331 (tetraacetyl glucose oxonium ion) and m/e 179 (2,6-dimethyl-N-hydroxyacetyl-aniline). The MS-data are compatible with the tetraacetyl derivative of



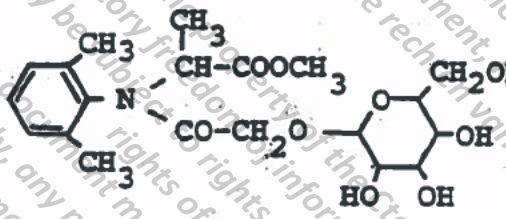
Fraction III (5.8 % of the total radioactivity) was resolved by reverse phase HPLC in two metabolite

fractions, designated as fractions III a (4.4 %) and III b (1.4 %).

Enzymatic cleavage of fraction III a by cellulase liberated a pesticide moiety, which was found to be identical with CGA 67 869.

MS-analysis of the conjugate was achieved by direct probe inlet of the acetyl derivative in the EI mode. The fragmentation pattern was identical with the one found for the methyl/acetyl derivative of fraction I.

Fraction III a has therefore the structure:



Enzymatic cleavage of fraction III b by cellulase liberated CGA 62 826 as pesticide moiety. The conjugate was analysed by MS (direct probe inlet, EI) after derivatization with Ac_2O/pyridine.

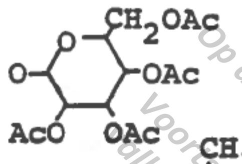
The spectrum shows the following main features: highest fragment at m/e 565, major fragments at m/e 347 (sugar moiety) and m/e 248 (pesticide moiety).

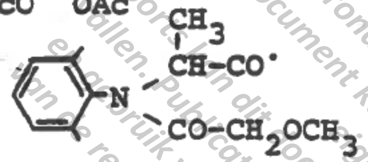
Fragmentation:

m/e 565 $M^+ - CH_2O$

509 $M^+ - O{=}C{=}CH{-}OCH_3$

Dit document is geen eigendom van de verplichting tot openbaarmaking. Op dit document kunnen rechten van derden rusten, waaronder intellectuele eigendomsrechten. Publicatie, verspreiding, vermenigvuldiging, commerciële exploitatie of gebruik van de inhoud hiervan zonder de toestemming van de rechthebbende van het document is niet toegestaan. This document is not the property of the tgb and only provided based on mandatory freedom of information legislation. The document may be subject to rights of third parties. Furthermore, this document may not be published, reproduced, distributed and/or regime. Consequently, any publication, distribution and use of this document or its contents without the permission of the owner of this document may therefore be prohibited and may violate the rights of the owner.

347 

248 

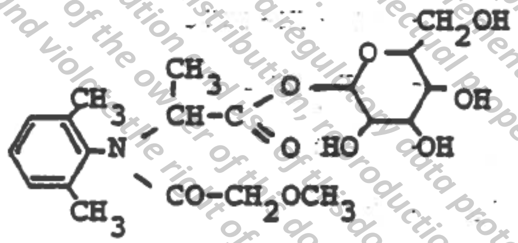
220 248 - Ċ=O

192 248 - CH_3-CH=CO

160 192 - CH_3OH

148 220 - CO=CH-OCH_3

The corresponding derivative of the reference compound CGA 119 860 shows practically identical spectra and confirms the structure of fraction III b as the glucose ester conjugate of N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-alanine.



<u>Fraction IV</u>(3.5 % of the total radioactivity) was obtained in a pure state. Enzymatical cleavage by cellulase liberated a pesticide moiety, which was found to be identical with the ring-hydroxylated

Dit document is geen eigendom van het Ctgb en wordt beschikbaar gemaakt op grond van een wettelijke verplichting tot openbaarmaking. Op dit document kunnen rechten van derden rusten, waaronder intellectuele eigendomsrechten en/of auteursrechten. Voorts kan dit document onder een regeling omtrent de bescherming vallen. Publicatie, verspreiding, vermenigvuldiging en/of exploitatie en gebruik van dit document of de inhoud hiervan zonder de toestemming van de rechthebbende kan derhalve een inbreuk opleveren van de rechten van de rechthebbende.
This document is not the property of the Ctgb and only provided based on mandatory freedom of information requirements. The document may be subject to third party intellectual property and copy rights. Furthermore, this document may be subject to a regulatory data protection regime. Consequently, any publication, distribution, reproduction and/or publishing and any commercial exploitation and use of this document or its contents without the permission of the owner of this document may therefore be prohibited and violate the rights of its owner.

derivative CGA 100 255.

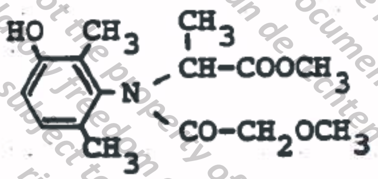
MS-analysis of the conjugate was achieved by direct probe inlet of the acetylated form in the EI mode:

The acetyl derivative showed no molecular ion (highest fragment at m/e 595) but major fragments at m/e 331 (tetraacetyl glucose oxonium ion) and m/e 295 (aglycone).

Fragmentation:

m/e 595 $M^+ - CH_2O$

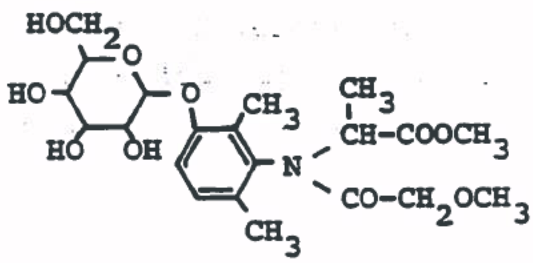
331 tetraacetyl glucose oxonium ion

295 

(aglycone + H)

250	295 - $\cdot CH_2OCH_3$	
236	295 - $\cdot COOCH_3$	
222	295 - $\cdot COCH_2OCH_3$	
208	295 - CH_3-$\dot{C}H$-$COOCH_3$	
190	236 - $\cdot CH_2OCH_3$	
176	208 - CH_3OH	
162	190 - CO	

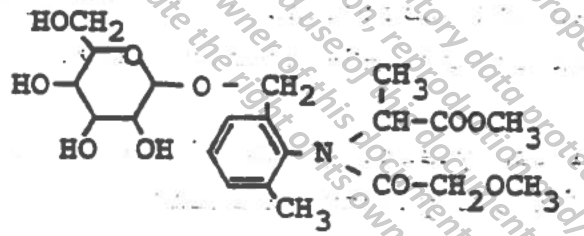
The MS-fragmentation pattern is well compatible with the tetraacetyl derivative of



Dit document is geen eigendom van het Ctgb en wordt beschikbaar gemaakt op grond van een wettelijke verplichting tot openbaarmaking. Op dit document kunnen rechten van derden rusten, waaronder intellectuele eigendomsrechten en/of auteursrechten. Voorts kan dit document onder een regeling omtrent gegevensbescherming vallen. Publicatie, verspreiding, vermenigvuldiging, commerciële exploitatie en ieder gebruik van dit document of de inhoud hiervan zonder de toestemming van de rechthebbende kan derhalve verboden zijn en een inbreuk opleveren van de rechten van deze rechthebbende.
This document is not the property of the Ctgb and only provided based on mandatory information requirements. The document may be subject to rights such as intellectual property and copy rights of third parties. Furthermore, this document may fall under a regulatory data protection regime. Consequently, any publication, distribution, reproduction and/or publishing and any commercial exploitation and use of this document or its contents without the permission of the owner of this document may therefore be prohibited and violate the rights of its owner.

<u>Fraction V</u> (17.0 % of the total radioactivity) was the major metabolite fraction of the water soluble radioactivity. Enzymatic cleavage by cellulase liberated a pesticide moiety, which consisted of two compounds in a ratio 9:1. This aglycones were found to be identical on TLC with the two atropisomeric forms of the benzylic alcohol derivative <u>CGA 94 689.</u>

MS-analysis of the conjugate was achieved by direct probe inlet of the acetylated form in the EI mode. The spectrum shows no molecular ion (highest fragment at m/e 595) but major fragments at m/e 294 (benzylic alcohol derivative) and m/e 331 (tetraacetyl glucose oxonium derivative). The fragmentation pattern was identical with that of metabolite fraction 1 (a small part of the conjugate had already been extracted with methylene chloride) and the synthetic reference compounds CGA 114 524/CGA 114 525, thus confirming the structure of fraction V as the two isomeric forms of the O-sugar conjugates of CGA 94 689



4. CONCLUSION

The metabolic pathways for the degradation of CGA 48 988 elucidated in this study in lettuce are represented in Figure 5.

Degradation of CGA 48 988 proceeds via

- hydroxylation of the aromatic ring yielding CGA 100 255, i.e. N-(2,6-dimethyl-3-hydroxyphenyl)-N-(methoxyacetyl)-alanine methylester

- oxydation of a ring methyl group yielding CGA 94 689, i.e. N-(2-hydroxymethyl-6-methylphenyl)-N-(methoxy-acetyl)-alanine methylester and subsequently CGA 108 905, i.e. N-(2-carboxy-6-methylphenyl)-N-(methoxyacetyl)-alanine methylester

- cleavage of the methylester and methylether bonds yielding CGA 62 826, i.e. N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-alanine and CGA 67 869, i.e. N-(2,6-dimethylphenyl)-N-(hydroxyacetyl)-alanine methylester and subsequently CGA 107 955, i.e. N-(2,6-dimethylphenyl)-N-(hydroxyacetyl)-alanine and

- N-dealkylation yielding CGA 37 734, i.e. 2,6-dimethyl-N-hydroxyacetyl-aniline.

All the metabolites thus formed - with exception of the benzoic acid derivative CGA 108 905 - were efficiently conjugated with glucose.

5. ACKNOWLEDGEMENT

The skilled technical assistance of Miss [redacted] is gratefully acknowledged. Thanks are due to Dr. [redacted] and Mr. [redacted] Residue Analysis, Agrochemicals Division, for the MS analysis.

Labelled CGA 48 988 as well as the non-labelled reference substances were synthesized by Dr. [redacted] Agricultural Division, CIBA-GEIGY Corporation, Greensboro, N.C., USA.

6. REFERENCES

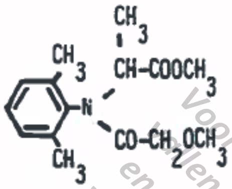
[1] Fate of CGA 48 988 in Lettuce, [redacted] CIBA-GEIGY LTD., Internal Project Report 38/79

This document is not the property of the Ctgb and may be subject to rights such as intellectual property and copy rights of third parties. The document may be subject to mandatory freedom of information requirements and only provided based on requirements. Furthermore, this document may fall under a regulatory data protection regime. Consequently, any publication, distribution, reproduction and/or publishing and any commercial exploitation and use of this document or its contents without the permission of the owner of this document may therefore be prohibited and violate the right of its owner.

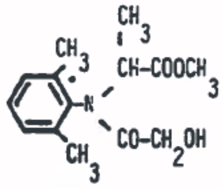
7. TABLES AND FIGURES

Table 1 Structures of reference compounds

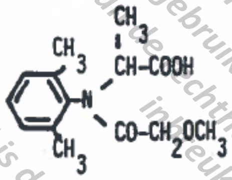
CGA 48 988



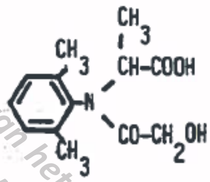
CGA 67 869



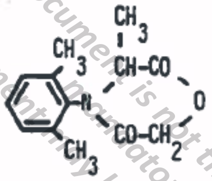
CGA 62 826



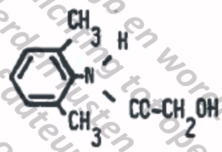
CGA 107 955



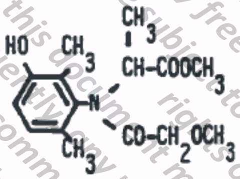
CGA 68 125



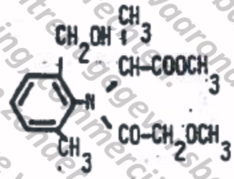
CGA 37 734



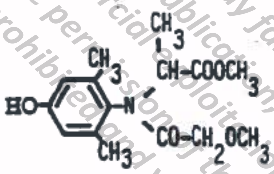
CGA 100 255



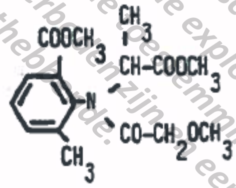
CGA 94 689
Isomers A+B



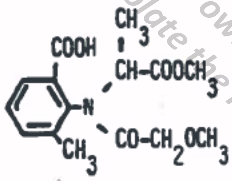
CGA 83 048



CGA 109 097

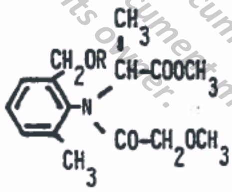


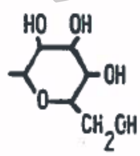
CGA 108 905



Isomer A = CGA 114 552 (R = R_1)
CGA 114 524 (R = R_2)

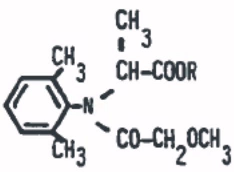
Isomer B = CGA 114 523 (R = R_1)
CGA 114 525 (R = R_2)

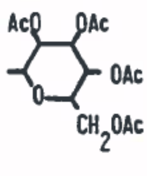




= R_1

CGA 119 860 (R = R_1)
CGA 119 861 (R = R_2)





= R_2

Dit document is geen eigendom van het Ctgb en wordt beschikbaar gemaakt op grond van een wettelijke verplichting tot openbaarmaking. Op dit document kunnen eigendomsrechten van derden rusten, waaronder intellectuele eigendomsrechten en/of auteursrechten. Voorts kan dit document onder een regeling omtrent gegevensbescherming vallen. Publicatie, verspreiding, vermenigvuldiging, commerciële exploitatie en gebruik van dit document, of de inhoud hiervan, zonder de toestemming van de rechthebbende van dit document, kan derhalve verboden zijn en/of een inbreuk opleveren van de rechten van de rechthebbende.
This document is not the property of the Ctgb and is provided based on the regulatory freedom of information requirements. The document may be subject to rights such as intellectual property and copyright of third parties. Furthermore, this document may fall under a regulatory data protection regime. Consequently, any publication, distribution, reproduction and/or publishing and any commercial exploitation and use of this document or its contents without the permission of the owner of this document may therefore be prohibited and violate the right of its owner.

<u>Table II</u> Pattern of metabolites in the aerial parts of lettuce after application of ^{14}C-CGA 48 988
(in percent of the radioactivity found by combustion)

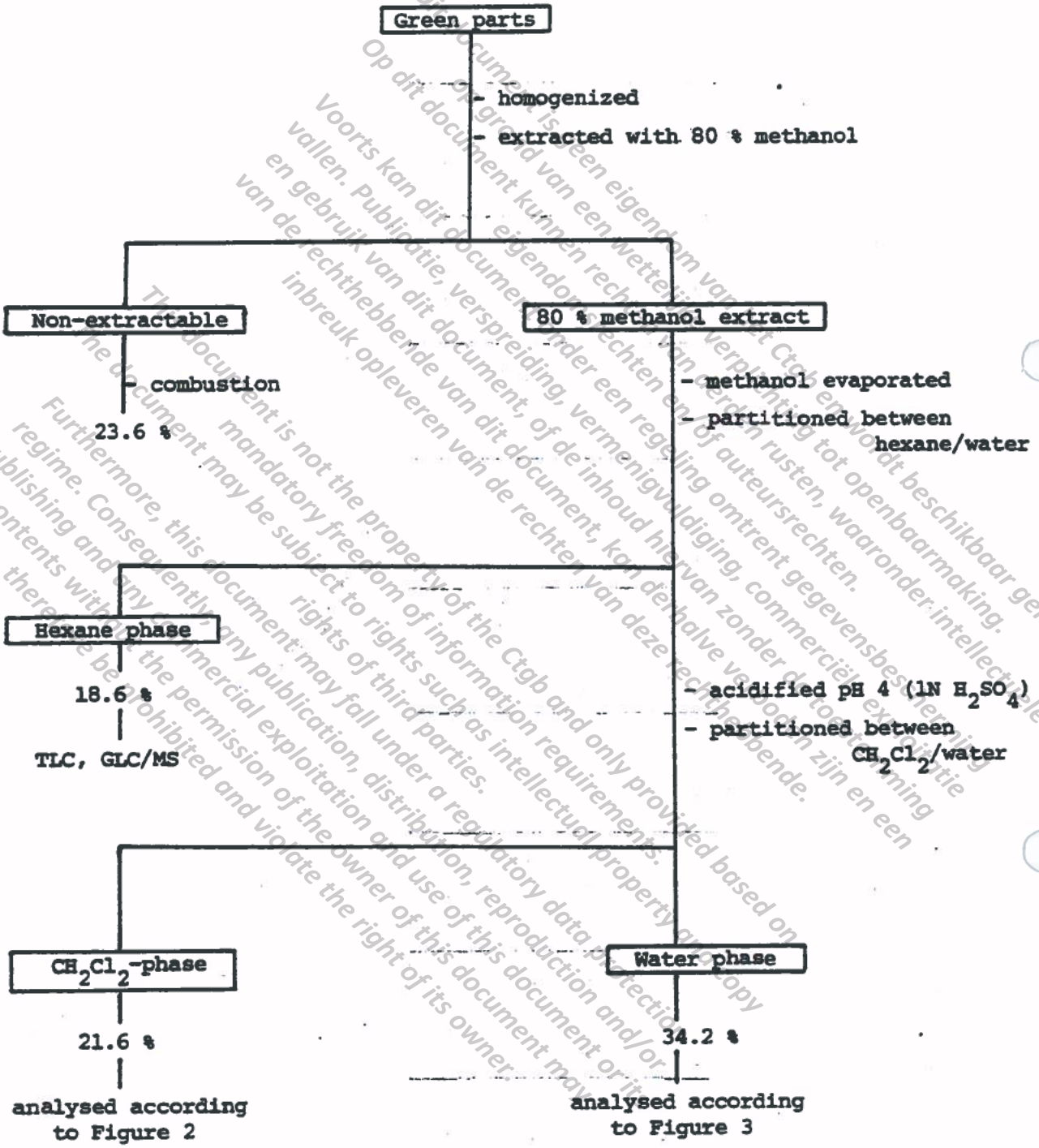
CGA 48 988	CGA 100 255		CGA 67 869		CGA 37 734		CGA 94 689				CGA 62 826		CGA 107 955		CGA 108 905	Unknown fraction 8
							Isomer A		Isomer B							
1)	1)	2)	1)	2)	1)	2)	1)	2)	1)	2)	1)	2)	1)	2)	1)	1)
18.6	2.7	3.5	4.5	4.4	1.2	1.7	0.6	1.7	4.5	15.3	4.6	1.4	3.9	6.2	1.2	0.4
18.6	6.2		8.9		2.9		2.3		19.8		6.0		10.1		1.2	0.4

1) In form of free metabolites

2) In form of glucose conjugates

Dit document is geen eigendom van het Ctgb en wordt beschikbaar gemaakt op grond van een wettelijke verplichting tot openbaarmaking. Op dit document kunnen rechten van derden rusten, waaronder intellectuele eigendomsrechten en/of auteursrechten. Voorts kan dit document onder een regeling omtrent gegevensbescherming vallen. Publicatie, verspreiding, vermenigvuldiging, commerciële exploitatie en gebruik van dit document, of de inhoud hiervan zonder de toestemming van de rechthebbende kan derhalve verboden zijn en een inbreuk opleveren van de rechten van deze rechthebbende.
This document is not the property of the Ctgb and only provided based on mandatory freedom of information requirements. The document may be subject to rights such as intellectual property and copy rights of third parties. Furthermore, this document may fall under a regulatory data protection regime. Consequently, any publication, distribution, reproduction and/or publishing and any commercial exploitation and use of this document or its contents without the permission of the owner of this document may therefore be prohibited and violate the right of its owner.

Figure 1 Fractionation of the radioactivity in the green parts of lettuce after application of ^{14}C-CGA 48 988



Dit document is een eigendom van wettelijke verplichting tot openbaarmaking. Op grond van een wettelijke verplichting tot openbaarmaking is dit beschikbaar gemaakt. Op dit document kunnen rechten van derden rusten, waaronder intellectuele eigendomsrechten en/of auteursrechten. Voorts kan dit document onder een regeling omtrent gegevensbescherming vallen. Publicatie, verspreiding, vermenigvuldiging, commerciële exploitatie en gebruik van dit document, of de inhoud hiervan zonder de toestemming van de rechthebbende van dit document, of de rechten van deze rechthebbende. inbreuk opleveren van de rechten van deze rechthebbende.
The document is not the property of the Ctgb and only provided based on mandatory freedom of information requirements. The document may be subject to rights such as intellectual property rights of third parties. Furthermore, this document may fall under a regulatory data protection regime. Consequently, any publication, distribution, reproduction and/or commercial exploitation and use of this document or its contents without the permission of the owner of this document may be prohibited and violate the right of its owner.

Figure 2 Procedure for the analysis of the methylene chloride soluble metabolites

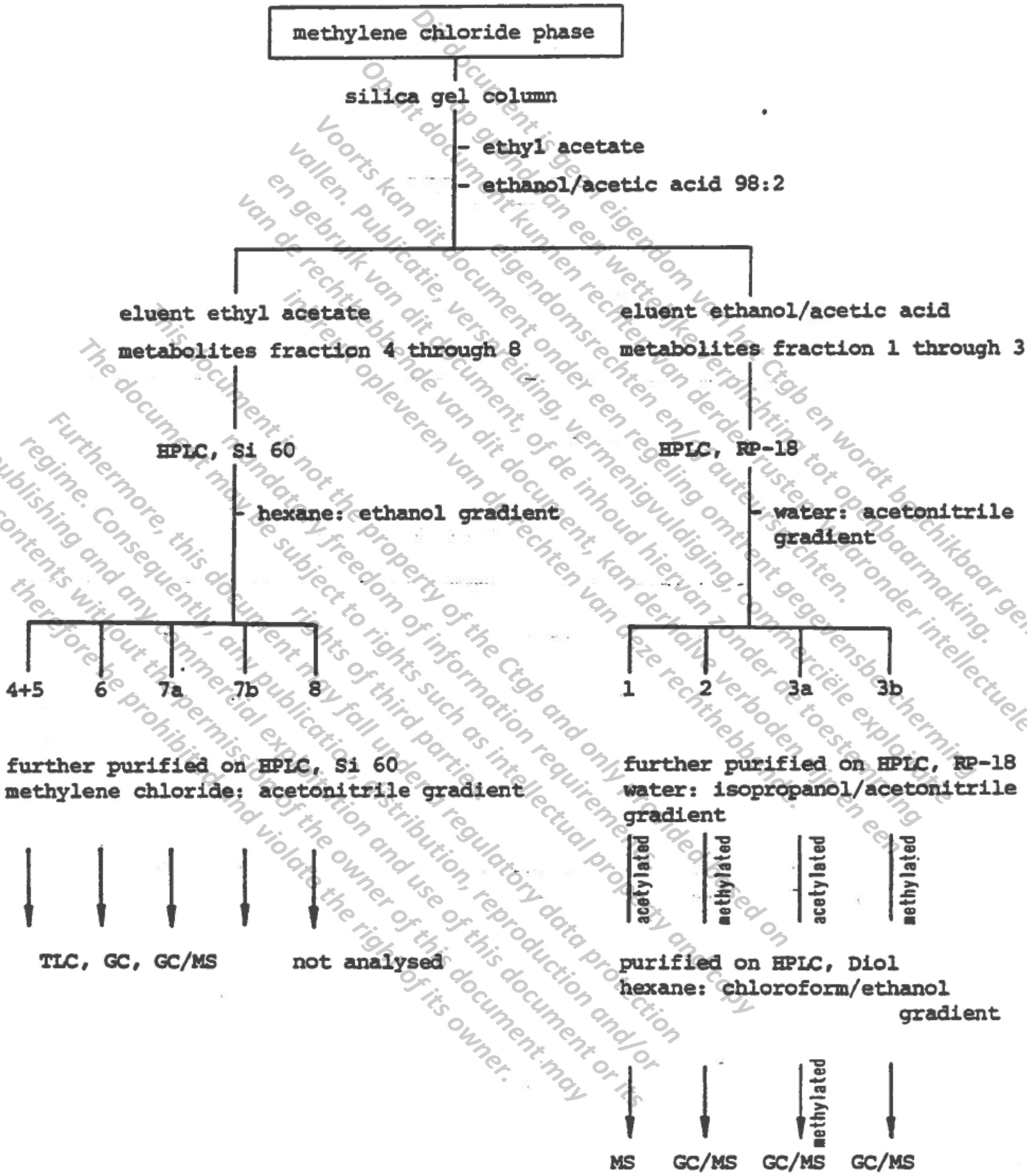


Figure 3 Procedure or the analysis of the water soluble metabolites

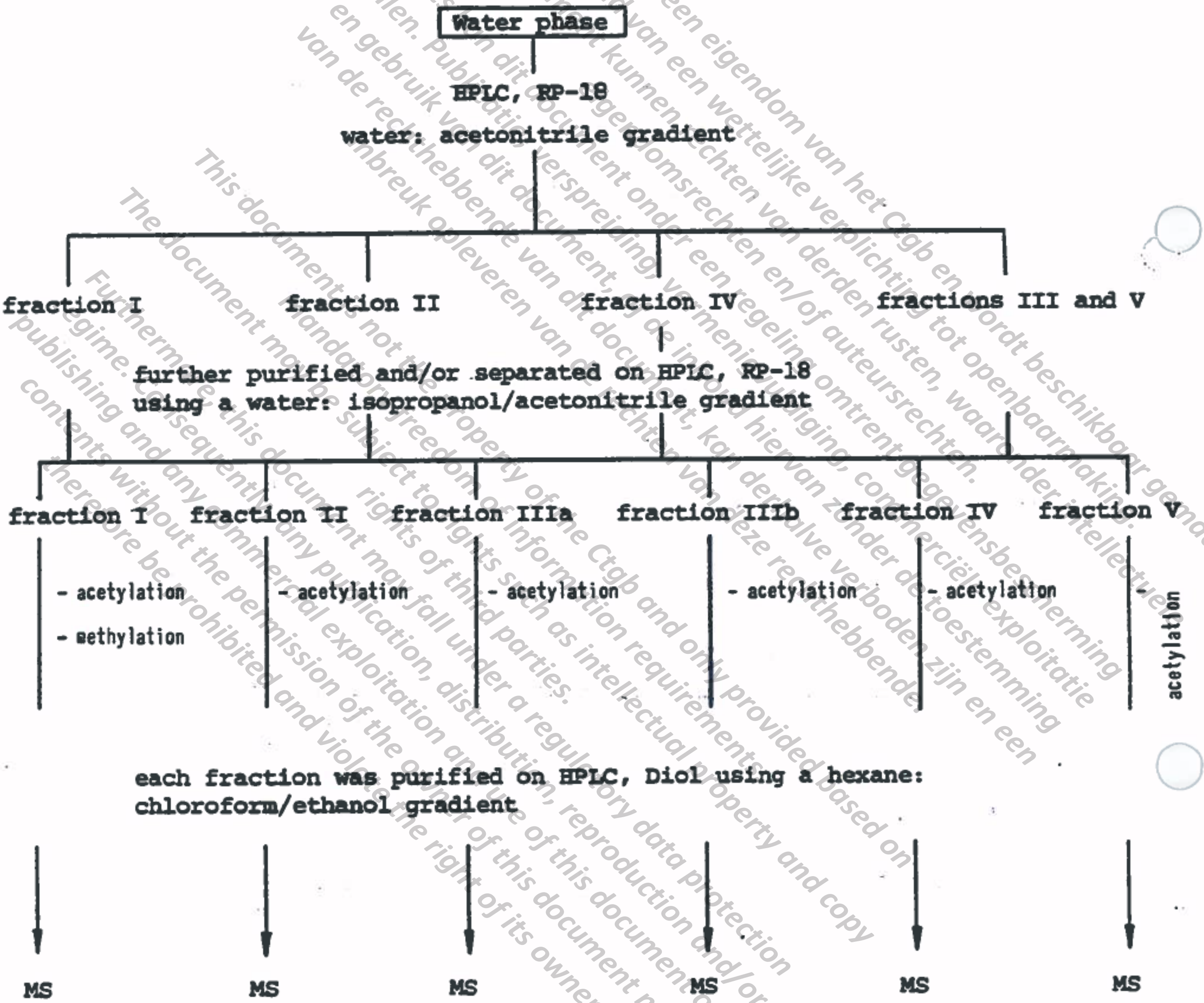
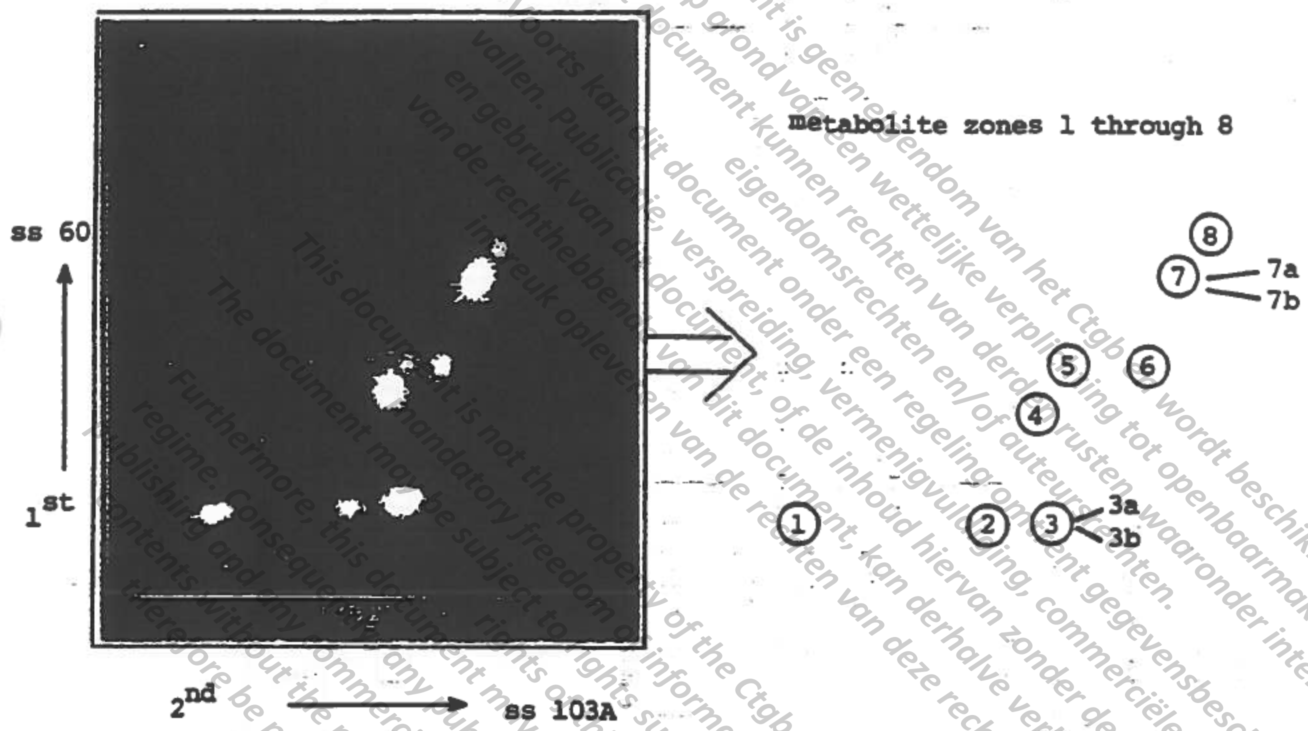
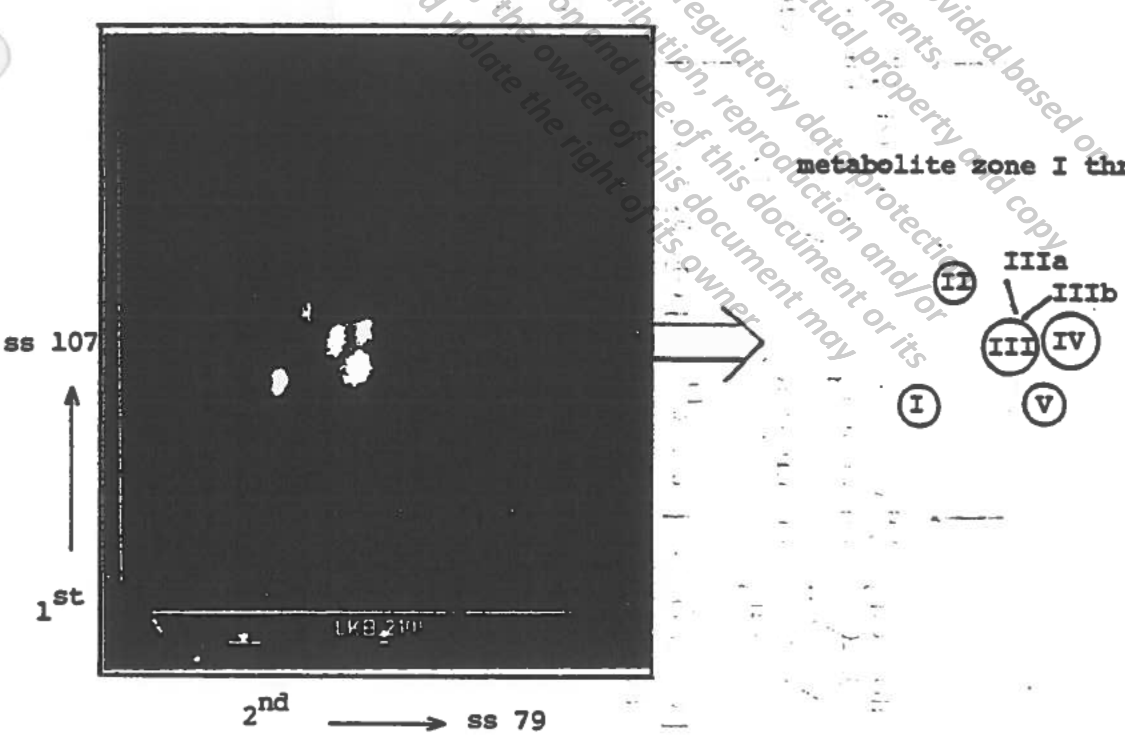


Figure 4 Two dimensional thin layer chromatograms of the ^{14}C-CGA 48 988 metabolites extracted from lettuce

methylene chloride phase



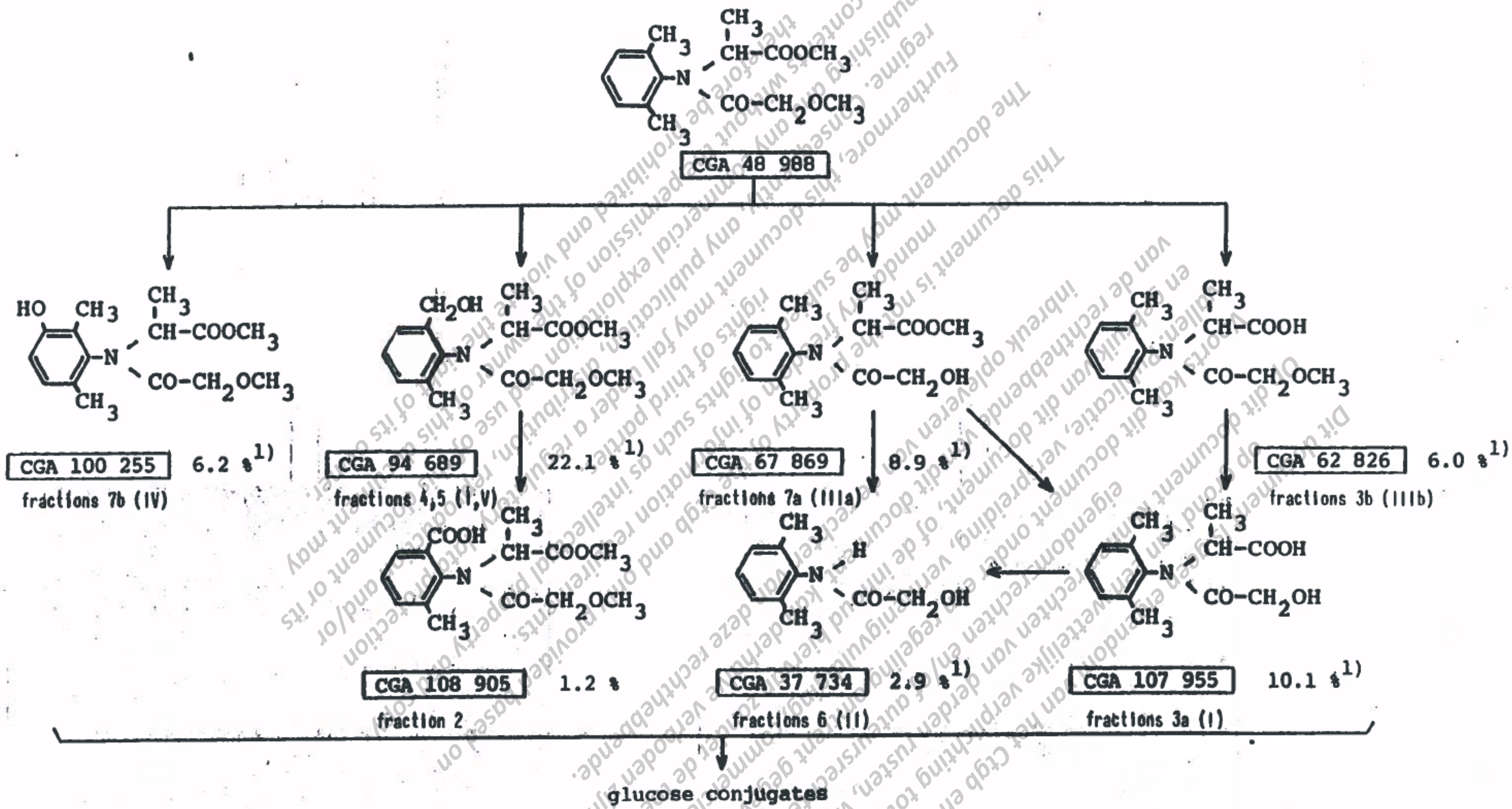
water phase



Dit document is geen eigendom van het Ctgb en wordt beschikbaar gemaakt op grond van een wettelijke verplichting tot openbaarmaking. Op dit document kunnen rechten van derden rusten, waaronder intellectuele eigendomsrechten en/of auteursrechten. Voorts kan dit document onder een regeling van gegevensbescherming vallen. Publicatie, verspreiding, vermenigvuldiging, commerciële exploitatie en gebruik van dit document, of de inhoud hiervan, kan derhalve zonder de toestemming van de rechthebbende verboden zijn en een inbreuk opleveren van de rechten van deze rechthebbende.

This document is not the property of the Ctgb and only provided based on information requirements. The document may be subject to rights such as intellectual property and copy rights of third parties. Furthermore, this document may fall under a regulatory data protection regime. Consequently, any publication, distribution, reproduction and/or publishing and any commercial exploitation and use of this document or its contents without the permission of the owner of this document may therefore be prohibited and violate the right of its owner.

Figure 5 Metabolic pathways proposed for the degradation of CGA 48 988 in lettuce



1) in the free and conjugated forms