Report on Study

CGA 329351

STUDY TITLE

Hydrolysis of ^{14}C-labelled CGA 329351 under Laboratory Conditions

DATA REQUIREMENT

EC-Directive 91/414/EEC: Annex II:7.2.1.1 Hydrolytic degradation.

For Further Requirements see Page 4

AUTHOR

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STUDY COMPLETED ON

January 3, 1996

PERFORMING LABORATORY

CIBA-GEIGY Limited
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LABORATORY PROJECT IDENTIFICATION

Project Number : 95EH05

SPONSOR

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CH-4002 Basle, Switzerland
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Total Number of Pages: 69

STATEMENT OF COMPLIANCE WITH GOOD LABORATORY PRACTICE

This study was performed in compliance with Good Laboratory Practice (GLP) in Switzerland, Procedures and Principles, March 1986 [Verfahren und Grundsaetze der Guten Laborpraxis (GLP) in der Schweiz, Maerz 1986] issued by the Federal Department of the Interior and the Intercantonal Office for the Control of Medicaments, Switzerland. These procedures are based on OECD Principles of GLP adopted on 12 May 1981 by Decision of the OECD Council concerning Mutual Acceptance of Data in the Assessment of Chemicals [C(81)30 (Final)].

January 3, 1996
Date

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Quality Assurance Statement

Ciba-Geigy Ltd., GLP Quality Assurance Product Safety, 4002 Basel

Project	95EH05
Test Substance	CGA 329351
Study Title	Hydrolysis of 14C-Labelled CGA 329351 under Laboratory Conditions
Study Director	Dr. 5.1.2.e Woo
QA-Inspector	5.1.2.e Woo

I hereby certify that the following Quality Assurance activities were performed:

Activity	Performed	Reported
Facility Inspection	March 23, 1995	April 06, 1995
Protocol Audit	July 26, 1995	July 26, 1995
Study Related Inspection	August 24, 1995	August 25, 1995
Facility Inspection	September 26, 1995	October 05, 1995
Final Report Audit	January 01, 1996	January 02, 1996

January 03, 1996
Date
Form. QSSTAT12

5.1.2.e Woo
Inspector Quality Assurance

GENERAL INFORMATION

Guidelines	The study was conducted to satisfy the: EC-Directive 91/414/EEC: Annex II: 7.2.1.1 Hydrolytic degradation. Pesticide Assessment Guidelines, Subdivision N, Chemistry: Environmental Fate, EPA-540/9-82-021, Section 161-1: Hydrolysis Studies; US Environmental Protection Agency, October 18, 1982 and under consideration of: OECD Guideline for Testing Chemicals, Hydrolysis as a Function of pH, 111, Adopted: 12 May 1981, Paris/ France Pruefung des Verhaltens von Pflanzenbehandlungsmitteln im Wasser, Merkblatt Nr. 55, Teil I und II; Biologische Bundesanstalt für Land- und Forstwirtschaft, Bundesrepublik Deutschland, Oktober 1980
Sponsor	Ciba-Geigy Ltd. Crop Protection Division Product Safety / Safety Evaluation CH-4002 Basel, Switzerland
represented by	Dr. 5.1.2.e Woo
Test Substance	Company Code: CGA 329351
Proposed Use	Fungicide
Project Number	95EH05
Study Director[1] *and* *Testing Facility*	Dr. 5.1.2.e Woo Ciba-Geigy Ltd. Crop Protection Division Product Safety / Ecochemistry CH-4002 Basel, Switzerland.
Technical Personnel[1]	5.1.2.e Woo (principal coworker)
External Cooperator	Mass Spectra (MS) for the identification of hydrolytic degradates were recorded and interpreted by: Ciba-Geigy Ltd. Crop Protection Division Product Safety / Metabolism CH-4002 Basel, Switzerland
represented by	Dr. 5.1.2.e Woo

[1] The job descriptions and the professional curricula vitae for all personnel participating in this study are archived in the testing facilities.

Supplier of the ^{14}C-labelled Test Substance	Ciba-Geigy Ltd. Crop Protection Division Divisional Unit Research and Development Research Services / Chemistry Support Isotope Laboratory CH-4002 Basel, Switzerland
represented by	Dr. 5.1.2.e Woo
Supplier of the Reference Substances	Ciba-Geigy Ltd. Crop Protection Division Product Safety / Chemistry Support CH-4002 Basel, Switzerland
represented by	Dr. 5.1.2.e Woo
Testing Period	Study Initiation: July 20, 1995 Experimental Start: July 28, 1995 Experimental Termination: October 18, 1995 Study Termination January 3, 1996
Archives	Protocols, raw data, correspondence, and the final report are archived in the test facilities at CIBA-GEIGY Limited,Basel.
Quality Assurance	Ciba-Geigy Ltd. Crop Protection Division Product Safety / Quality Assurance CH-4002 Basel, Switzerland
represented by	Dr. 5.1.2.e Woo
Integrity of the Study	No circumstances were observed affecting the integrity of the study.

For the report:

January 3, 1996
Date:

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Ciba-Geigy Ltd.
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LIST OF AMENDMENTS TO PROTOCOL

Amendment No.	Date	Concerning	Reason for Alteration
No Amendments were performed			

Table of Contents

Summary

The hydrolytic behaviour of CGA 329351, i.e. *(R)-2-(N-(2,6-dimethyl-phenyl)-methoxyacetylamino)-propionic acid methyl ester* labelled in the phenyl-ring was investigated at four different pH's (pH's 1,5,7 and 9) and at three different temperatures(pH 9).Under acidic or environmentally relevant conditions, i.e. up to pH 7 even under elevated temperatures (50°C) the compound proved to be hydrolytically stable. Only under alkaline conditions the compound was hydrolysed with half-lives of 116.4, 7.7 and 2.7 days for 25, 50 and 60°C. For 20°C a half-life of 216 days was calculated.
Hydrolysis proceeded via cleavage of the methylester bond leaving (R)-2-[N-(2,6-dimethyl-phenyl)-methoxyacetylamino]-propionic acid.

1. Introduction

The objectives of the hydrolysis study are to provide information on the rate of hydrolysis of the test substance and on the rates of formation and decline of hydrolysis products.

For this purpose, the ^{14}C-phenyl-ring labelled test substance was incubated in aqueous medium at various temperatures and pH-values under sterile conditions in the dark.

2. Materials and Methods

2.1 Chemicals

2.1.1 Unlabelled Test Substance

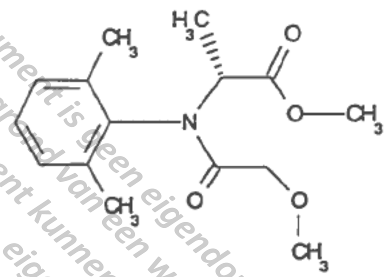
As reference material unlabelled CGA 329351 was used. The below given data were taken from Ciba-Geigy "Status Report"May 9, 1994.

Company Code	CGA 329351
Chemical Name (IUPAC)	(R)-2-(N-(2,6-dimethyl-phenyl)-methoxyacetylamino)-propionic acid methyl ester
CAS Registry Number	70630-17-0
Empirical Formula	$C_{15}H_{21}NO_4$
Molecular Weight	279.34 g/mole
Physical State at 20°C	viscous liquid
Specific rotation	α_D=-54.8°
Colour	colourless
Vapour Pressure at 20°C	2.9 x 10^{-3} Pa
Solubility (at 20 °C)	water: 26 g/l acetone: > 100% ethanol: > 100 % (w/v) acetonitrile: > 100 %
Partition Coefficient n-octanol/water	log P_{OW} = 1.53 (flask-method)
pK$_a$-Value	< 0 (basic)
Amount	0.05 g
Batch Number	AMS 758/101
Purity	99.8 %
Expiration Date	11/96
Storage Conditions	0-5 °C in the dark
Max. recommended Field Rate per Application	100 g a.i./ha

Toxicity	ALD 50 rat: >200-500 mg/kg
Special Precautions	Routine hygiene procedures

2.1.2 ^{14}C-Labelled Test Substance

Structure
(label: 14C-U-ring)



Amount	7.5 mg
Batch Number	MSR-II-89
Specific Activity	1.45 MBq/mg or 39.3 µCi/mg
Purity	98.8 % as certified (but see also under 4.1.1)
Expiration Date	9/95

2.2 Test System

2.2.1 Aqueous Systems

The hydrolytic behaviour of the test substance was investigated at various pHs and temperatures. For this purpose suitable aqueous solutions/buffers were used.
The buffers' ionic strength was ≤0.01 M to keep pH constant and buffer effects negligible.

The following buffer pH's and types were used (for details see Table 1):

pH 5 (0.01M acetate)
pH 7 (0.01M phosphate)
pH 9 (0.01M borate)

In addition to the buffers given above 0.1 M HCl (pH 1) was tested.

The buffers/solutions were sterilized by sterile filtration and glassware by autoclaving.

2.2.2 Test Apparatus and Experimental Conditions

Samples to be incubated at the different temperatures were placed into tightly sealed brown test tubes of 10 ml volume:

Sample volume:	5 ml
Sample concentrations:	5 mg/l

Experimental conditions:	Samples (tightly closed with head space, light protected) were shaken under temperature controlled conditions. Temperature accuracy: ±1°C; Temperature monitoring (reading): ±0.1°C
Temperatures:	25, 50 and 60 °C

2.3 Study Procedure

The study was performed in two steps, i.e. a pre-test and a final test were performed.

2.3.1 Pre Test

A pre-test based on OECD test guideline was performed at 50°C at pH 1, 5, 7, and 9. For this purpose, duplicate samples were set-up. The purpose of the pre-test was to determine whether a test compound is stable at a given pH or in case that degradation is observed to determine the experimental conditions (duration of the final test and sampling times). If less than 10% of parent degradation was observed after 5 days at a certain pH, the test substance was considered to be hydrolytically stable at this particular pH.

Degradation of 10% in 5 days at 50°C (OECD) corresponds to a half-life of 33 days (k=0.021) assuming exponential decay (see under 2.6.1):

$$lnC_t = C_0 * e^{-kt} \quad ln(90) = ln(100) - k*5$$

$$(ln(100) - ln(90))/5 = k = 0.021$$

$$T/2 = ln(2)/k = 33 \text{ days}$$

When transferring to 25°C (Arrhenius) the half-life would be 6 times higher, i.e. it would be 198 days. Based on this estimate the rate constant would be:

$$ln(2)/(T/2) = k = 0.0035$$

This criteria exactly corresponds to the EPA interpretation considering compounds with a degradation of ≤10% within 30 days at 25°C (half-life 198 days; k= 0.0035) as hydrolytically stable. For compounds showing this behaviour EPA requires only a confirmatory test at 25°C for 30 days with sampling dates at 0, 15 and 30 days.

However when the test results of the 5 days pre-test showed a degradation of < 5% no additional testing was performed, since no reasonable data were expected from a prolonged pre-test.

2.3.1.1 Treatment of Test Samples

2.3.1.1.1 Preparation of Stock Solution

The test substance solution in toluene was made up to 2 ml with acetone and thereafter the radioactivity determined by LSC to be 7.364 mg.

2.3.1.1.2 Preparation of Application Solution (Pre-test)

From the stock solution a volume of about 0.4 ml was taken the solvents evaporated under a gentle stream of nitrogen and the residual radioactivity dissolved in 5 ml sterile, distilled water. The total amount of radioactivity in the aqueous solution was determined by LSC to be 1.28 mg.

2.3.1.1.3 Treatment (Pre-test)

For each pH-condition 1.17 ml of the application solution were placed into a sterile Erlenmeyer flask and the volume made up with the corresponding sterile buffer solution to 60 ml. The final amount of radioactivity in the buffer solutions was determined by LSC to be 0.3 mg. This figure corresponded to a test concentration of 5 mg/l. From the corresponding treated buffer solutions aliquots of 5 ml each were placed into the test tubes.

2.3.1.2 Incubation (Pre-test)

Samples were incubated in a shaking water bath at 50 °C for 5 days.

2.3.1.3 Sampling

For each pH duplicate samples were taken daily. After sampling the pH was determined and thereafter for acidic and alkaline samples the pH adjusted to about 7 prior to storage or analysis.

2.3.2 Final Test

The final test was performed only for pH 9 at three temperatures.

2.3.2.1 Preparation of Application Solution

From the stock solution a volume of 0.424 ml was taken the solvents evaporated under a gentle stream of nitrogen and the residual radioactivity dissolved in 5ml acetone. The total amount of radioactivity in the aqueous solution was determined by LSC to be 1.60 mg.

2.3.2.1.1 Treatment

For 25, 50 and 60°C samples 3.12, 0.78 and 0.78 ml of the application solution were placed into a sterile Erlenmeyer flasks and the volume made up with the corresponding sterile buffer solution to 200, 50 and 50 ml, respectively. The final amount of radioactivity in the corresponding buffer solutions was determined by LSC to be 1.01, 0.248 and 0.249 mg. This

figures corresponded to test concentrations of 5.05, 4.97 and 4.97 mg/l. From the corresponding treated buffer solutions aliquots of 5 ml each were placed into the test tubes.

2.3.2.2 Incubation

Sample test tubes were incubated in a shaking water baths at 25, 50 and 60°C for 32, 15 and 11 days respectively.

2.3.2.3 Sampling

For samples exposed to 25°C duplicate test tubes were taken. For 50 and 60 °C one test tube each was taken at all sampling times except for time zero where duplicates were taken. After sampling the pH was determined and thereafter for acidic and alkaline samples the pH adjusted to about 7 prior to storage or analysis. For more details see Table 7 - Table 9.

2.4 Analysis

2.4.1 Test Solutions

For each sample to be analyzed, the solution pH was measured at ambient temperature immediately after the samples were taken. Acidic and basic samples were stabilized at pH 7 by adding diluted NaOH or HCl solution. The ^{14}C-activity was measured by LSC.
Subsamples of the neutralised test solutions were directly analysed by HPLC for test substance and degradation products.
Thereafter the parent molecule and its major degradate were isolated by HPLC and submitted to further characterization by TLC, HVE and mass spectrometry.

2.4.2 Sterility Testing

At the end of the experiment subsamples of the final test solution of pH 9 at each temperature condition were tested for possible microbial contamination.

The number of microorganisms present in buffer solution was determined by the total plate count method. For this purpose, 2 ml of the test solution were made up with sterile water to 50 ml and filtered over membrane filters (0.45 µm). Additionally sterilized tap water was prepared in the same way. For each dilution, filters were placed into petri dishes containing total count growth medium. Incubation of the petri dishes was performed for up to 48 h at room temperature.

At the end of the incubation the developed colonies were counted. The following materials were used for the test:

Materials:

Filtration device:	Millipore XX 10 047 00 IV XX1004705
Membranefilter HA 47 mm, 0.45 µm:	Millipore M00000HABG 047S1
Petri dishes (sterile):	Petrislides, Millipore PDMA 04700

Medium:

Total Count Standard Medium
M-TGE broth: Millipore M00000P2T

2.4.3 Measurement of Radioactivity

All measurements were performed at least in duplicate using a model CA 2200 Liquid Scintillation Counter (LSC)[2]. All measurements were corrected for background and counting efficiency (a quench curve was established using Packard[2] quenched standards).

The following scintillation cocktails were used:

Scintillator I: Irgascint® A-300[3]

Scintillator II: OptiPhase 'HiSafe'®[4]

Liquid samples were directly measured using *Scintillator I* or *Scintillator II* (up to 0.8 ml).

2.4.4 High Performance Liquid Chromatography (HPLC)

HPLC analysis was carried out on a Spectra Physics[5] Liquid Chromatograph assembled with the following components:

Instruments:

HPLC-System:	Pump:	SP 8800
	Autosampler:	SP 8880
	Autoinjector:	SP 8880
	Integrator:	Chromjet
	Oven:	SP 8792
	Data System:	Spectra Physics Chromstation
Detectors:	UV/VIS:	Spectra 200
	RAM:	Berthold LB 506 C-1 equipped with a 150 µl solid flow cell (YG 150) and the Berthold "Winflow" data system[6].

Radioactive fractions were quantified using the Berthold evaluation software.

The software was validated by fractionation of the eluent and LSC. The data are presented in Appendix A.

Operating Conditions:

Column:	Nucleosil C-18, length 25 cm, inner diameter 4.6 mm, particle size 5 µm[7].
Oven:	30°C

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2 Packard Instrument Company Inc., Downers Grove Ill., U.S.A.
3 CIBA-GEIGY LTD, Basle, Switzerland
4 Wallac Oy, Turku, Finland
5 *Spectra-Physics AG*, Allschwil, Switzerland
6 *Berthold AG*, Regensdorf, Germany
7 Bischoff, Leonberg, Germany

Mobile Phases: Eluent A: Water
Eluent B: Acetonitrile
Flow: 1 ml/min.

Elution :

The following linear gradient was performed:

Time [min]	Eluent A [%]	Eluent B [%]
0-3	75	25
3-10	75-->5	25-->95
10-14	5	95
14-15	5-->75	95-->25
15-25	75	25

Injection Volume: 70-100 µl

Detectors: UV/VIS: Wavelength: 210 nm
Range: 0.1 AUF's
Cell Volume: 150 µl

RAM: -

Retention Times determined for Reference Compound and Metabolites:

Compound Code-Number	Retention Time (min)
CGA 329351	13.07
[redacted]	2.02-4.12
[redacted]	2.62-7.15
[redacted]	11.17
[redacted]	12.43
[redacted]	13.38

Retention times varied strongly depending on the the properties of the samples (adjustment of pH of samples).

2.4.5 Thin Layer Chromatography (TLC)

Thin-layer chromatography was carried out using the following equipment:

Analytical TLC was performed using the following methods and materials:

Mode: Adsorption chromatography
Plates: Silica gel plates (KG 60, F254)[8]
Size: 5x20 cm or 20x20 cm
Particle size: 60 µm
Layer thickness: 0.25 mm

For one dimensional and for two dimensional TLC 5 x 20 cm and 20 x 20 cm plates were used, respectively.

For analysis the following solvent systems were used:

Solvent System	Eluents	RF-Value	
		CGA 329351	M1
1	CH2Cl2/MeOH/CH3COOH/H2O (70+20+10+5)	.93	.90
2	Ethyl acetate/EtOH/NH3(25%)/H2O (65+23+4+2)	.91	.03
3	CHCl3/Acetone (9+1)	.53	.01
4	CHCl3/MeOH/HCOOH/H2O (75+20+4+2)	.93	.75

The plates were developed without chamber saturation in both dimensions.

For two-dimensional TLC solvent system 3 was used for the first dimension and solvent system 4 for the second dimension.

Radioactive spots were detected and quantified with a Berthold Linear analyzer CHROMA 2D[9], equipped with computer system running the Berthold evaluation software.

Non-radioactive spots were detected under UV light at 254 nm.

The TLC evaluation software was validated by scraping off the silica containing the radioactive spots and the residual silica of TLC-pates and measurement of the radioactivity present by LSC. For this purpose the silica gel was placed into scintillation vials, homogenized, thereafter methanol/water (2 ml; ratio 4:1) added and the mixture shaken for about 10 secs. The silica gel-methanol/water mixture was then suspended in *Scintillator II* and submitted to LSC. Representative data are presented in **Appendix A**.

2.4.6 High Voltage Electrophoresis (HVE)

High voltage electrophoresis was performed with a CAMAG HVE model 63051 (CAMAG, CH-4132 Muttenz/Switzerland) equipped with a water cooling system.

[8] *E. Merck*, Darmstadt, Germany
[9] *Berthold AG*, Regensdorf, Germany

Support material	20x40 cm paper sheets, Schleicher + Schüll 2043B (Schleicher + Schüll, Seelze, Hannover/FRG)
Buffer pH10	0.030 M disodium tetraborate + 0.042 M sodium hydroxide
Electrical power	75 V/cm 95 mA
Time of development	20 min.

2.5 Mass Spectrometry (MS)

The identity of degradates was confirmed by mass spectrometry. The instrument used was a Finnigan mass spectrometer equipped with a LC-System (TSQ 7000 LC-MS/MS) or a Hewlett-Packard 5988A GC-MS.

LC-MS/Conditions:

Ionisation technique	APCI (Atmospheric Pressure Chemical Ionization)
Ion polarity	negative
Aquisition mode	centroid
Scan mode	single quadrupole scan
APCI offset	15 Volt
Electron multiplier	1300 Volt
Quadrupole 1 scan range	100-600 amu

LC-Conditions:

Column	Packing material	Nucleosil 100; RP 18
	Length (cm)	10
	Diameter (mm)	2
	Particle size (µm)	5
	Temperature	RT
Mobile phase	Eluent A: Eluent B:	water acetonitrile
	Flow (ml/min.)	0.4

Gradient (min.)	Eluent A	Eluent B
0-3	100	0
3-30	100-->0	0-->100
30-40	0	100

Detector	RAM (Berthold)	LB 507; 50µl cell

GC-MS/Conditions:

Apparatus	Hewlett-Packard 5988A GC-MS
Ionisation technique	EI
Ionisation	70 eVolt
Electron multiplier	1300 Volt
Scan range	60-500 amu

GC-Conditions:

Column	Packing material	DB 17Nucleosil 100; RP 18
	Length (m)	15
	Diameter (mm)	0.33
Temperatures	Injector temp.(°C)	300
	Program	initial: 100°C gradient: 100-->250°C (15 °C/min.)
Derivatisation	on-column	Methelute® Pierce

2.6 Calculations

2.6.1 Degradation of the Test Substance

Total ^{14}C-material balance was calculated for each sample. All values given refer to the radioactivity applied. An example of representative data and calculation is given in Appendix A.

The degradation rate of the test compound was calculated by assuming pseudo first-order reaction kinetics.

$$-\frac{dC}{dt} = k_1 \bullet C$$

solution of the differential equation:

$$C_t = C_1 \bullet e^{-k_1 \bullet t}$$

where:

C_1 denotes the maximum concentration of the test compound, C_t its concentration at depuration time t and k_1 its rate constant. The rate constant was calculated by applying the MicroCal Origin least squares parameter estimation program[10].

Half-life:

Half-lives were calculated according the equation:

$$t_{0.5} = \frac{\ln 2}{k_1}$$

2.6.2 Rate Constants and Half-lives at other Temperatures

Rate constants and half-lives others than those experimentally determined were calculated using the Arrhenius equation:

$$k=A*e\text{-}(Ea/RT) \qquad \text{or } Ln\ k=-Ea/R*1/T+Ln\ A$$

where A is the frequency factor (s-1), Ea the activation energy ($J*mole^{-1}$), R the gas constant (8.3144 $J*mole^{-1}*K^{-1}$) and T the absolute temperature (°K).

The figures for Ea and A were obtained by correlation of the experimentally determined rate constants (lnk) with the reciprocal temperatures.

Determination of Activation Entropy (ΔS#) and Enthalpy (ΔH#).The activation entropy and enthalpy were calculated using the following formulas:

$$\Delta H\# = Ea - RT$$

and the transformed Eyring relationship:

$$\Delta S\# = \Delta H\#/T + R*Ln\,(k_r/T) - R*Ln(KB/h)$$

where R is the gas constant, k_r the reaction constant (s-1), T the absolute temperature, KB the Boltzmann constant (1.3807-23 J*K-1) and h the Planks constant (6.6266-34 J*sec).

[10] *MicroCal Software Inc.*, Northhampton, USA.

2.6.3 Region of Interest (ROI)

Values given in ROI % (ROI: Region Of Interest) were calculated as follows:

$$ROI\ \%\ of\ fraction\ x = \frac{radioactivity\ of\ fraction\ x}{radioactivity\ of\ all\ selected\ fractions} \bullet 100$$

2.6.4 Limits of detection/quantitation

Limits of detection and quantitation were calculated for the various methods applied. The results are presented in **Appendix B**.

3. Storage

As far as possible aqueous samples were analyzed directly after sampling. If not directly analyzed samples were stored at 4°C under conditions where minimum further degradation was expected (neutralization). Storage time did not exceed four weeks.

4. Results and Discussion

4.1 General Information

4.1.1 Radiochemical Purity and Stability of the Test Substance

The purity of the radiolabelled CGA 329351 in the stock-solution in toluene/acetone was found to be 98.6[11] (Figure 1)

The compound proved to be stable under storage conditions (refrigerator) for more than one month as shown by HPLC-analysis of the treated, unexposed samples set up August 17, 1995 (buffer pH 9) showing still a purity of 97.95 % (see e.g. Table 9).

4.1.2 Microbial Plate Counts

No colonies were detected in the buffer solutions at the end of the exposure thus proving the sterility of the samples.

[11] Analysis date 7/07/1995

4.1.3 Temperature

The temperature of the buffer solution for the pre-test and the final test was at the preset value. The standard deviation was for all experiments below 0.05°C. For more infomation see Figure 13.

4.1.4 pH of Buffer Solutions

The pH of the buffer solutions of the pre-test and final test were at the preset value (see Table 1 and Table 2

4.2 Test Results

4.2.1 Pre-test

4.2.1.1 Recovery and Rate of Degradation

The balances of radioactivity and distribution rates of CGA 329351 and its hydrolytic degradates are given in Table 3 to Table 6 and in Figure 8.

The recovery of radioactivity ranged for all samples from 98.53 to 100 %.

Practically no degradation of the parent compound was observed within 5 days under acidic and neutral conditions conditions (pH 1 to 7) at a temperature of 50 degrees centigrade, i.e. 4.74, 0.68, 2.25 % of the applied amount of the test compound was degraded at pH 1, 5 and 7 respectively. Only at pH 9 a significant degradation was observed with 34.02 % in 5 days.

From these data it can be concluded that the test compound is stable under acidic and neutral conditions. Therefore, no further testing was performed at these pH's.

For alkaline conditions further testing was performed according to the scheme given in Table 10. Based on the degradation of 34 % in five days at 50 °C the following half-lives (Figure 8) were estimated for exposure at pH 9:

Temperature (°C)	Half-life (days)	Rate constant [k (d^{-1})]	Intended Duration	Sampling Times	No of Samples
25	76	0.00917	30 days	17	34
50	8	0.0824	2*T/2	9	16
60	4	0.165	2*T/2	9	16

4.2.2 Final Test at pH 9

The balances of radioactivity and distribution rates of CGA 329351 and its hydrolytic degradates are given in Table 7 to Table 9 and in Figure 9 to Figure 11. The rate constants and half-lives are summarised in Table 11. The Arrhenius and Eyring parameters are given in Table 12 and Figure 12.

4.2.2.1 Recovery and Rate of Degradation of CGA 329351

The recovery of radioactivity was, on average, for all samples 98.8+/-1.9%.

The compound degraded at pH 9 with half-lives of 116.4, 7.7 and 2.7 days for 25, 50 and 60 °C, respectively(Table 11). The corresponding Rate constants were calculated to be 6.89E-08, 1.04E-06 and 2.97E-06 s^{-1}. Based on these findings the Arrhenius parameters were calculated (Table 12) resulting in the following equation:

lnk=-Ea/RT+lnA = lnk = -88336*(1/(8.3144*T)) + 19

From the above given equation the rate constant for 20°C (=293°K) would be:

$\ln k_{20°C}$ = -17.11 --> k = 3.72E-08--> T/2 = 215.8 days

4.2.3 Formation of Degradates and Identification

4.2.3.1 Pattern of Formation and Decline of Degradates

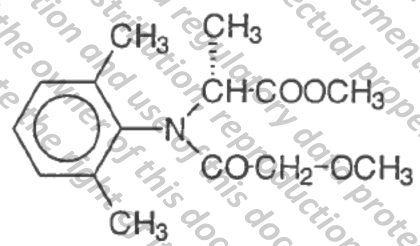
The patterns of degradates are given in Figures 2-4, 6,7 and in Figures 17-19.

Besides three minor degradates (M2-M5) not exceeding 2.16 % of the radioactivity applied only the parent molecule and degradate M1 were found in the alkaline buffer solutions. The latter compound reached its highest concentration at the end of the corresponding experiment. It amounted to 15.97, 69.46 and 91.30 % for samples incubated at 25, 50 and 60°C, respectively.

4.2.3.2 Identity of radioactive Fractions

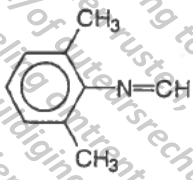
4.2.3.2.1 Parent Fraction

The identity of the parent fraction with CGA 329351 was shown by co-chromatography of the radioactive fraction on HPLC (Figure 7) and on 2D-TLC with the authentic reference compound (Figure 6) and by comparitative mass spectrometry (GC-MS). The result is shown in Figure 16.
The following fragments were obtained:



$C_{15}H_{21}NO_4$

EI-MS

AMU	Rel. Intensity	Ion Registration	Assignments	Fragments
279	34	positive	M	
249	100	positive	M - 30	⇒ CH_2O
234	36	positive	M - 45	⇒ CH_2OCH_3
220	37	positive	M - 59	⇒ $COOCH_3$
206	83	positive	M - 73	⇒ $COCH_2OCH_3$
192	80	positive	M - 87	⇒ $CH(CH_3)COOCH_3$
190	33	positive	249 - 59	⇒ $COOCH_3$
174	24	positive	206 - 32	⇒ CH_3OH
160	56	positive	192 - 32	⇒ CH_3OH
146	67	positive	220 - 72	⇒ $CO{=}CHOCH_3$
132	90	positive	M - 147	= 

DCI-MS/Methane

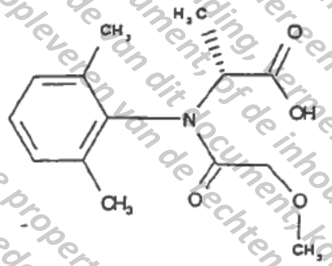
AMU	Rel. Intensity	Ion Registration	Assignments	Fragments
280	100	positive	M + H	
248	12	positive	M - 31	⇒ CH_3O

AMU	Rel. Intensity	Ion Registration	Assignments	Fragments
278	20	negative	M - H	
264	8	negative	M - 15	⇒ CH_3
263	30	negative	278 - 15	⇒ CH_3
246	100	negative	278 - 32	⇒ CH_3OH
233	14	negative	278 - 45	⇒ CH_3OCH_2

AMU	Rel. Intensity	Ion Registration	Assignments	Fragments
192	17	negative	M - 87	⇒ CH_3-CH-$COOCH_3$
174	13	negative	246 - 72	⇒ O=C=CH-OCH_3

4.2.3.2.2 Degradate M1

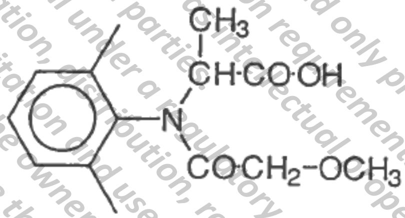
On HPLC, M1 proved to be polar i.e. it was eluted already after 4 minutes. On TLC with neutral solvents M1 remained at the start but when developed with polar acidic solvents M1 showed Rf-values of 0.75-0.90. When submitted to HVE at pH 10 M1 migrated versus the anode (Figure 5) Based on these findings M1 was assumed to represent an acid. When submitted to LC-MS M1 showed a molecular ion (M+) of 265. After methylation (M1-methyl) proven on GC-MS to be identical with the Parent molecule (Figure 14 and Figure 15). Based on these findings the following structure was proposed for M1:



(M1: MW 265; (R)-2-(N-(2,6-dimethyl-phenyl)-methoxyacetylamino)-propionic acid)

Fragments and interpretation:

M1:

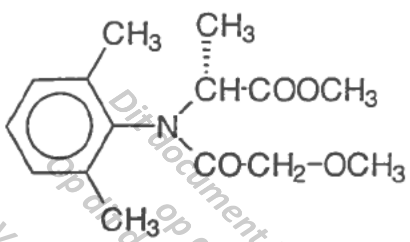


$C_{14}H_{19}NO_4$

APCI

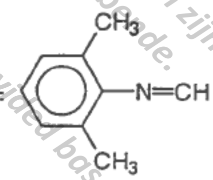
AMU	Rel. Intensity	Ion Registration	Assignments	Fragments
264	100	negative	M - H	

M1 after on-column derivatization:



$C_{15}H_{21}NO_4$

EI-MS

AMU	Rel. Intensity	Ion Registration	Assignments	Fragments
279	34	positive	M	
249	100	positive	M - 30	⇒ CH_2O
234	36	positive	M - 45	⇒ CH_2OCH_3
220	37	positive	M - 59	⇒ $COOCH_3$
206	83	positive	M - 73	⇒ $COCH_2OCH_3$
192	80	positive	M - 87	⇒ $CH(CH_3)COOCH_3$
190	33	positive	249 - 59	⇒ $COOCH_3$
174	24	positive	206 - 32	⇒ CH_3OH
160	56	positive	192 - 32	⇒ CH_3OH
146	67	positive	220 - 72	⇒ $CO{=}CHOCH_3$
132	90	positive	M - 147	= 

5. Conclusions

CGA 329351 was found to be stable under acidic and neutral conditions (pH 1-7) even under elevated temperatures (50°C). Only under alkaline conditions (pH 9)the compound was hydrolysed with half-lives of 116.4, 7.7 and 2.7 days for 25, 50 and 60°C. For 20°C a half-life of 216 days was calculated. Hydrolysis proceeded via cleavage of the methylester bond leaving (R)-2-[N-(2,6-dimethyl-phenyl)-methoxyacetylamino]-propionic acid.

6. Acknowledgements

The skilled technical assistance of Mr. 5.1.2.e Woo is gratefully acknowledged.

Table 1: Composition of Buffers and initial pH Values

Test	pH (after dilution)	Composition	Dilution
Pretest	1.012	0.1 M HCl	None
	5.022	1.795 M CH_3COONa + 0.825 M CH_3COOH	1:200
	7.011	0.041 M Na_2HPO_4 + 0.028 M KH_2PO_4	1:7
	9.065	0.043 M disodiumtetraborate + 0.017 M KH_2PO_4	1:6
Final Test	9.021	0.043 M disodiumtetraborate + 0.017 M KH_2PO_4	1:6

Table 2: pH-Values at the Sampling Time

Incubation Time (days)	pH-Condition							
	1		5		7		9	
	Series							
	A	B	A	B	A	B	A	B
1	1.01	1.01	5.02	5.02	7.10	7.10	9.07	9.07
2	1.11	1.10	4.90	4.91	7.19	7.10	8.99	8.99
3	1.01	1.00	4.89	4.91	7.19	7.12	9.00	9.01
4	1.08	1.07	4.99	4.94	7.19	7.14	8.99	8.98
5	0.98	1.09	5.05	5.01	7.19	7.10	9.02	9.03
Mean	**1.04**	**1.05**	**4.97**	**4.96**	**7.17**	**7.11**	**9.01**	**9.01**
Stand. Dev.	0.05	0.04	0.07	0.05	0.04	0.02	0.03	0.04

Final Test

Incubation Time (days)	Temperature (°C)			
	25		50	60
	Series			
	A	B	A	A
0	9.02	9.02	9.02	9.02
2	9.04	9.03	9.06	9.03
4	9.03	9.03	-	9.04
5	-	-	9.04	9.05
6	9.01	9.01	9.03	9.03
7	-	-	-	9.00
8	9.03	9.03	9.04	9.03
11	9.02	9.04	9.04	9.04
13	9.03	9.03	9.02	-
15	9.03	9.02	9.03	-
18	9.01	9.02	-	-
20	9.03	9.02	-	-
22	9.02	9.03	-	-
25			-	-
26			-	-
27			-	-
28			-	-
29			-	-
32			-	-
32			-	-
Mean	**9.02**	**9.02**	**9.03**	**9.03**
Stand. Dev.	0.01	0.01	0.01	0.01

Table 3: Degradation of CGA 329351 in Buffer Solution pH 1 at 50°C (Pre-test)

(Mean of duplicate samples)

Time (days)	Degradate/Code-No.						Total
	Parent	M1	M2	M3	M4	M5	
	Retention Time (min.)						
	12.97	2.02	2.62	11.17	12.43	13.38	
	Identity/CGA-No.						
	329351	UK	UK	UK	UK	UK	
1.00	97.40	0.00	0.00	0.82	0.95	0.83	100.00
2.00	89.78	8.08	0.00	0.46	0.41	0.57	99.30
3.00	94.66	0.78	3.08	0.58	0.10	0.76	99.95
4.00	93.60	4.39	0.00	0.51	0.28	0.68	99.45
5.02	92.66	2.54	1.70	0.82	0.49	0.70	98.91

Table 4: Degradation of CGA 329351 in Buffer Solution pH 5 at 50°C (Pre-test)

(Mean of duplicate samples)

Time (days)	Degradate/Code-No.					Total
	Parent	M1	M3	M4	M5	
	Retention Time (min.)					
	12.97	2.02	11.17	12.43	13.38	
	Identity/CGA-No.					
	329351	UK	UK	UK	UK	
0.00	97.75	0.00	0.00	0.45	1.81	100.00
1.00	96.49	0.24	0.59	0.72	0.99	99.03
2.00	97.41	0.30	0.42	0.16	1.29	99.58
4.00	96.93	0.00	0.94	0.43	1.13	99.43
5.02	97.07	0.00	0.67	0.40	1.07	99.21

Table 5: Degradation of CGA 329351 in Buffer Solution pH 7 at 50°C (Pre-test)

(Mean of duplicate samples)

Time (days)	Degradate/Code-No.						Total
	Parent	M1	M2	M3	M4	M5	
	Retention Time (min.)						
	12.97	2.02	2.62	11.17	12.43	13.38	
	Identity/CGA-No.						
	329351	UK	UK	UK	UK	UK	
0.00	98.13	0.00	0.00	0.42	0.39	1.07	100.00
1.00	96.43	1.03	0.00	0.46	0.37	1.03	99.31
2.00	97.00	0.88	0.00	0.97	0.05	0.86	99.76
4.00	95.69	1.17	0.82	0.57	0.37	0.98	99.60
5.02	95.88	0.98	0.83	0.24	0.36	0.80	99.08

Table 6: Degradation of CGA 329351 in Buffer Solution pH 9 at 50°C (Pre-test)

(Mean of duplicate samples)

Time (days)	Degradate/Code-No.						Total
	Parent	M1	M2	M3	M4	M5	
	Retention Time (min.)						
	12.97	2.02	2.62	11.17	12.43	13.38	
	Identity/CGA-No.						
	329351	UK	UK	UK	UK	UK	
0.00	97.27	0.00	0.00	0.75	0.59	0.89	99.49
1.00	86.29	9.71	0.26	0.73	0.17	1.37	98.53
2.00	79.34	17.65	0.00	0.68	0.00	1.82	99.49
4.00	69.91	4.33	23.61	0.62	0.00	0.52	99.00
5.02	63.25	4.25	29.79	0.65	0.08	0.53	98.54

Table 7: Degradation of CGA 329351 in Buffer Solution pH 9 at 25°C (Final-test)

(Values given represent mean of two samples.)

Time (days)	Degradate/Code-No.					Total
	Parent	M1	M3	M4	M5	
	Retention Time (min.)					
	12.97	2.02 -3.50	11.17	12.43	13.38	
	Identity/CGA-No.					
	329351		UK	UK	UK	
0.00	97.97	0.00	0.00	0.87	1.17	100.00
2.00	95.16	2.25	0.80	0.24	0.63	99.08
4.00	94.27	3.67	0.68	0.22	0.78	99.63
6.00	92.79	4.64	0.65	0.21	0.92	99.20
8.00	91.22	5.61	0.56	0.45	1.02	98.85
11.00	90.92	5.93	0.42	0.38	1.24	98.89
13.00	88.43	7.87	0.57	0.10	1.70	98.68
15.00	88.22	8.21	0.66	0.21	1.49	98.79
18.00	86.62	10.75	0.81	0.63	0.57	99.37
20.00	85.70	11.65	0.64	0.20	0.84	99.02
22.00	84.26	11.87	0.90	0.00	1.48	98.51
25.00	83.76	13.47	0.51	0.00	1.26	99.01
26.00	83.78	12.84	0.52	0.13	1.43	98.70
27.00	82.18	14.35	0.60	0.19	1.99	99.30
28.00	81.89	14.59	1.00	0.19	1.42	99.09
29.00	80.99	14.73	0.99	0.20	1.82	98.73
32.00	79.74	15.97	0.60	0.30	2.16	98.76

Table 8: Degradation of CGA 329351 in Buffer Solution pH 9 at 50°C (Final-test)

Time (days)	(Seconds)	Degradate/Code-No. Parent	M1	M2	M3	M4	M5	Total
		Retention Time (min.)						
		12.97	2.02 -3.50	7.15	11.17	12.43	13.38	
		Identity/CGA-No.						
		329351		UK	UK	UK	UK	
0.00	0	98.25	0.00	0.00	0.48	0.51	0.76	100.00
2.00	173100	80.22	17.96	0.00	1.04	0.00	0.82	100.04
5.00	432000	60.62	38.33	0.00	0.17	0.00	0.60	99.72
6.00	518400	57.97	40.42	0.00	0.73	0.00	0.44	99.56
8.00	691200	46.95	50.86	0.00	0.92	0.00	0.24	98.96
11.00	950400	35.09	62.37	0.00	0.81	0.18	0.73	99.19
13.00	1123200	30.83	67.59	0.00	0.61	0.32	0.56	99.92
15.00	1296000	26.15	69.46	1.77	0.29	0.00	1.13	98.79

Table 9: Degradation of CGA 329351 in Buffer Solution pH 9 at 60°C (Final-test)

Time		Degradate/Code-No.						Total
(days)	(Seconds)	Parent	M1	M2	M3	M4	M5	
		Retention Time (min.)						
		12.97	2.02 -3.50	4.48	11.17	12.43	13.38	
		Identity/CGA-No.						
		329351	UK	UK	UK	UK	UK	
0.00	0	97.95	0.90	0.00	0.00	0.37	0.78	100.00
2.00	173100	58.82	39.08	1.13	0.00	0.00	0.57	99.59
4.00	345600	34.27	63.89	0.00	0.81	0.00	0.44	99.40
5.00	432000	27.06	71.32	0.00	0.45	0.00	0.47	99.28
6.00	518400	20.93	76.17	0.00	0.56	0.22	0.66	98.54
7.00	604800	17.18	81.16	0.00	0.51	0.49	0.47	99.80
8.00	691200	11.90	86.51	0.00	0.36	0.00	0.75	99.52
11.00	950400	7.07	91.30	0.00	0.61	0.00	0.32	99.29

Table 10: Criteria for further Testing after the Pre-test

pH	Degradation at 50°C	Final test/Temp.	Half-life Estimate (days)	No. Samplin g times	Duration	Replicates	No. of Samples/Temperature[12]		
							25/37°C	50°C	60°C
1	≥ 10%	Yes/37°C		8	1-2 half-lives or 30 days	yes	16	-	-
	< 10%	No					-	-	-
5	≥ 10%	Yes/25, 50, 60°C	≤ 15	8	2 half-lives	yes for 25°C	16	9	9
			>15-≤ 30	8	1 half-life		16	9	9
			>30 - <198	17	30 days		34	18	18
	< 10%	Final test at 25°C		3	30 days	yes	6	-	-
7	≥ 10%	Yes/25, 50, 60°C	≤ 15	8	2 half-lives	yes for 25°C	16[13]	9	9
			>15-≤ 30	8	1 half-life		16[13]	9	9
			>30 - <198	17	30 days		34[14]	18	18
	< 10%	Final test at 25°C		3	30 days	yes	6[15]		
9	≥ 10%	Yes/25, 50, 60°C	≤ 15	8	2 half-lives	yes for 25°C	16	9	9
			>15-≤ 30	8	1 half-life		16	9	9
			>30 - <198	17	30 days		34	18	18
	< 10%	Final test at 25°C		3	30 days	yes	6	-	-

12 For all conditions time zero samples in duplicate.
13 At least 6 sampling times between 20 and 70% of degradation (pH's 5, 7and 9).
14 At least 15 samples between 10 and 30% of degradation (pH's 5, 7and 9).
15 If no degradation is observed in the pre-test (<5 %) no further testing.

Table 11: Rate constants of Hydrolysis of CGA 329351 in Buffer Solution pH 9 at various Temperatures

Temperature (°C)	(°K)	k (s-1)	Half-life (days)	Chi^2
25	298	6.89E-08	116.4	0.39
50	323	1.04E-06	7.7	1.36
60	333	2.97E-06	2.7	0.60

Table 12: Calculated Arrhenius (Ea, A) and Eyring (ΔH#, ΔS#) Parameters for pH 9

Arrhenius		Eyring (25°C)	
Ea	A	ΔH#	ΔS#
J*mole-1	s-1	J*mole-1	J*°K-1
88336	2.08E+08	85900	73

Figure 1: Purity of ^{14}C-CGA 329351

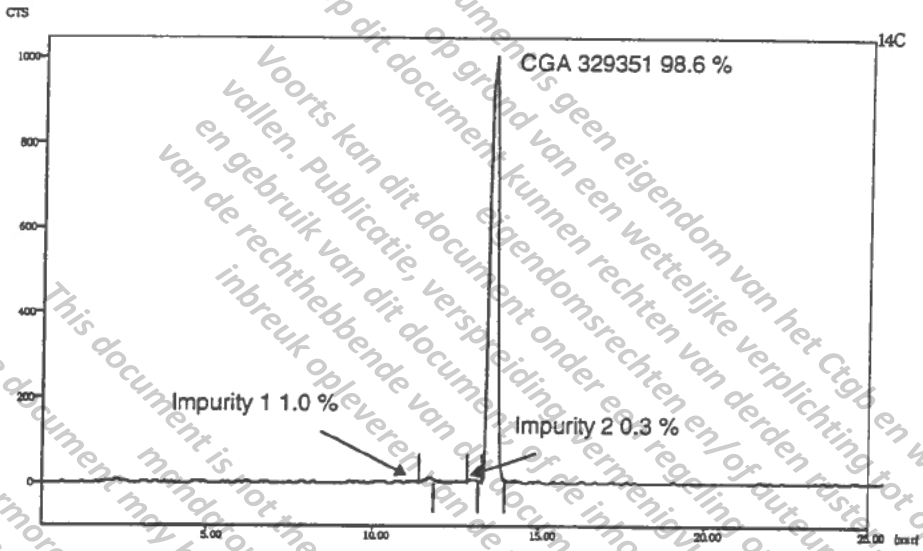


Figure 2: HPLC of Hydrolysis Solution of CGA 329351 at pH 9 and 25°C

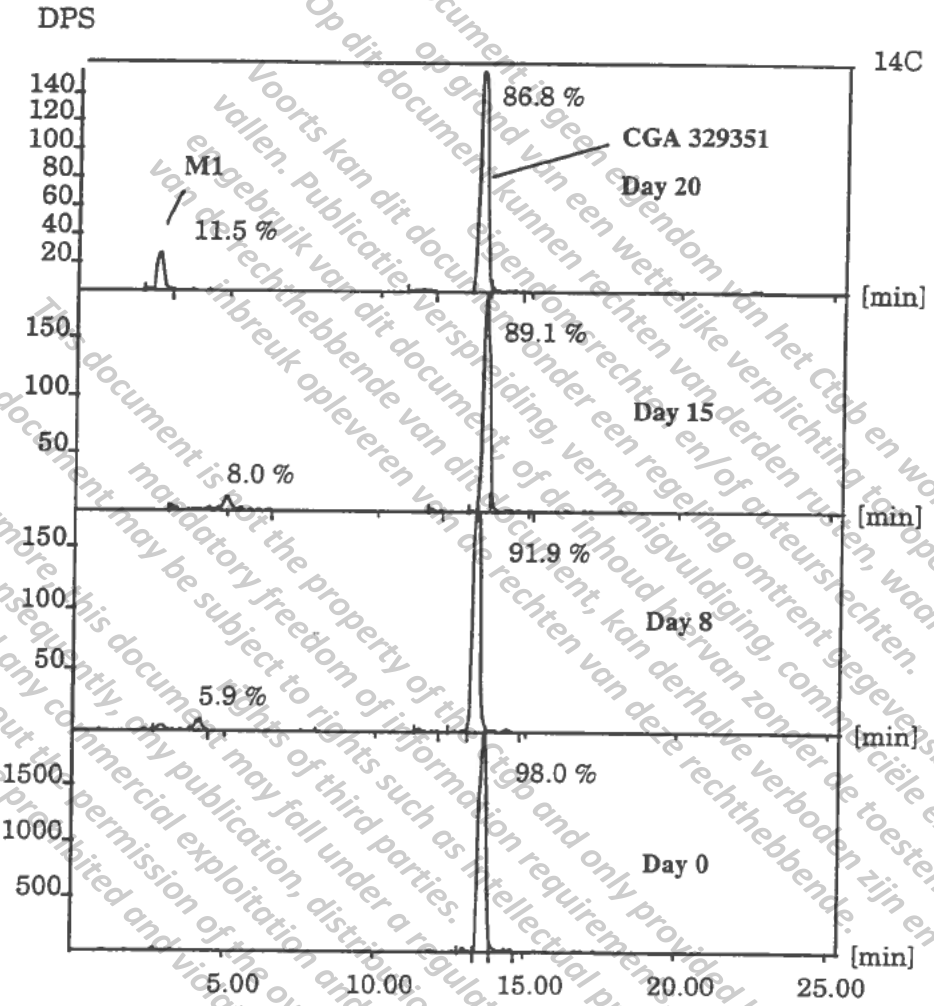
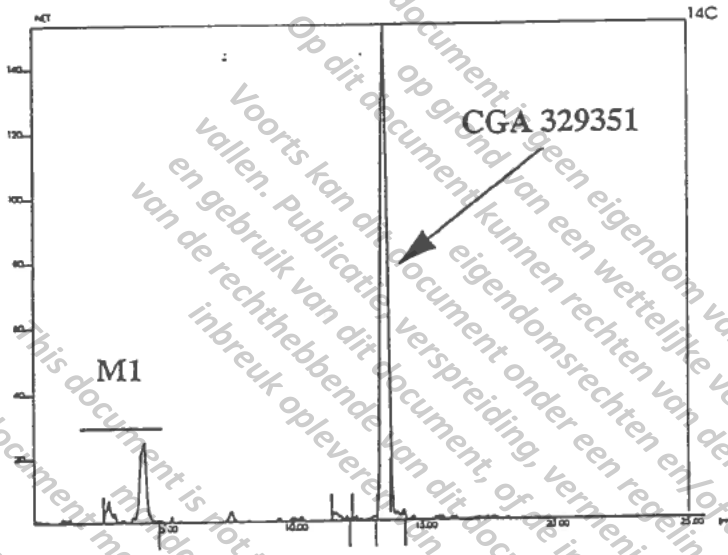


Figure 2 continued: HPLC of sample exposed for 32 days at pH 9 and 25°C (Series A)



File name : 25M17001.CH2 User : 14C Curr. Date : 4-Oct-95 17:00:34
Run Length : 25.00 [min]Acqu. Date : 2-Oct-95 19:20:18
Sample 17A/32 days/pH 9 25C/0.1 ml = 33997 dpm
Control Method : YG_150
LL = 25 UL = 750
Bkg (Cpm) = 15 Channel Delay(Sec) = 16
Efficiency % = 75

#	Name	Rt	NET	%Area
1	M1	4.12	345	15.16
2	M3	11.27	17	0.76
3	M4	12.05	7	0.32
4	CGA329351	13.15	1851	81.36
5	M5	13.40	54	2.40

Total Area of Peak = 2275.00

Injection Volume = 100.00 µl

Figure 3: HPLC of Hydrolysis Solution of CGA 329351 at pH 9 and 50°C

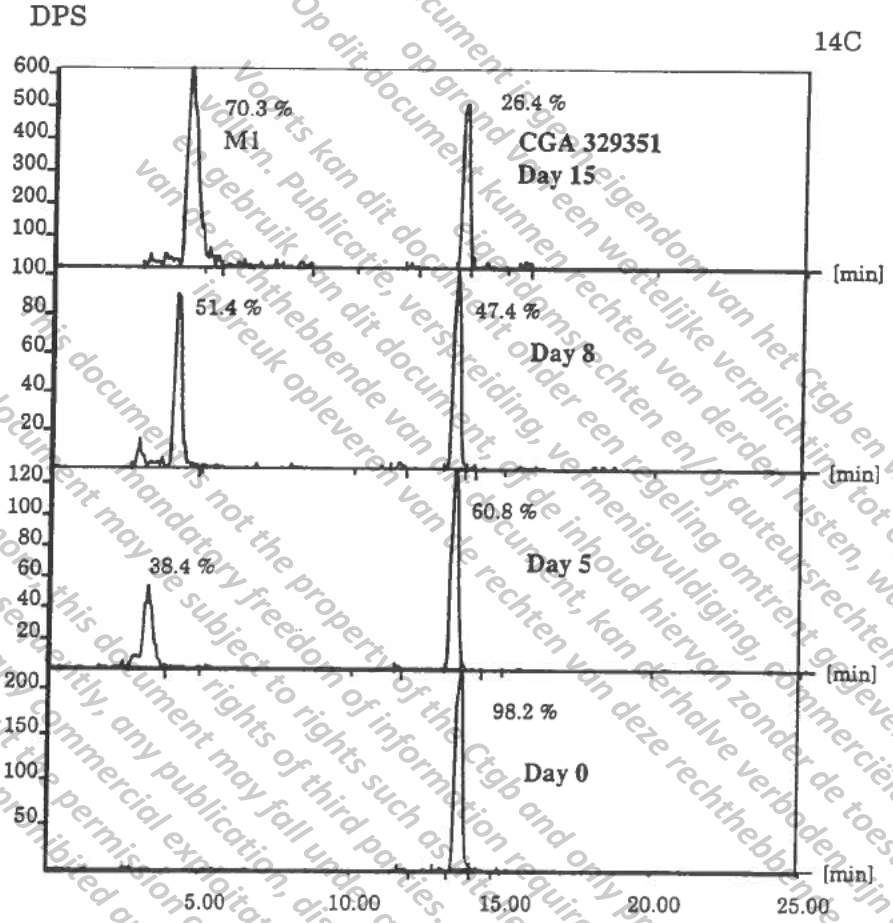


Figure 4: HPLC of Hydrolysis Solution of CGA 329351 at pH 9 60°C

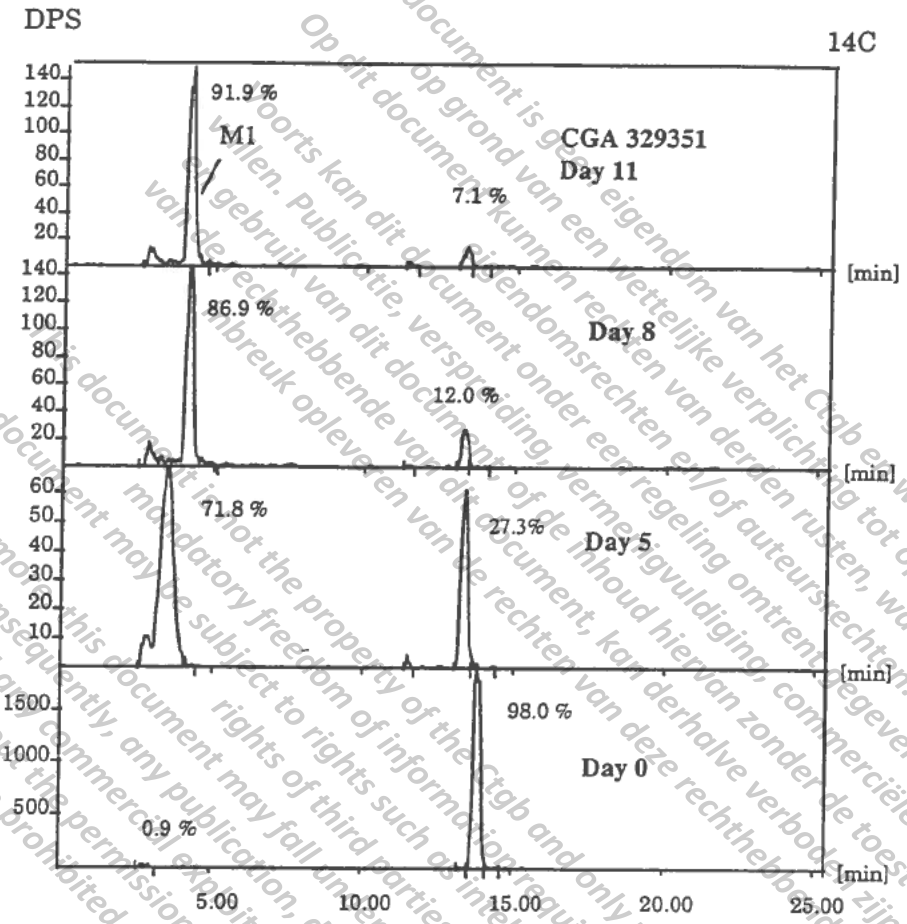


Figure 5: High Voltage Electrophoresis at pH 10 with Degradate M1

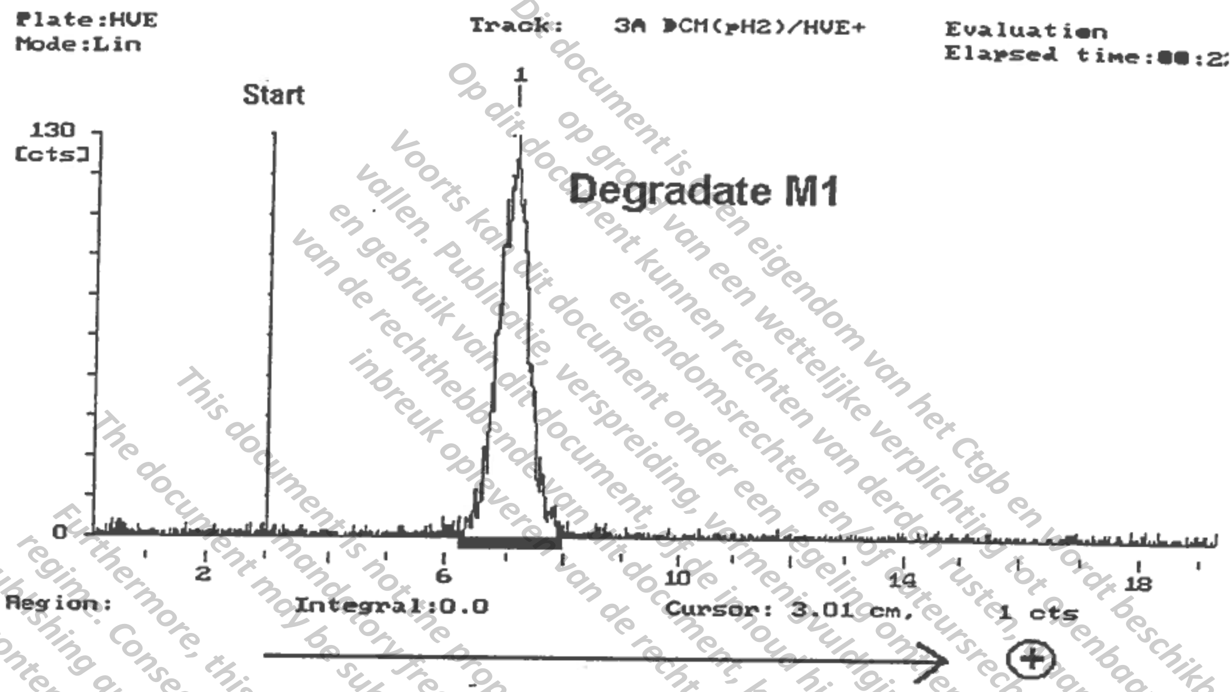
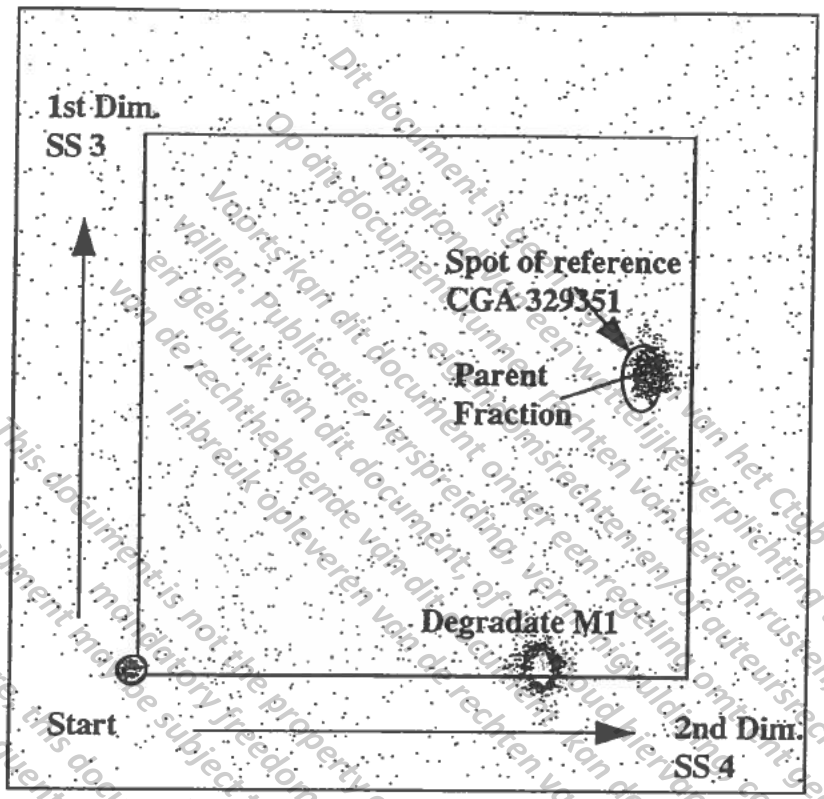


Figure 6: Co-Chromatography on two-dimensional TLC: Parent Fraction from Hydrolysis with Reference CGA 329351



DAR Region Integration Report: EG&G Berthold

Plate name: 95EH05 Meas date: 6 Sep 1995 14:04:39
File name: 95EH05M1.DAR Run time: 01 hrs 00 min 10 sec
Gain: High resolution Z-calibration: None
Start x: 3.00 Front x: 17.00 Instr backgrnd: 213.7872 cpm/cm²
Start y: 3.00 Front y: 17.00 Dead time: 8.4 sec (inactive)
Comments: Metaboliten Ansatz Abbruch Analysis of 100% region 1: AllPlate

Label	Rf(x)	Rf(y)	x(cm)	y(cm)	cts	cpm	cpm/cm²	SD	ROIs	All
B1	0.82	0.28	14.41	6.85	1415	23.5	1.5	2.7	52.70	
B2	0.44	0.55	9.20	10.76	1270	21.1	1.6	2.8	47.30	
Average background = 88.004				**cts/cm²**		**1.46**	**cpm/cm²**			
M1	0.02	0.74	3.28	13.41	16108	267.7	163.3	0.8	72.64	77.8
CGA329351	0.56	0.93	10.84	16.04	6066	100.8	71.8	1.3	27.36	29.3

Figure 7: HPLC of Sample 17A (32 days; 25°C) and Reference Substance CGA 329351

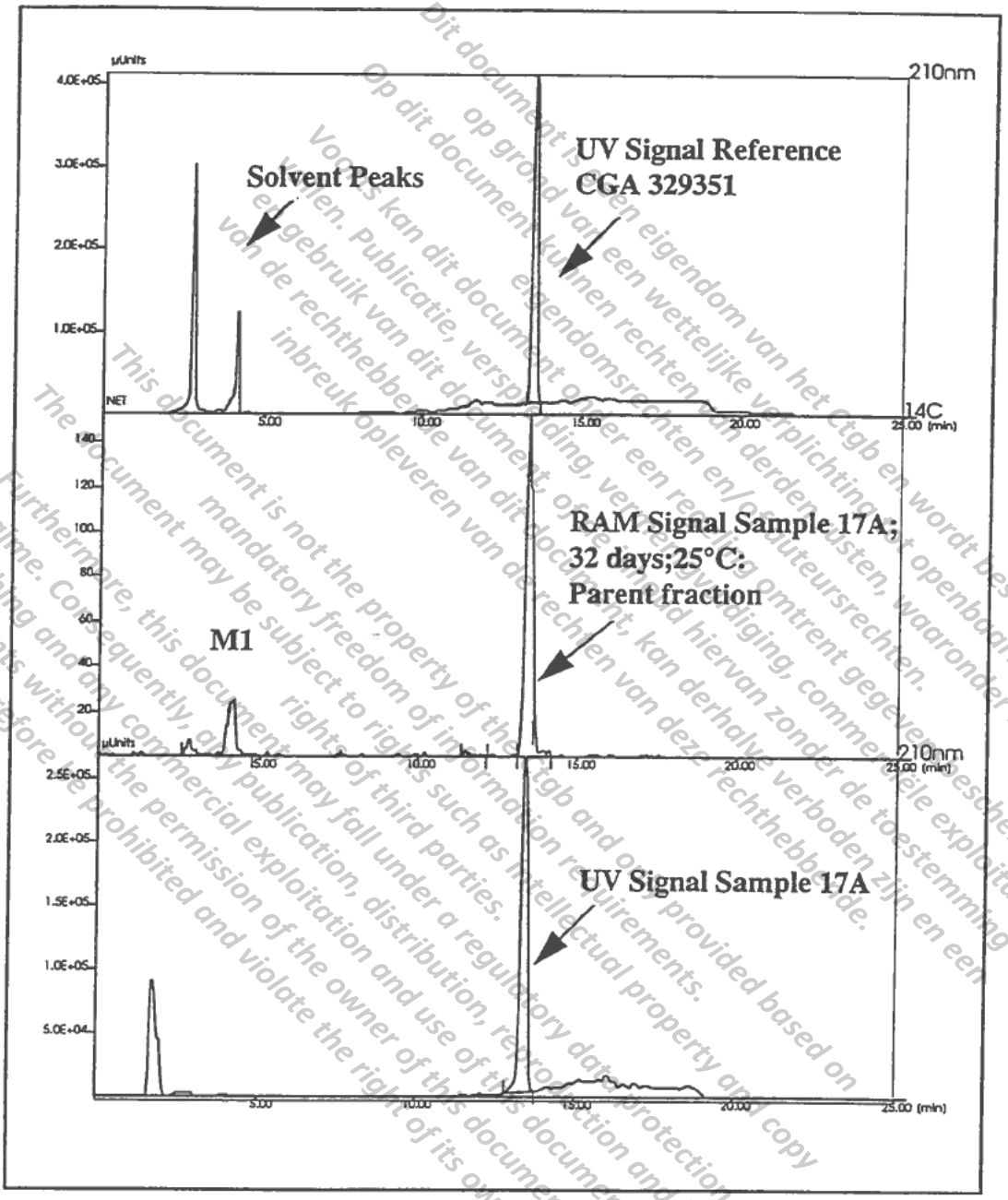


Figure 8: Degradation of CGA 329351 at pH 9 and 50°C (Pre-test) and estimated Degradation Curves for 25, 50 and 60°C on the Basis of the Pre-test Data

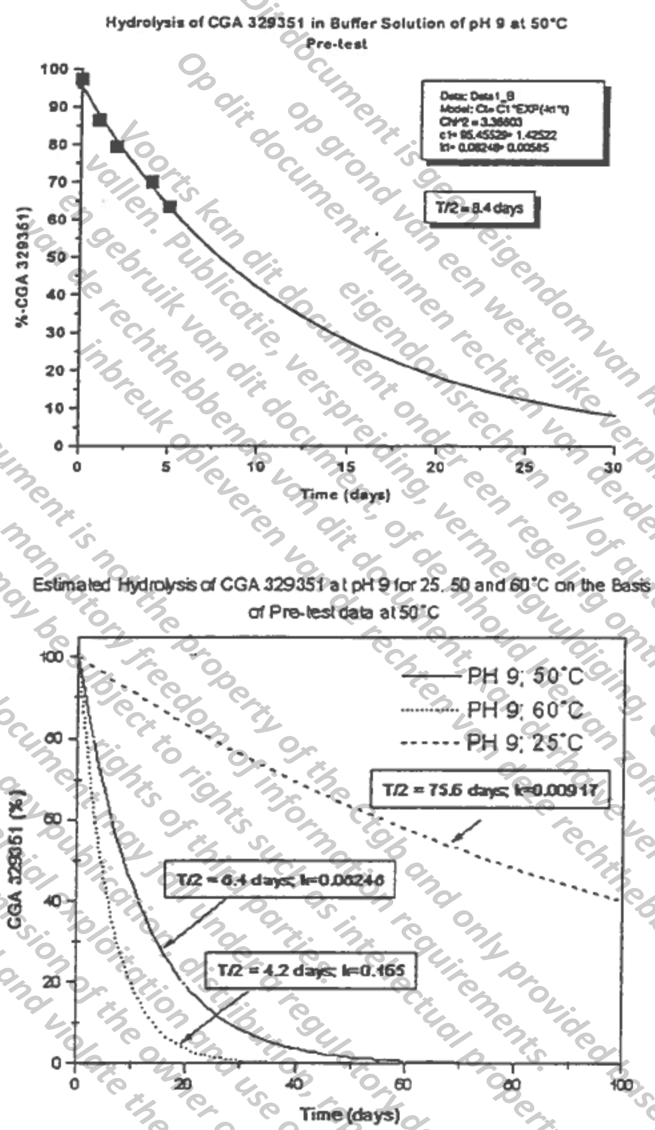
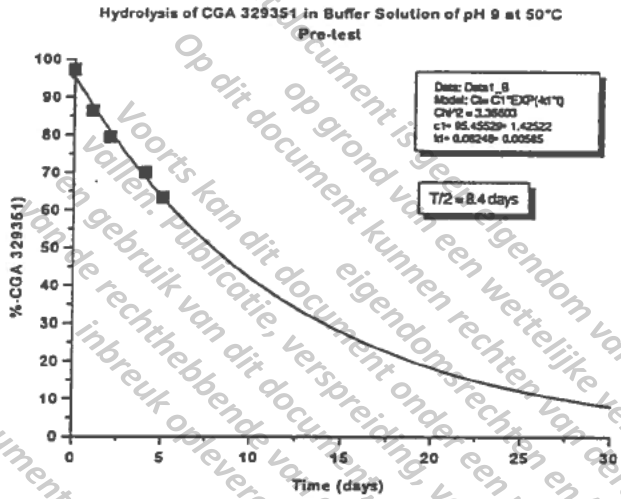


Figure 8: Degradation of CGA 329351 at pH 9 and 50°C (Pre-test) and estimated Degradation Curves for 25, 50 and 60°C on the Basis of the Pre-test Data



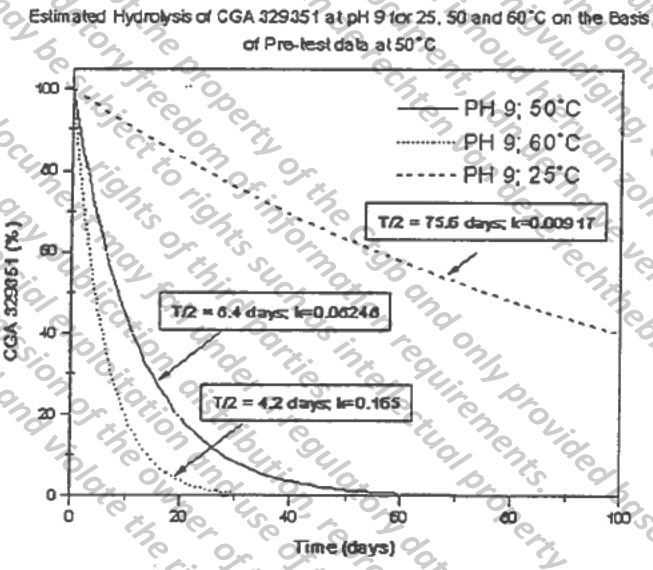


Figure 9: Hydrolysis Rate of CGA 329351 at pH 9 and 25 °C (Final Test)

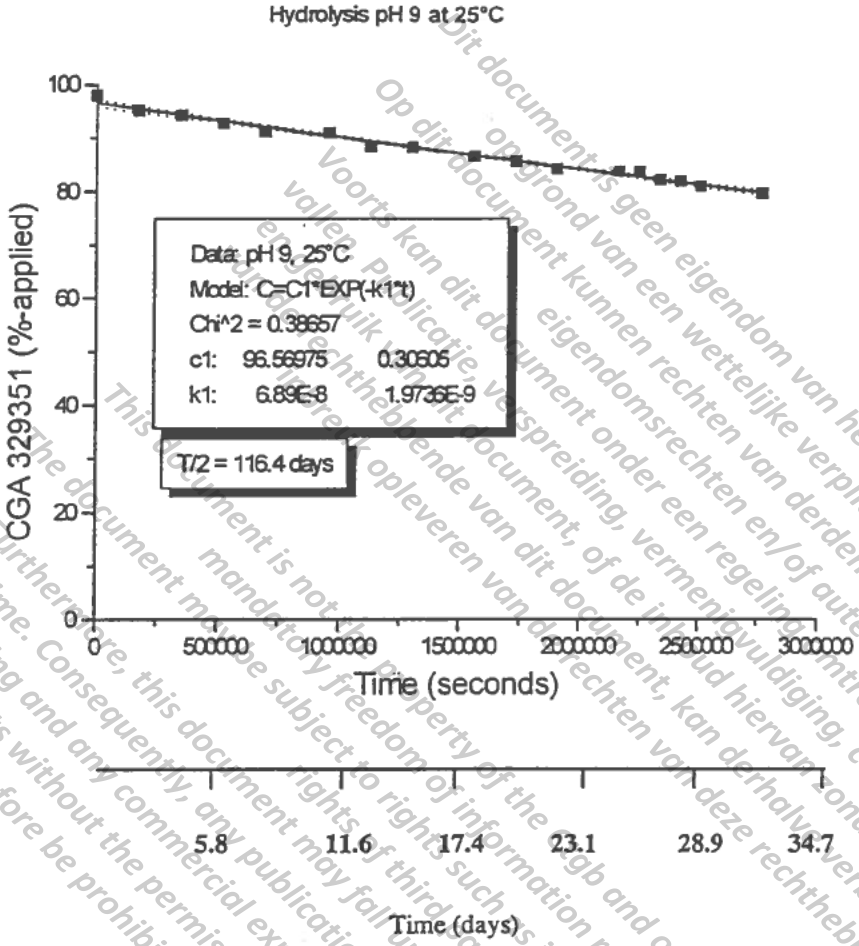


Figure 10: Hydrolysis Rate of CGA 329351 at pH 9 and 50 °C (Final Test)

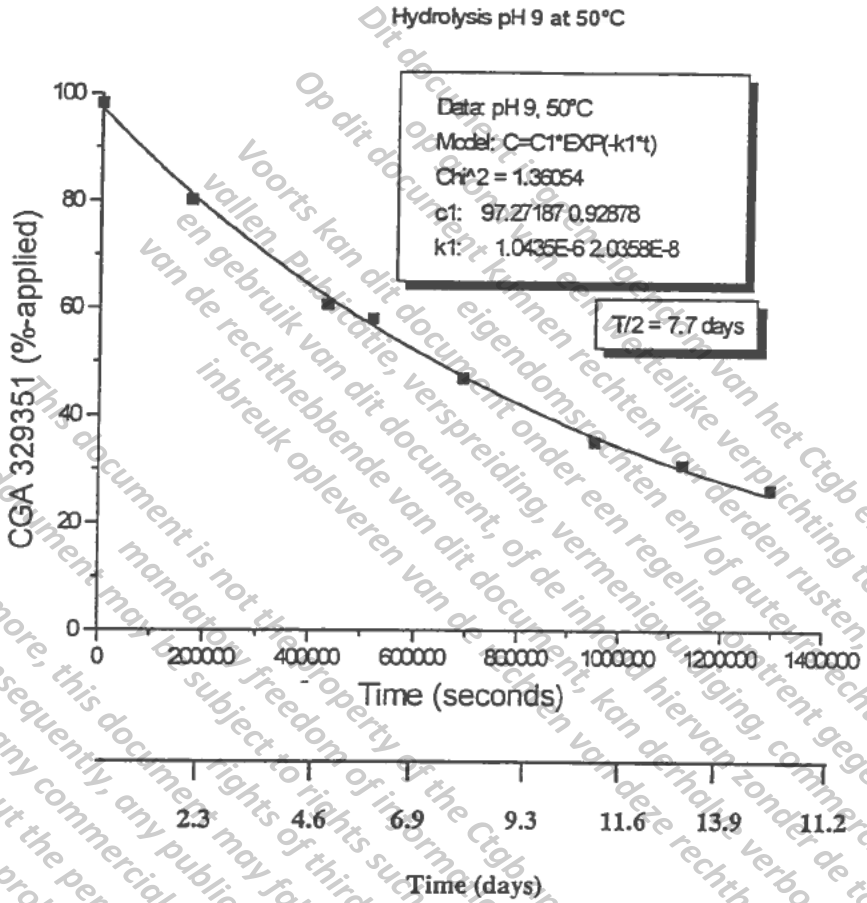


Figure 11: Hydrolysis Rate of CGA 329351 at pH 9 and 60 °C (Final Test)

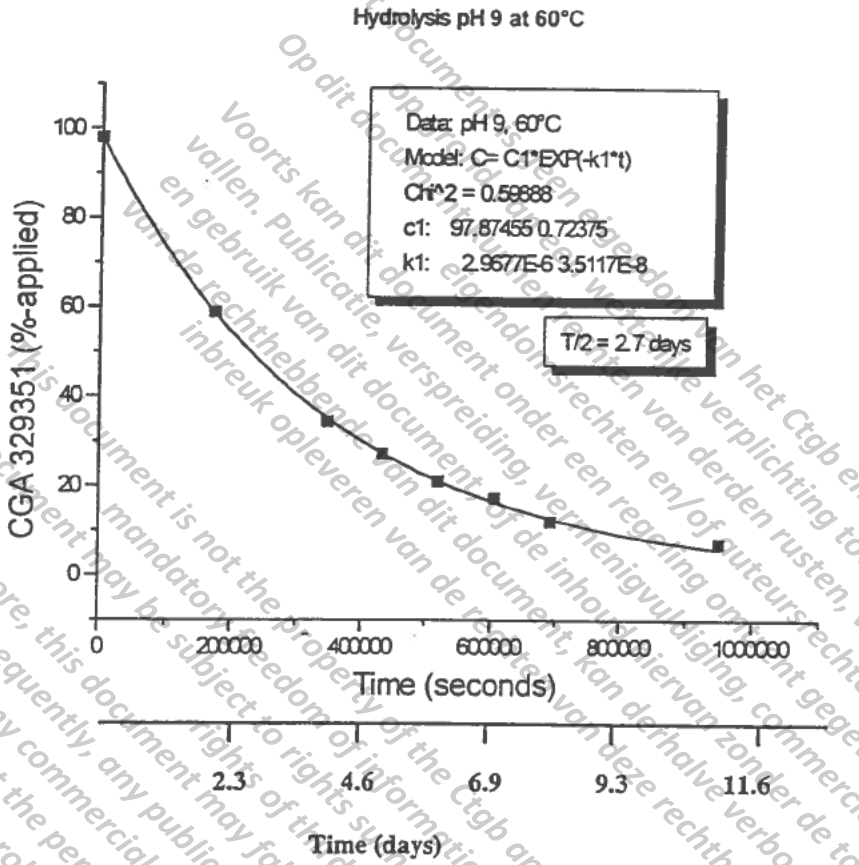


Figure 12: Arrhenius Plot of Reaction Constants (lnk) versus inverse Temperature (1/T)

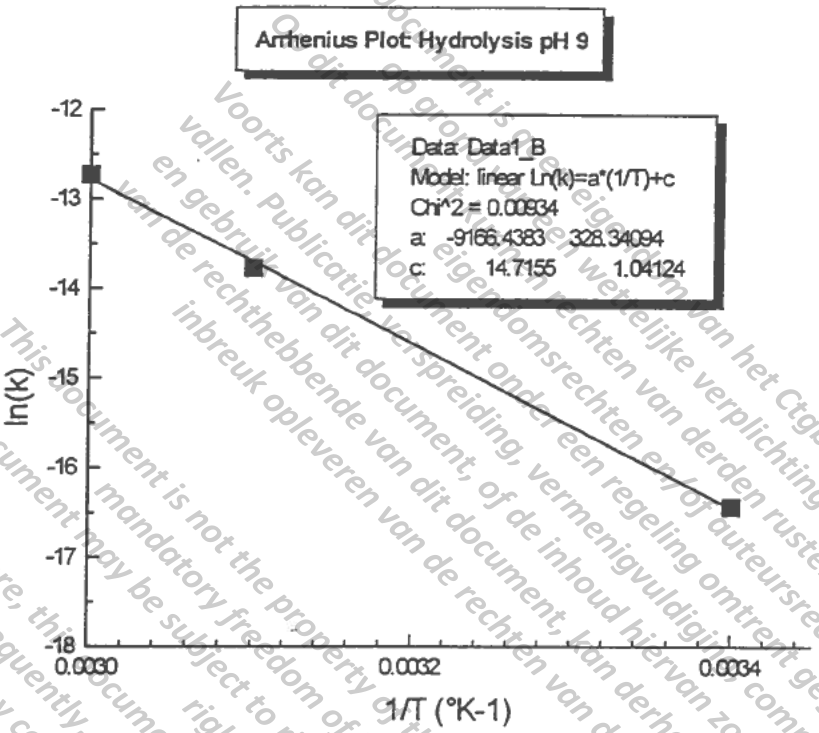


Figure 13: Temperature Control of Pre-Test and Final Test at pH 9

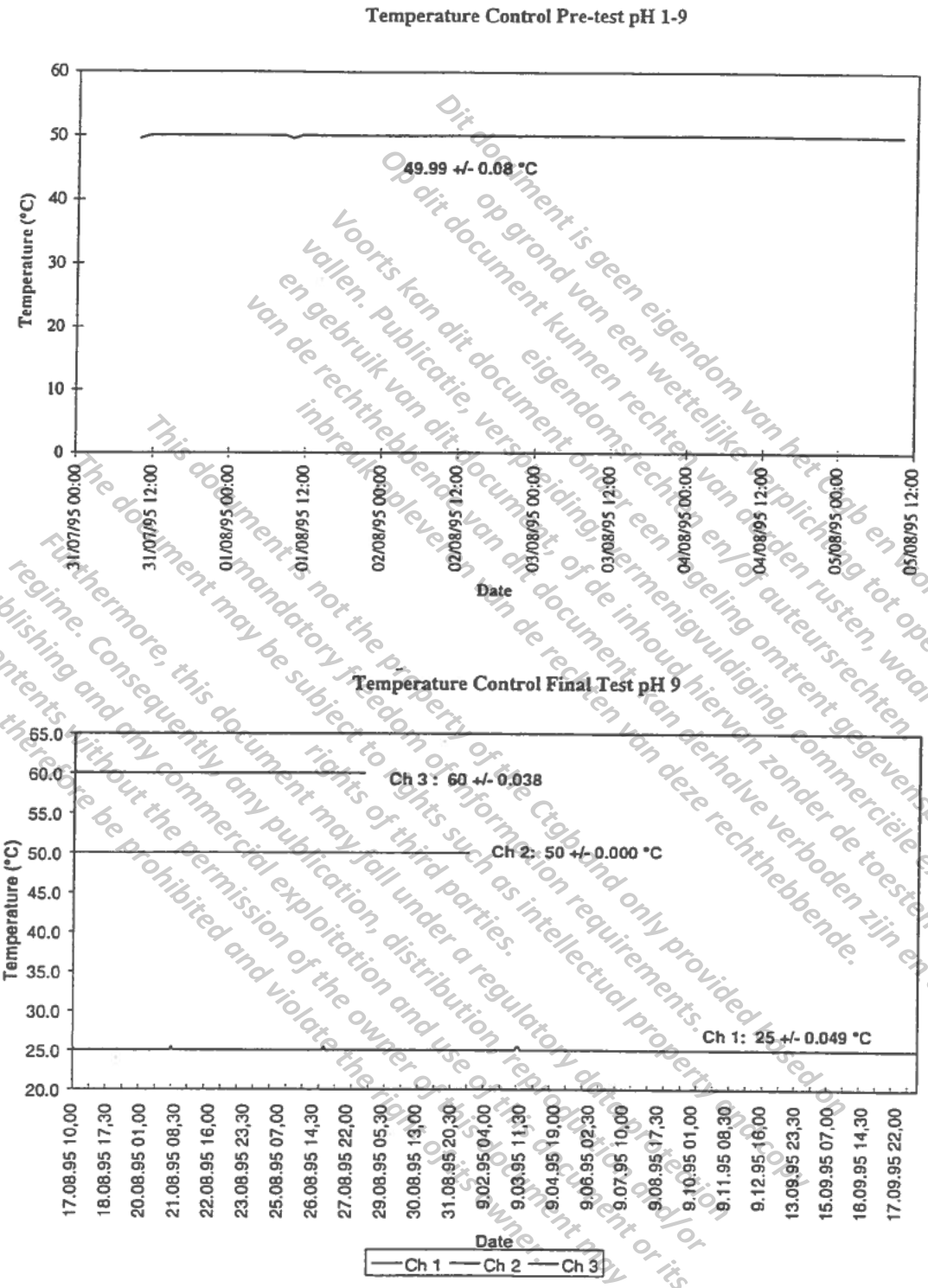
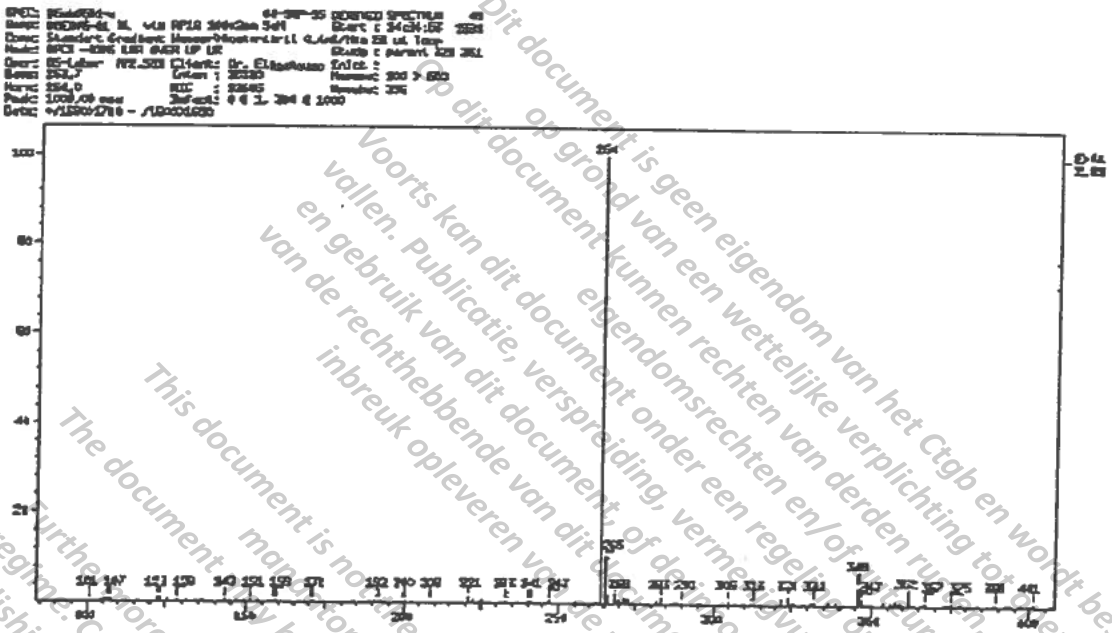


Figure 14: Mass Spectrum of Degradate M1 (negative ion) on LC-MS after APCI and of Reference CGA 62826

APCI mass spectrum negative mode



APCI mass spectrum negative mode: reference CGA 62 826

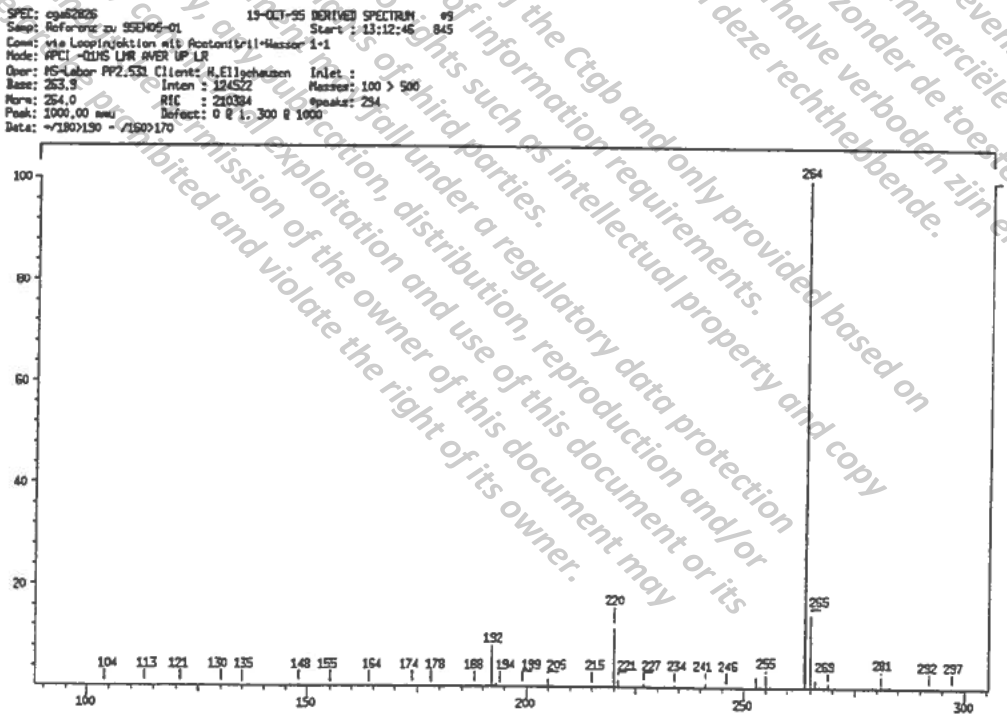
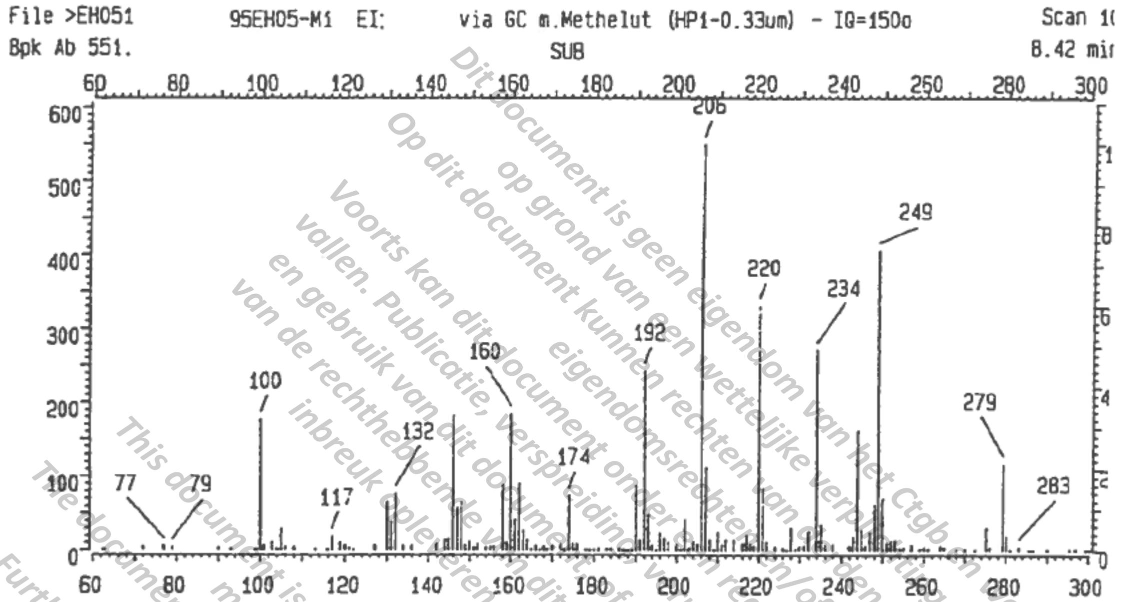


Figure 15: Mass Spectrum of Degradate M1 (EI Mode; on Column Methylation) and of Reference CGA 329351

Mass spectrum EI-mode: metabolite M1; on column methylation



Mass spectrum EI-mode: reference CGA 329 351

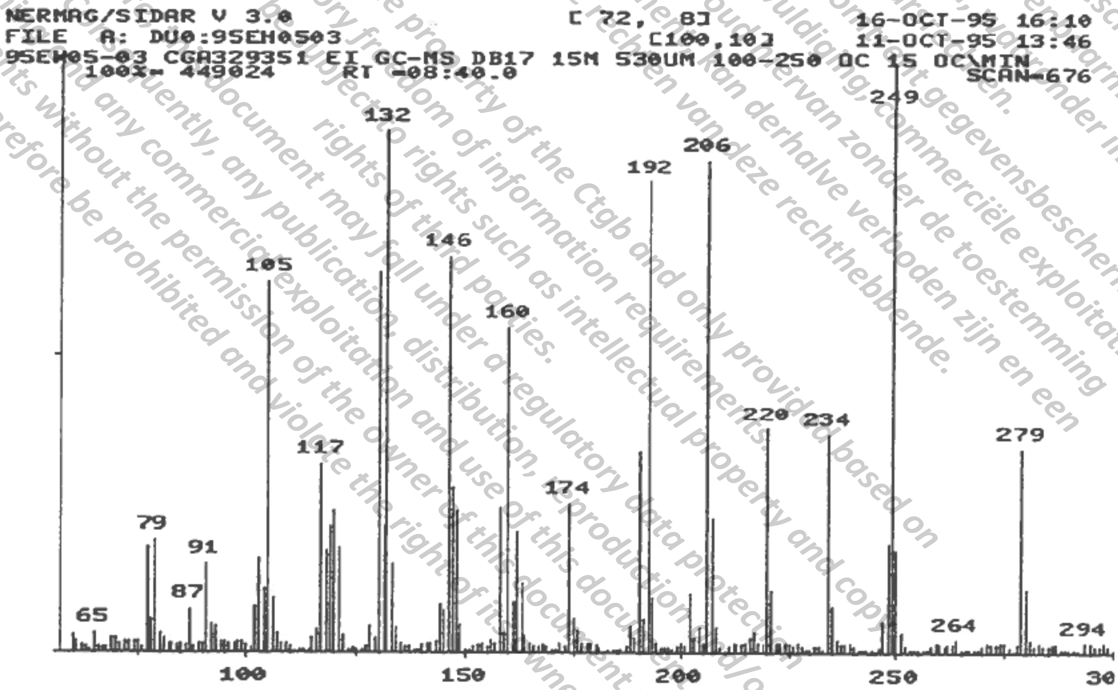
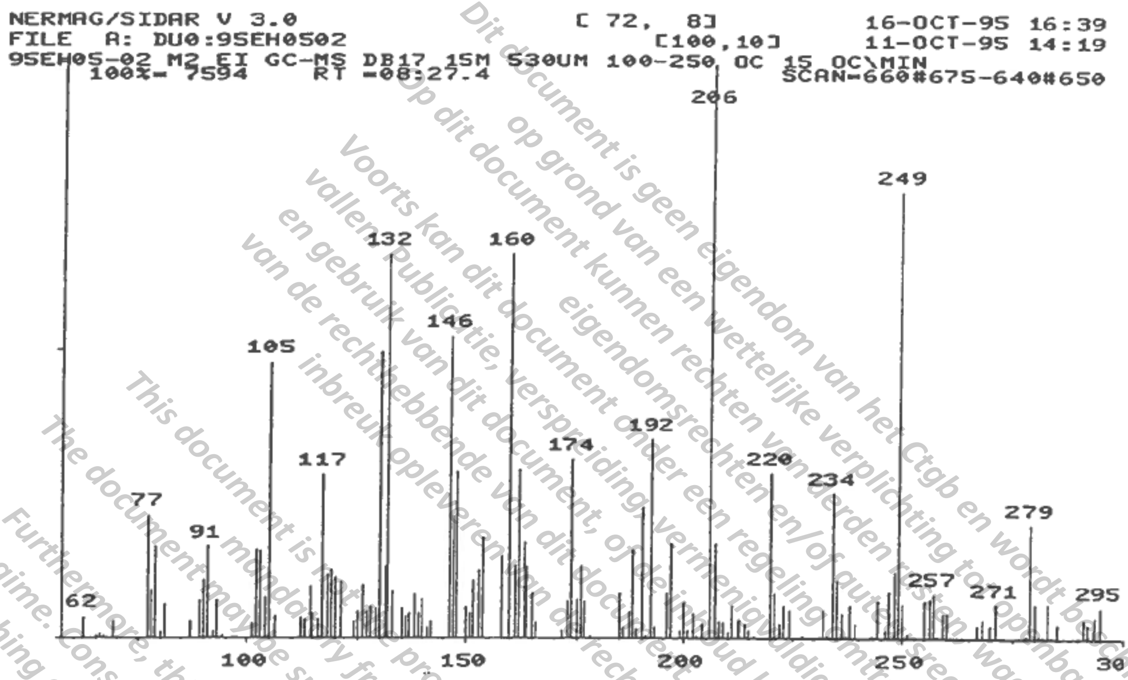


Figure 16: Mass Spectrum of Radioactive Parent Fraction (M2) isolated from Sample 17a (pH 9, 25°C, 32 days)

Mass spectrum EI-mode:



Mass spectra CI-mode with methane:

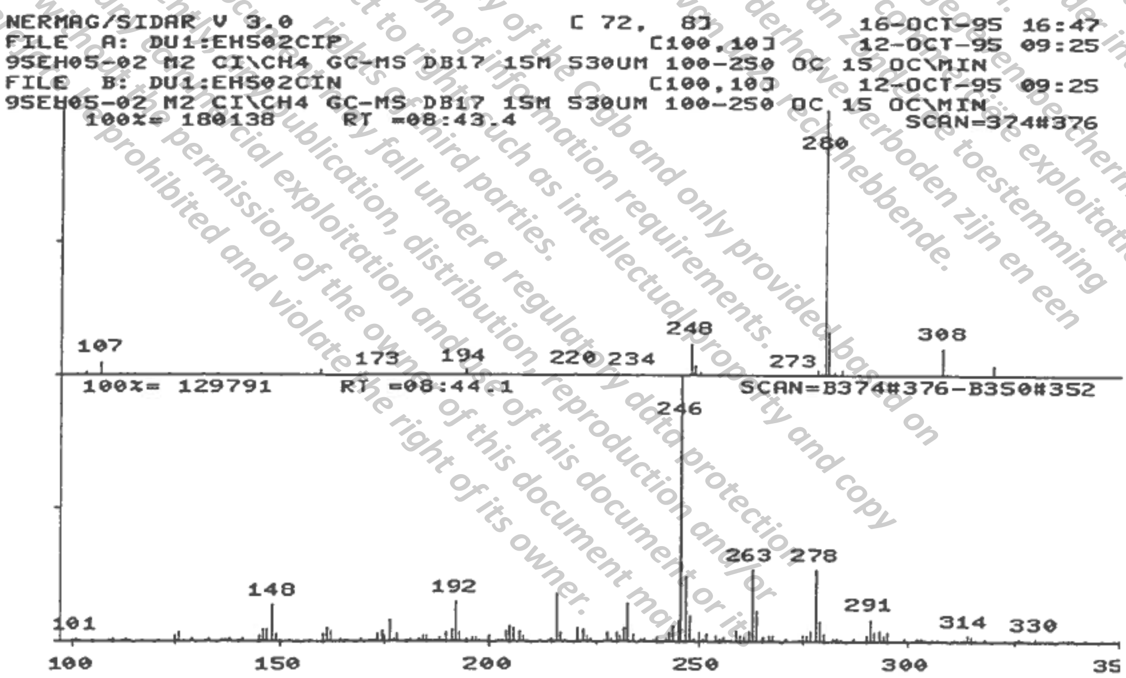
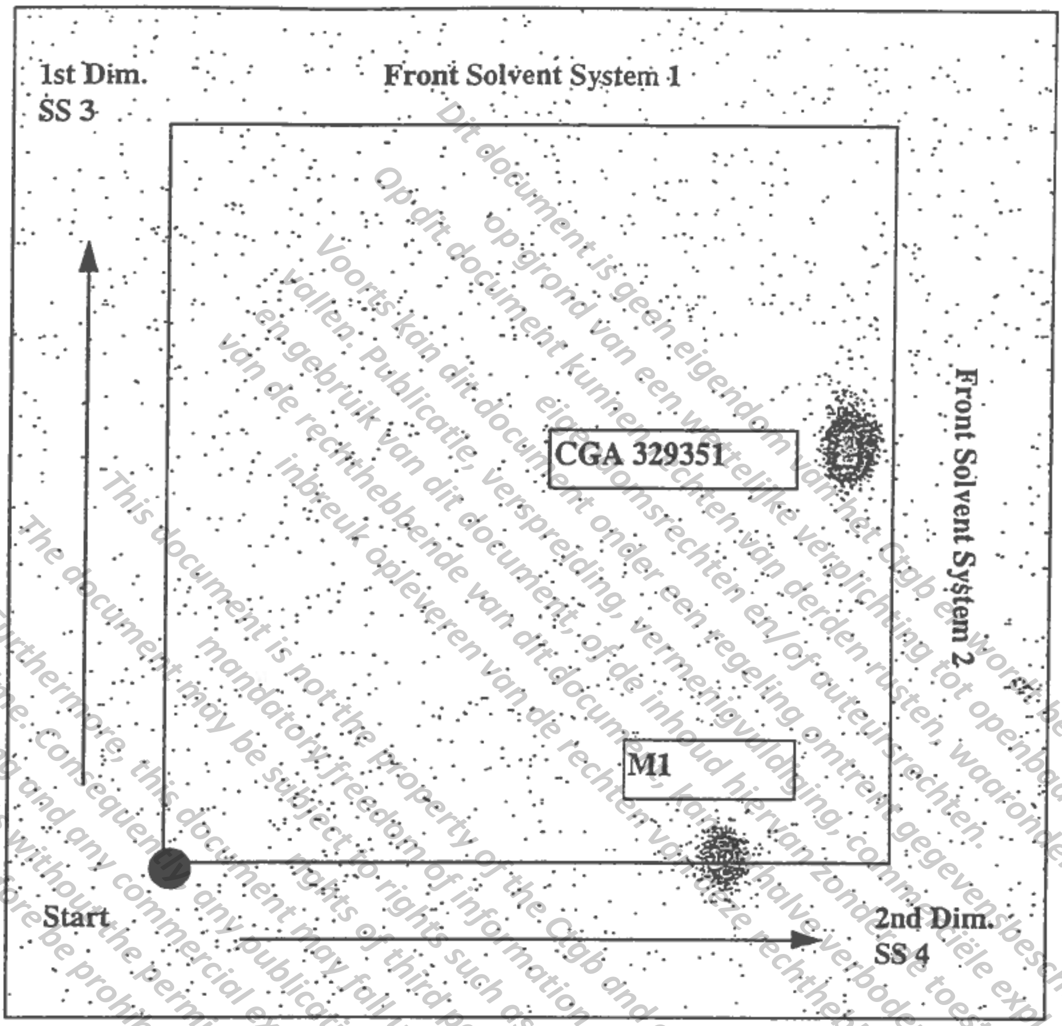


Figure 17: 2D-TLC with Buffer Solution of CGA 329351 exposed to pH 9 for 32 days at 25°C (Sample 17A)



DAR Region Integration Report

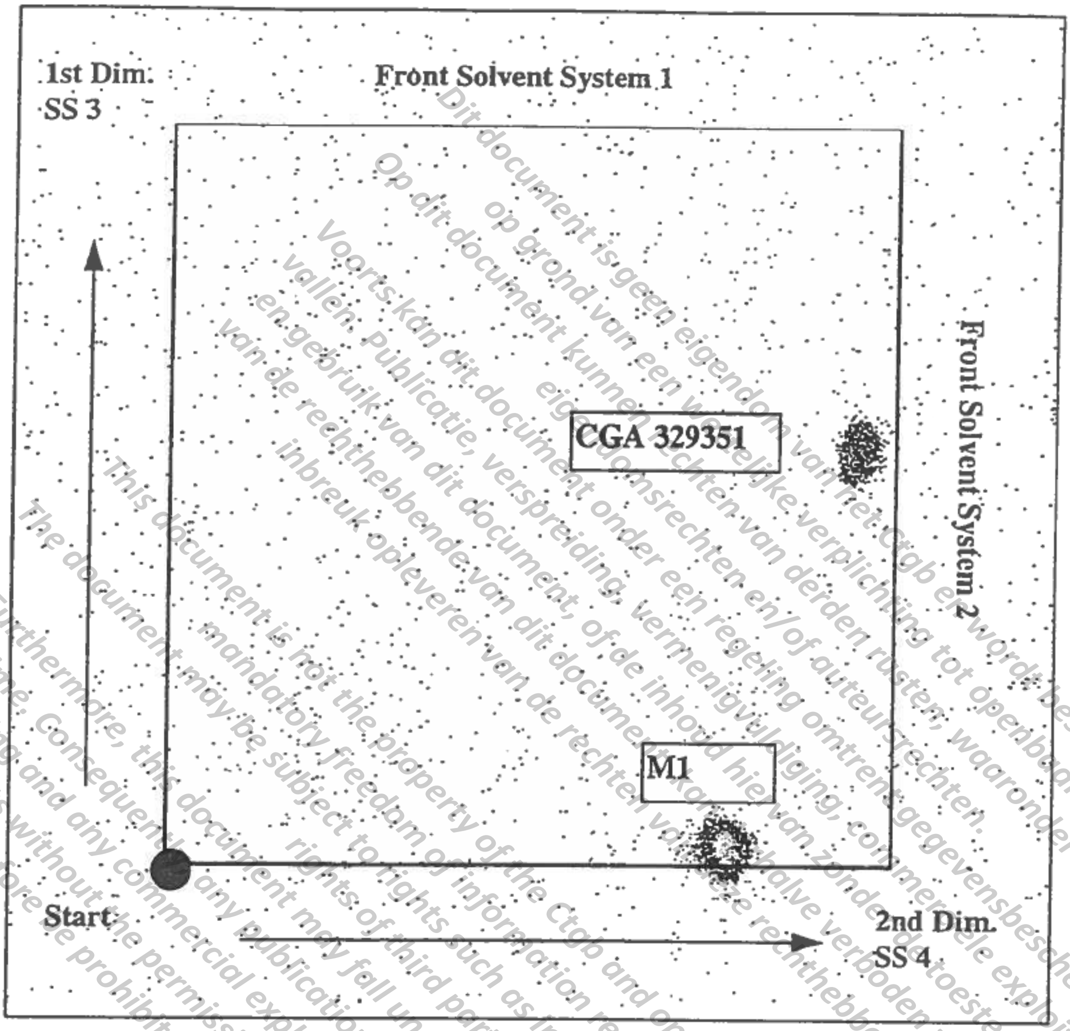
5.1 2.e Woo

Plate name: TLC18 Meas date: 4 Oct 1995 18:30:03
File name: 95EH05_1.DAR Run time: 01 hrs 00 min 10 sec
Gain: High resolution Z-calibration: None
Start x: 3.00 Front x: 16.00 Instr backgrnd: 214.2794 cpm/cm²
Start y: 3.00 Front y: 17.00 Dead time: 8.3 sec (active)
Pk search params: n/a
Comments: M17A pH9 25°C
30µl = 10199dpm
Analysis of 100% region 1: AllPlate

Rgn	Label	Position Rf(x)	Rf(y)	Net x(cm)	Net y(cm)	Net cts	% cpm	% cpm/cm²	% SD	ROIs	All	Shape
	B1		0.74	0.22	12.68	6.09	2032	33.8	1.5	2.2	51.30	
	B2		0.45	0.57	8.87	11.03	1929	32.1	1.4	2.3	48.70	
Average background =86.836 cts/cm²					1.44	cpm/cm²						
1	M1		0.02	0.77	3.28	13.80	3855	64.1	37.5	1.6	18.51	
2	CGA329351		0.64	0.94	11.35	16.12	16973	282.1	112.3	0.8	81.49	

	Counts	Cpm
Total gross:	54546	906.6
Total net:	19811	329.3
Gross in ROIs:	21196	352.3
Net in ROIs:	20829	346.2

Figure 18: 2D-TLC with Buffer Solution of CGA 329351 exposed to pH 9 for 32 days at 50°C



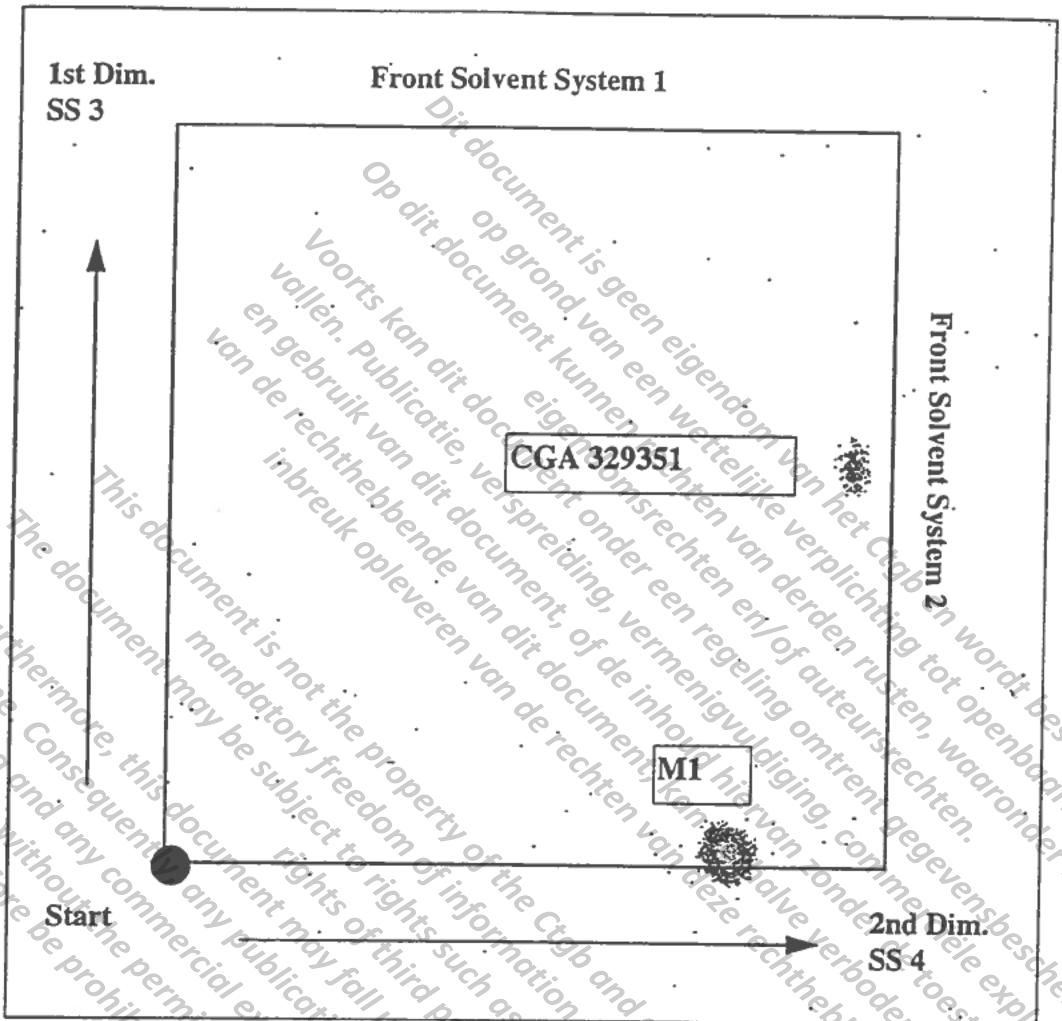
DAR Region Integration Report
5.1.2.e Woo [redacted]

Plate name: TLC19 Meas date: 4 Oct 1995 19:39:35
File name: 95EH05_2.DAR Run time: 01 hrs 00 min 10 sec
Gain: High resolution Z-calibration: None
Start x: 3.00 Front x: 17.00 Instr backgrnd: 214.2527 cpm/cm²
Start y: 3.00 Front y: 17.00 Dead time: 7.8 sec (active)
Pk search params: n/a
Comments: M8 pH9 50°C 25µl = 9785dpm
Analysis of 100% region 1: AllPlate

Rgn	Label	Rf(x)	Rf(y)	Position x(cm)	y(cm)	Net cts	Net cpm	Net cpm/cm²	% SD	% ROIs	
B1		0.79	0.23	14.02	6.26	1888	31.4	1.6	2.3	51.35	
B2		0.40	0.61	8.55	11.50	1788	29.7	1.4	2.4	48.65	
Average background = 88.074 cts/cm²					1.46	cpm/cm²					
1 M1			0.01	0.77	3.21	13.80	14833	246.5	106.6	0.8	77.90
2 CGA32935	1		0.58	0.95	11.18	16.24	4209	70.0	41.6	1.6	22.10

	Counts	Cpm
Total gross:	51964	863.7
Total net:	16734	278.1
Gross in ROIs:	19394	322.3
Net in ROIs:	19042	316.5

Figure 19: 2D-TLC with Buffer Solution of CGA 329351 exposed to pH 9 for 32 days at 60°C



DAR Region Integration Report
[redacted]

Plate name: TLC20 Meas date: 4 Oct 1995 20:49:07
File name: 95EH05_3.DAR Run time: 01 hrs 00 min 10 sec
Gain: High resolution Z-calibration: None
Start x:3.00 Front x: 17.00 Instr backgrnd: 214.2330 cpm/cm²
Start y:3.00 Front y: 17.00 Dead time: 7.5 sec (active)
Pk search params: n/a
Comments: M8 pH9 60°C 25µl = 9574dpm
Analysis of 100% region 1: AllPlate

Rgn	Label	Position Rf(x)	Rf(y)	x(cm)	y(cm)	Net cts	Net cpm	Net cpm/cm²	% SD	% ROIs	%
B1		0.80	0.24	14.17	6.39	1894	31.5	1.6	2.3	46.07	
B2		0.41	0.63	8.77	11.84	2218	36.9	1.4	2.1	53.93	

Average background =89.864 cts/cm² 1.49 cpm/cm²

Rgn	Label	Rf(x)	Rf(y)	x(cm)	y(cm)	Net cts	Net cpm	Net cpm/cm²	% SD	% ROIs	%
1	M1	0.03	0.78	3.43	13.96	16219	269.6	148.2	0.8	94.2	
2	CGA329351		0.58	0.95	11.07	16.28	992	16.5	18.0	3.3	5.76

	Counts	Cpm
Total gross:	49838	828.3
Total net:	13892	230.9
Gross in ROIs:	17457	290.1
Net in ROIs:	17211	286.1

APPENDIX A: Representative Data

Analytical Data

Series A Samples 1-17: pH 9, 25°C

Proj. :		95EH05	Puffervol. (ml)	
Substance:	CGA	329351	AnfangspH	
Batch :		MSR-II-89	Series	A
Buffer			Temperature	25°c
Applied (Date)		17/08/95 10:00		
Experiment	Final Test			

Analyses

Dry outside of sample tube and after removal of cap weigh.
After the temperature of the sample reaches room temperature determine pH:
Depending on pH the sample must be neutralized. In case of neutralization the sample must be weighed a second time.
Finally two aliquots must be taken for LSC and a part must be taken for HPLC analysis.

Sample No	Sampling Date	Incubation Time (days)	pH End	Sample ml	Total Weight (g) Brutto	Total Weight (g) Netto	Total Vol. (ml)	Sample dpm	Total dpm	Applied %	Counter
1	17/08/95 10:00	0.00	9.021	0.10	23.000	5.000	4.947	44036	2178562	100.00	12
2	19/08/95 10:05	2.00	9.040	0.10	26.985	8.919	8.825	24001	2118057	99.02	14
3	21/08/95 10:00	4.00	9.032	0.10	23.549	5.515	5.457	39249	2141737	99.28	14
4	23/08/95 10:00	6.00	9.009	0.10	23.604	5.518	5.460	39434	2153003	99.12	16
5	25/08/95 10:00	8.00	9.025	0.10	23.576	5.526	5.468	39435	2156179	99.19	18
6	28/08/95 10:00	11.00	9.018	0.10	23.435	5.446	5.389	39912	2150667	99.02	19
7	30/08/95 10:00	13.00	9.030	0.10	23.658	5.414	5.357	39445	2113013	98.37	20
8	01/09/95 10:00	15.00	9.028	0.10	24.026	5.418	5.361	39696	2128030	98.49	21
9	04/09/95 10:00	18.00	9.005	0.10	24.144	5.420	5.363	40126	2151876	99.61	22
10	06/09/95 10:00	20.00	9.031	0.10	23.691	5.426	5.369	39527	2122099	98.79	24
11	08/09/95 10:00	22.00	9.015	0.10	23.781	5.588	5.529	37841	2092238	98.04	26
12	11/09/95 10:00	25.00	9.140	0.10	23.477	5.374	5.317	39795	2116013	98.13	27
13	12/09/95 10:00	26.00	9.130	0.10	24.210	6.254	6.188	34477	2133435	98.96	27
14	13/09/95 10:00	27.00	9.140	0.10	24.481	6.369	6.302	33693	2123259	99.05	27
15	14/09/95 10:00	28.00	9.140	0.10	23.986	6.346	6.279	33731	2117978	98.84	27
16	15/09/95 10:00	29.00	9.120	0.10	24.193	6.172	6.107	34706	2119447	98.65	27
17	18/09/95 10:00	32.00	9.140	0.10	24.537	6.372	6.305	33997	2143426	98.94	27

HPLC-ANALYSIS
(Results % ROI acc. RAM)

PROJEKT: 95EH05

Sample No.	Time (days)	Degradate/Code-No. Parent	M1	M3	M4	M5	Total	HPLC-No
		Retention Time (min.)						
		12.97	2.02 -3.50	11.17	12.43	13.38		
		Identity/CGA-No.						
		329351		UK	UK	UK		
1	0.00	97.95	0.00	0.00	0.86	1.19	100.00	7
2	2.00	95.97	2.48	0.92	0.00	0.63	100.00	8
3	4.00	94.54	3.50	0.90	0.24	0.82	100.00	8
4	6.00	93.81	4.42	0.65	0.20	0.92	100.00	9
5	8.00	91.86	5.92	0.64	0.70	0.88	100.00	10
6	11.00	91.59	6.94	0.25	0.00	1.22	100.00	11
7	13.00	89.65	8.46	0.45	0.08	1.36	100.00	12
8	15.00	89.13	7.96	0.68	0.21	2.02	100.00	13
9	18.00	87.46	10.22	1.05	1.27	0.00	100.00	14
10	20.00	86.78	11.49	0.85	0.00	0.88	100.00	16
11	22.00	85.92	11.73	0.84	0.00	1.51	100.00	17
12	25.00	84.62	13.50	0.67	0.00	1.21	100.00	18
13	26.00	85.48	13.09	0.53	0.00	0.90	100.00	18
14	27.00	82.53	14.94	0.32	0.39	1.82	100.00	18
15	28.00	82.43	15.03	1.02	0.38	1.14	100.00	18
16	29.00	82.05	14.73	1.22	0.40	1.60	100.00	18
17	32.00	81.36	15.16	0.76	0.32	2.40	100.00	18

Validation of HPLC Data by Fractionation and LSC

(Sample 17A; day 32)

Sample	Injected Volume (ml)	DPM
M17A	0,10	33997

Fraction	Recovered dpm	%	Counter-No	HPLC-No
M1	4879	14,35	28	19
CGA329351	27854	81,93	28	19
Remainder	566	1,66	28	19
Total	33299	97,95		

Validation of TLC Data

Validation by scraping off radioactive zones and LSC (Sample 17A; day 32)

Applied to TLC

17A/pH9/25°C	0.03	ml =	10199	dpm

Recovered from TLC

		Recovered LSC		RAM
Spot	Identity	dpm	% Rec.	% ROI
BKG1		0	–	
BKG2		0	–	
1	M1	1727	16.93	18.51
2	CGA 329351	7648	74.99	81.49
A		1	0.01	
B		22	0.22	
C		42	0.41	
D		88	0.86	
Total		9528	93.42	

Cofirmation of HPLC Data by 2-D TLC

Sample	M1 TLC	Parent TLC	M1 HPLC	Parent HPLC	M3 HPLC	M4 HPLC	M5 HPLC
17A/pH9/25°C	18.51	81.49	15.16	81.36	0.76	0.32	2.40
8/pH9/50°C	77.90	22.10	72.10	26.47	0.29	0.00	1.14
8/pH9/60°C	94.24	5.76	91.95	7.12	0.61	0.00	0.32

HPLC-ANALYSIS
(Results % applied)

Sample-No.	Time (days)	Degradate/Code-No.					Total
		Parent	M1	M3	M4	M5	
		Retention Time (min.)					
		12.97	2.02 -3.50	11.17	12.43	13.38	
		Identity/CGA-No.					
		329351		UK	UK	UK	
1	0.00	97.95	0.00	0.00	0.86	1.19	100.00
2	2.00	95.03	2.46	0.91	0.00	0.62	99.02
3	4.00	93.86	3.47	0.89	0.24	0.81	99.28
4	6.00	92.99	4.38	0.64	0.20	0.91	99.12
5	8.00	91.12	5.87	0.63	0.69	0.87	99.19
6	11.00	90.69	6.87	0.25	0.00	1.21	99.02
7	13.00	88.19	8.32	0.44	0.08	1.34	98.37
8	15.00	87.78	7.84	0.67	0.21	1.99	98.49
9	18.00	87.12	10.18	1.05	1.27	0.00	99.61
10	20.00	85.73	11.35	0.84	0.00	0.87	98.79
11	22.00	84.23	11.50	0.82	0.00	1.48	98.04
12	25.00	83.04	13.25	0.66	0.00	1.19	98.13
13	26.00	84.59	12.95	0.52	0.00	0.89	98.96
14	27.00	81.74	14.80	0.32	0.39	1.80	99.05
15	28.00	81.47	14.86	1.01	0.38	1.13	98.84
16	29.00	80.94	14.53	1.20	0.39	1.58	98.65
17	32.00	80.50	15.00	0.75	0.32	2.37	98.94

Calculation e.g. amount CGA 329351 sample 11, day 22:

Radioactivity in aqueous phase: 98.04 % applied
Result of HPLC for CGA 329351: 85.92 % ROI (Region of Interest)
CGA 329351 (in %-applied): (98.04 *85.92)/100 = 84.23%

APPENDIX B: Limit of Detection and Quantitation

Calculation of Limit of Detection and Quantitation

Limit of Detection and Quantitation

Limit of Detection for HPLC (Sample 9; pH 9; 25°C; Series A)
(Figure A)

Amount applied (mg)	0.025
Amount applied (dpm)	2160262
Volume of buffer (ml)	4.91
Recovery (%-applied)	99.61

	cpm	% applied
Background:	15	
Parent molecule:	2120	87.47
DL = 87.1/2120*15 =		0.62

(DL: Detection Limit)

	%-ROI	%-applied	Parent equiv. (mg)	mg/l Buffer (ppm)
Background HPLC (DL):	0.62	0.62	0.0002	0.031
Limit of Detection (2XDL):	1.24	1.23	0.0003	0.062
Limit of Quantitation (3xDL):	1.86	1.85	0.0005	0.093

Limit of Detection and Quantitation for 2-D TLC
(Figure 6)

	cpm/cm2	% applied
Background:	1.46	
Parent molecule:	71.8	72.59
DL = 87.1/2120*15 =		1.48

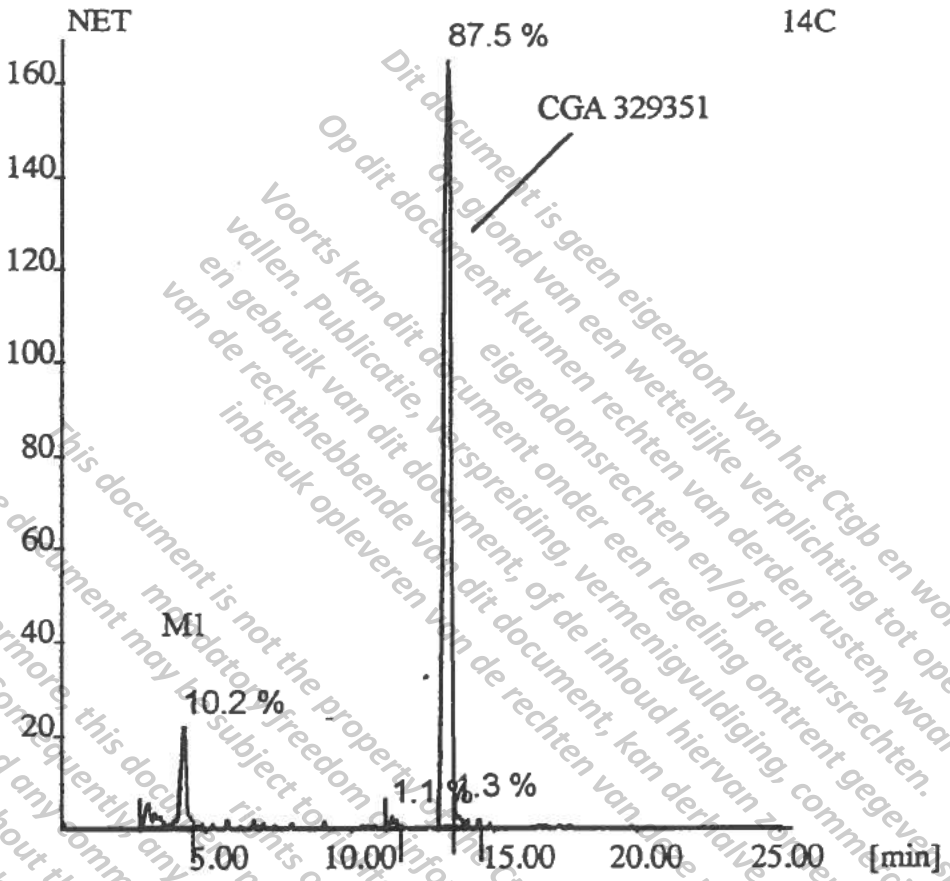
	%-ROI	%-applied	Parent equiv. (mg)	mg/l Buffer (ppm)
Background TLC (DL):	1.48	1.47	0.0004	0.074
Limit of Detection (2XDL):	2.95	2.94	0.0007	0.148
Limit of Quantitation (3xDL):	4.43	4.41	0.0011	0.223

Limit of Detection and Quantitation for Liquid Scintillation Counter (LSC)

	ml	dpm	Total dpm	%-applied	Parent equiv. (mg)	mg/l Buffer (ppm)
Sample size measured (ml)	0.10					
Buffer Volume (ml)	4.91					
Background LSC (BGK LSC):		16	785	0.04	0.0009	0.183
Limit of Detection (2*BGK):		32	1570	0.07	0.0018	0.367
Limit of Quantitation (3*BGK):		48	2355	0.11	0.0027	0.550

Figure A: HPLC of Buffer Solution pH 9 exposed for 18 days at 25°C

(Sample 9; Series A)



File name : 25M9_001.CH2 User : 14C Curr. Date : 2-Oct-95 10:07:30
Run Length : 25.00 [min]Acqu. Date : 4-Sep-95 10:41:24
Info :
Sample 9A/18 Days/pH 9 25C/0.1 ml = 40126 dpm
Control Method : YG_150
LL = 25 UL = 750
Bkg (Cpm) = 15 Channel Delay(Sec) = 16
Efficiency % = 75

#	Name	Rt	NET	%Area
1	M1	4.13	248	10.22
2	M3	11.22	25	1.05
3	CGA329351	13.10	2120	87.46
4	M5	13.38	31	1.27

Total Area of Peak = 2423.75