Bayer AG
Crop Protection Development
Institute for Metabolism Research
51368 Leverkusen
Germany

Monheim, December 7, 1993

ADDENDUM

Addendum to NTN 33893 Cotton Report PF No.: 3675
(Study Number M 173 0311-6)
Metabolism of NTN 33893 in Cotton after Seed Treatment

Authors: [redacted]

PF3675 / MO-00-002815

[redacted]

TABLE OF CONTENTS

page No.

I. SUMMARY

The extracts of cotton seeds of the soil drench experiment from the original NTN 33893 metabolism study, report No. 3675, were further investigated.

The major metabolite (metabolite 15, 27.1 % of the radioactivity in the seeds, 2.54 mg/kg) in the methanol/6N HCl reflux extract was identified as 6-hydroxynicotinic acid methyl ester by co-chromatography with the authentic reference compound using two-dimensional TLC and GC/MS after methylation. Metabolite 16 (1.6 %, 0.15 mg/kg) was identified as 6-hydroxynicotinic acid and metabolite 19.1 (0.7 %, 0.06 mg/kg) as 6-chloronicotinic acid methyl ester by co-chromatography with the corresponding authentic reference compound using two-dimensional TLC.

Furthermore, the residues in the methanol/water phase (19.9 %, 1.86 mg/kg) and in the methanol reflux extract (44.5 %, 4.16 mg/kg) were characterized as being based mostly on 6-chloronicotinic acid, 91 % and 87 % of the radioactivity, respectively.

II. INTRODUCTION

This study is an addendum to the BAYER-PF report No. 3675 "Metabolism of NTN 33893 in Cotton after Seed Treatment", Study No. M 173 0311-6. The purpose of this study was to provide further identification of metabolites in the seeds of the soil drench experiment, especially the main metabolite in the methanol/6N HCl reflux extract, as requested by EPA (memorandum of Griffith, 1993).

In the original cotton study two experiments were conducted, firstly a normal seed dressing experiment (experiment 1) and secondly an overdose experiment (experiment 2) performed by pouring a solution of NTN 33893 onto the soil surrounding the cotton plants. The residues obtained from the seeds in the seed dressing experiment were very low, only sufficient to quantitate the amount of radioactivity in the various extracts.

Identification of metabolites in the seeds was conducted using the seeds obtained from the overdose experiment. Due to the low amount of total residue in the seeds from the seed treatment experiment, only a limited amount of identification work with the overdose experiment was conducted. This involved co-chromatography (TLC) of the extracts with reference compounds of known plant metabolites. In this way some metabolites in the methanol/water phase and methanol reflux extract were identified. However, although in the original study no metabolites were identified in the methanol/6N HCl reflux extract, the presence of four significant metabolites (metabolites 15, 16, 17 and 19) was shown by TLC, of which metabolite 15 was by far the major component.

The total amount of seeds obtained from the overdose experiment (33.5 g) had been used for the identification work in the original study and no seeds were left for additional investigation. However, the three original extracts, methanol/ water phase, methanol reflux extract and methanol/6N HCl reflux extract, were still available so that the additional identification work for metabolite 15 was conducted using these extracts.

The study began on October 7, 1993 and finished on December 3,1993.

III. METHODS

A. Thin layer chromatography (TLC)

The radioactive solutions were investigated by one and two-dimensional TLC using silica gel plates (Kieselgel 60 F_{254}, Merck, Darmstadt, Germany) which were eluted with the following solvent systems:

Solvent system SS I	:	Ethyl Acetate / Propan-2-ol / Water 65:23:12
Solvent system SS II	:	Ethyl Acetate / Toluene / Methanol / Acetic Acid 80:20:20:1
Solvent system SS III	:	Butan-1-ol / Acetic Acid / Water 80:20:20
Solvent system SS IV	:	Chloroform / Methanol / Acetic Acid / Water 65:25:3.5:3.5
Solvent system SS V	:	n-Hexane / Ethyl Acetate 80:20

The radioactive compounds were detected using a BAS 2000 Bio-Imaging Analyzer (Fuji). This instrument employs an ultra sensitive imaging plate (IP) for detecting radioactive energy which when exposed accumulates and stores irradiated radioactive energy. The plate is then scanned with a fine laser beam and emits luminescence in proportion to the recorded radiation intensity. The quantitative distribution of the metabolites was determined using the Image Analyze software package.

The reference compounds used in co-chromatography were visualised by UV light (254 nm).

B. High performance liquid chromatography (HPLC)

HPLC was used for the isolation and purification of metabolite 15 from the methanol/6N HCl reflux extract

HPLC Instrument	: HP 1090 Hewlett Packard
Column	: 1. LiChrosorb RP 18 (7 µm), 250 x 4 mm (Merck) 2. LiChrospher RP 8 Select B (5 µm), 250 x 4 mm (Merck)

Gradient	: Methanol in water
	0-5 min. 0 % methanol
	5-25 min. 0-100 % methanol
	25-30 min. 100 % methanol
	30-35 min. 0 % methanol
Detectors	: Diode Array Detector, wavelength 265 nm,
	Radioactivity Flow-Through Detector "Ramona D" (Raytest)
Fraction Collector	: Gilson, Model 202

C. Preparation and use of diazomethane-d_2

2-(2-Ethoxyethoxy)ethan(ol-d) (carbitol-d, 50g) and anhydrous diethyl ether (20 ml) were added to a solution of 30 % sodium deuteroxide in D_2O (20 g) in a 250 ml distillation flask. The flask was equipped with a dropping funnel, an efficient condenser and a magnetic stirring bar and was heated in a water bath to 70°C. A solution of Diazald (N-methyl-N-nitroso-p-toluene-sulfon amide) in anhydrous diethyl ether (10 ml per gram of Diazald) was added over a period of 20 minutes and aliquots of this solution were added to the sample until the yellow color remained. An aliquot, taken directly from the reaction mixture, was injected into the GC/MS.

D. Mass spectrometry (MS)

The GC/MS spectra (electron impact) were recorded on a mass selective detector HP 5970 combined with a GC 5880A (Hewlett - Packard). The capillary column used was a 15 m SE 54 (CS), inner diameter 0.25 mm, film thickness 0.25 µm. The oven temperature program was 60°C for 1 minute, heating rate 10°/minute up to 280°C, the end temperature was kept constant for 20 minutes. Injection was splitless at 260°C.

E. Determination of 6-chloronicotinic acid using the residue method based on 6-CNA

The residue method for NTN 33893, developed by [redacted] (1990 a and b), was used to determine the total residues in the methanol/water phase and in the methanol reflux extract.

Aliquots of the two phases were reduced to the aqueous remainder (3-4 ml) to which 5 ml of a 32 % sodium hydroxide solution and 100 ml of a 5 % potassium permanganate solution were added. This mixture was heated just to the point of boiling (ca. 7-10 min.) and then cooled in an ice-bath (to ca. 15°C). To the cooled solution

50 ml of a 10 % sulfuric acid solution was added followed by enough 40 % sodium hydrogensulfite to make the solution colorless and finally conc. sulfuric acid was added until pH <1 was obtained. The resultant solution was extracted with t-butyl methyl ether (3 x 100 ml) and the combined ether phases reduced using a rotary evaporator (35-40°C) to ca. 5 ml. This solution was evaporated to a volume of ca. 0.2 ml in a small vial under a stream of nitrogen and the solution was made up to 1 ml with acetonitrile.

After measuring the radioactivity content of the solution the amount of 6-chloronicotinic acid formed by this procedure was determined using two-dimensional TLC in solvent system II/I.

IV. RESULTS AND DISCUSSION

A. Stability of metabolites in the stored extracts

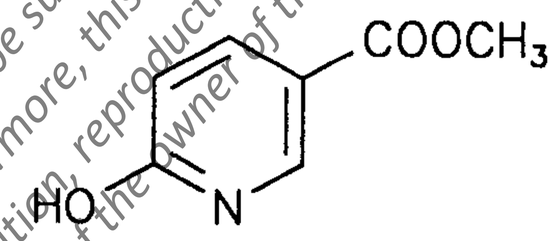
The methanol/water phase and the methanol reflux extract from the 1. analysis (extraction date 4/7/90, day 21) were once more analyzed using TLC (Figures 1 and 2). The metabolic pattern did not significantly alter during the storage period of over 3 years.

The methanol/6N HCl reflux extracts from the 1. and 2. analyses (extraction dates 4/7/90, day 21 and 19/3/91, day 279) were also once more analyzed using TLC. The extracts had the same pattern of metabolites and therefore were combined for purification. TLC investigation showed that the metabolic pattern of the combined extracts was nearly the same as in the original extracts from the 1. and 2. analyses (Figure 3). However, minor quantitative differences in the metabolite composition were noticed (Table I).

B. Identification of metabolites in the methanol/6N HCl reflux extract

B.1. Metabolite 15

Metabolite 15 was isolated and purified from the combined methanol/6N HCl reflux extracts (see section IV. A.) using HPLC (Figure 4). It was identified as 6-hydroxy-nicotinicacid methyl ester by two-dimensional TLC co-chromatography with the authentic reference compound BNF 8125 D in two solvent systems (Figures 5 and 6).



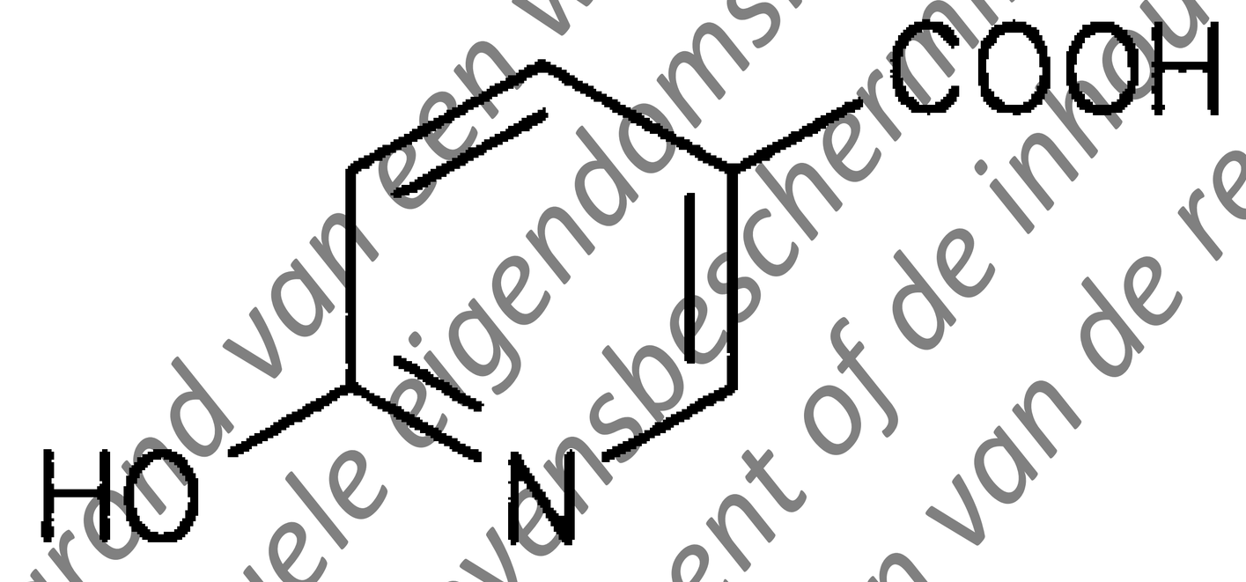
Metabolite 15 = 6-hydroxynicotinic acid methyl ester

The structure of metabolite 15 was confirmed by GC/MS after methylation. Methylation was necessary to separate the metabolite from plant constituents. In order to determine the position of methylation CD_2N_2 was used. The mass spectrometric analysis showed that one CD_2H-group was present after methylation. The electron impact mass spectrum showed a molecular ion at m/z 169 (^{12}C-isotope, Figure 7). Loss of the methoxy group from the ester moiety (31 amu) yielded ion m/z 138. Further cleavage of

the carbonyl group (28 amu) resulted in m/z 110. The spectrum was in good accordance to that of the reference compound. The isotope patterns of the ions m/z 110, 138 and 169 were due to the high specific radioactivity of the active ingredient applied.

B.2. Metabolite 16

Metabolite 16 was identified as 6-hydroxynicotinic acid by two-dimensional TLC of the combined methanol/6N HCl reflux extract with the authentic reference compound GBH 4315 (Figures 8 and 9).



Metabolite 16 = 6-hydroxynicotinic acid

B.3. Metabolite 19

Further TLC investigation of the methanol/6N HCl reflux extract in solvent system IV/V showed that metabolite 19 consisted of two components, namely metabolite 19.1 and metabolite 19.2, 0.7 % and 3.2 % in the seeds, respectively (Figure 10). Metabolite 19.1 was identical with the authentic reference compound 6-CNA methyl ester (BNF 5535 D).

B.4. Stability of the metabolites in methanol/6N HCl

To explain the formation of the 6-hydroxynicotinic acid metabolites, the metabolites present in the methanol/water phase (see Figure 1) and in the methanol reflux extract (see Figure 2) were subjected to the same stringent conditions as were used to release recalcitrant residues from the cottonseed solids - a 6 hour reflux in methanol/6N HCl. The respective extracts were evaporated to dryness, dissolved in methanol/6N HCl (1:1) and refluxed for 6 hours. TLC investigation of these solutions showed that nearly all metabolites were converted to metabolites 15 and 16 (Figures 11 and 12). This confirmed that these two metabolites were artifacts formed under the drastic conditions used and were not genuine metabolites.

C. Characterization of residues using the 6-CNA method

In order to further characterize the constituents of the methanol/water phase and of the methanol reflux extract investigations were conducted using the 6-CNA method. The results showed that the major proportion of the residue was based on 6-chloronicotinic acid. In the case of the methanol/water phase 91 % of the radioactivity was characterized as being based on 6-CNA and in the case of the methanol reflux extract 87 % was characterized.

V. CONCLUSIONS

In the original study the drastic extraction procedure for the various plant fractions with methanol/6N HCl (1:1) under reflux was used to release further components from the solid material which had been previously extracted with methanol/water and methanol under reflux. The two major metabolites, metabolite 15 and metabolite 16, in the methanol/6N HCl reflux extract were identified in this study as 6-hydroxynicotinic acid methyl ester and 6-hydroxynicotinic acid, respectively. It was assumed that these metabolites were methanol/HCl artifacts formed under the drastic conditions. This was confirmed by investigating the stability of the metabolites present in the methanol/water phase and in the methanol reflux extract under the same conditions. It was shown that nearly all metabolites were converted to metabolites 15 and 16. Therefore, it can be concluded that metabolites 15 and 16 originally had the same structures as the metabolites present in the methanol/water phase and in the methanol reflux extract, but which were bound to the plant matrix.

Further characterization of the residues in the methanol/water phase and in the methanol reflux extract showed that a high proportion of the residue (91 % and 87 %, respectively) was based on 6-chloronicotinic acid using the 6-CNA residue method. Therefore, a proportion of the unknown metabolites must be based on 6-chloroncotinic acid.

The total amount identified in the seeds was approximately 74 %, a further 12 % was characterized.

VI. SIGNATURES

[redacted]

7/12/93
Date

8.12.93
Date

7/12/93
Date

10.12.93
Date

Acknowledgement:

The authors gratefully acknowledge the excellent technical assistance of [redacted].

VII.

Referat GLP

[redacted]

VIII. REFERENCES

Memorandum from [redacted] September 21, 1993.
PP# 3F04169/3H05655 - Imidacloprid (Confidor®) on apples, cottonseed, potatoes, meat, milk, poultry, and eggs.
Review of residue data and analytical method. pp. 3-4 and 17-19.

[redacted] (1990 a)
Method for the Determination of the Total Residues of Imidacloprid in Vegetable Samples and Drinking Water.
Bayer AG, Plant Protection Research, Institute for Residue Analysis.
Report No. RA-418/90 from 31.05.1990

[redacted] (1990 b)
Validation of the Residue Method for the Total Imidacloprid Residue in Vegetable Samples using Radioactive Residues.
Bayer AG, Plant Protection Research, Institute for Residue Analysis.
Report No. RA-419/90 from 31.05.1990

Table I:

Metabolites in the methanol/6N HCl reflux extract from the original study (1. and 2. analysis) and from the present study (combined extracts)

Radioactivity in the seeds = 100 %, mg/kg values expressed as NTN 33893 equivalents

Metabolites	1. Analysis Extraction date 4/7/1990 (day 21)		2. Analysis Extraction date 19/3/1991 (day 279)		Present Study	
	%	mg/kg	%	mg/kg	%	mg/kg
methanol/6N HCl 1:1 reflux extract	(32.4)	(3.03)	(37.2)	(3.48)	(34.8)	(3.26)
metabolite 15 = 6-hydroxynicotinicacid methyl ester	17.3	1.62	16.7	1.56	27.1	2.54
metabolite 16 = 6-hydroxynicotinicacid	4.7	0.44	6.7	0.63	1.6	0.15
metabolite 17 = unknown	1.9	0.18	2.8	0.26	1.7	0.16
metabolite 18 = unknown	0	0	2.5	0.24	0	0
metabolite 19 = unknown	3.6	0.33	2.6	0.24	-	-
metabolite 19.1 = 6-CNA methyl ester	-	-	-	-	0.7	0.06
metabolite 19.2 = unknown	-	-	-	-	3.2	0.30
metabolite 20 = unknown	-	-	-	-	0.5	0.05
minor unknown components	4.9	0.46	5.9	0.55	0	0
total identified	*	*	*	*	29.4	2.75

* not identified in the original study

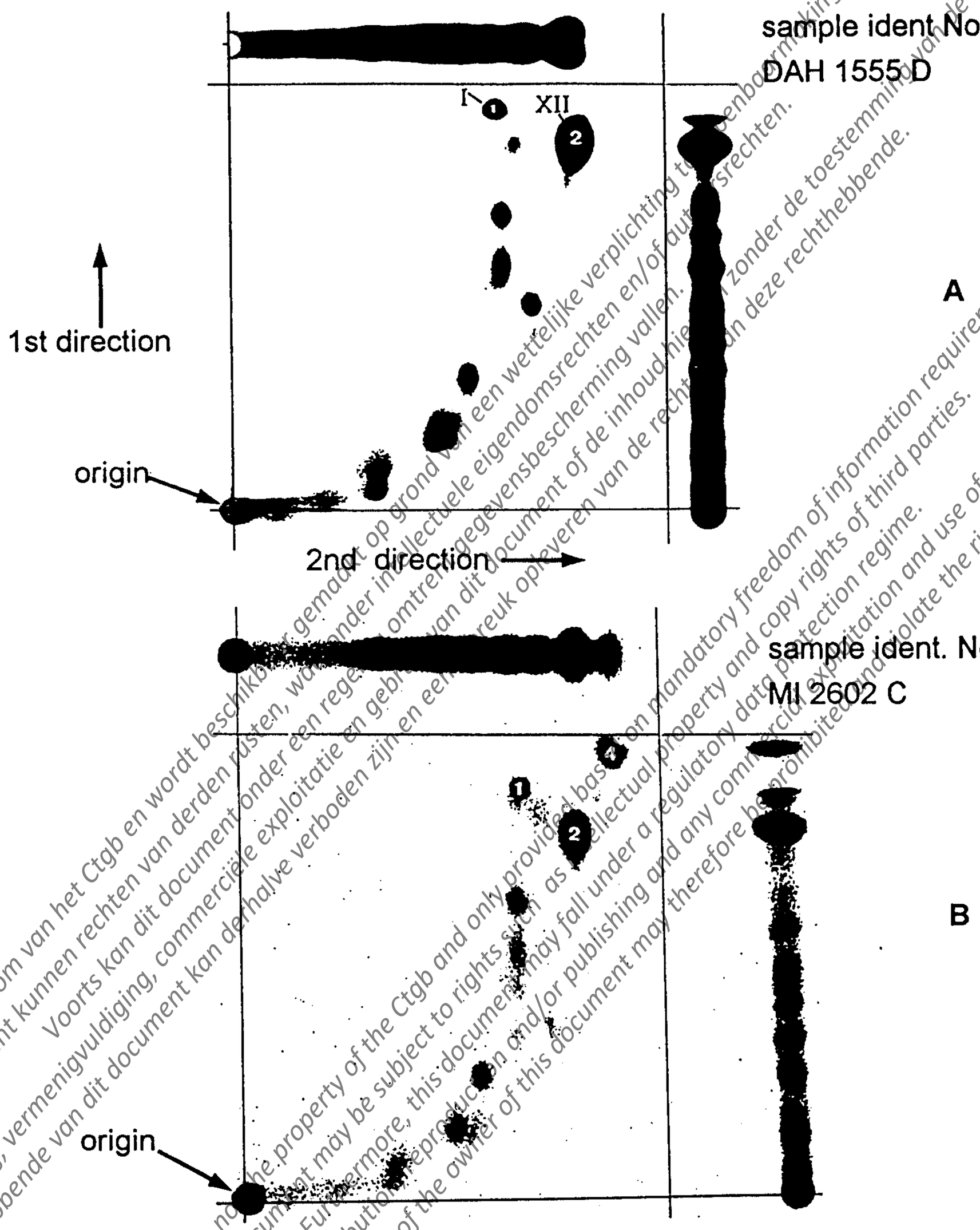


Figure 1:

Two-dimensional and one-dimensional TLC of the methanol/water phase of seeds,
A from the original study (1. analysis), B from the present study

1st Direction: SS IV = chloroform/methanol/acetic acid/water 65:25:3.5:3.5
2nd Direction: SS III = butan-1-ol/acetic acid/water 80:20:20
The Arabic numbers refer to the metabolites, the Roman numbers refer to reference compounds

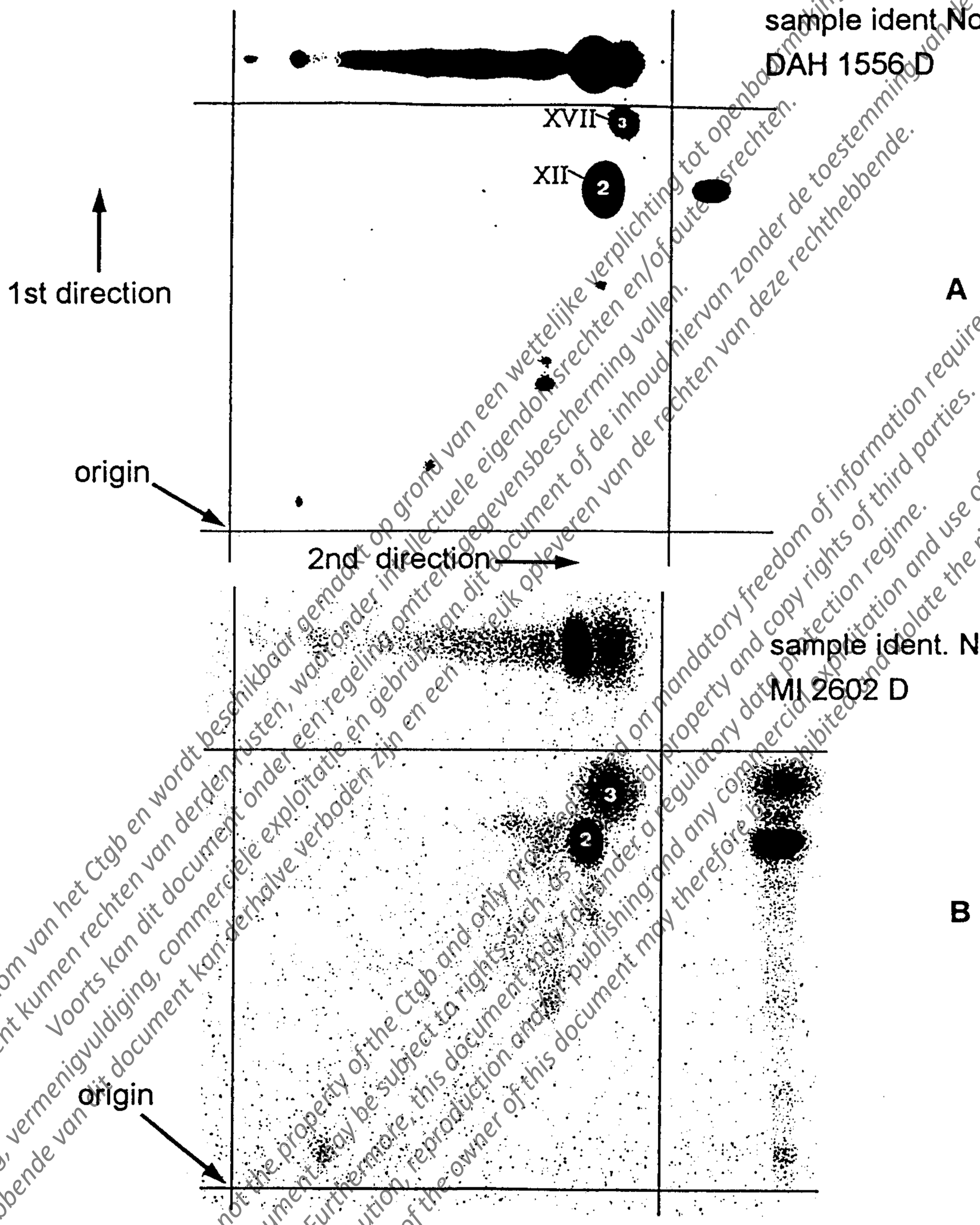


Figure 2:

Two-dimensional and one-dimensional TLC of the methanol reflux extract of seeds, A from the original study (1. analysis), B from the present study

1st Direction: SS IV = chloroform/methanol/acetic acid/water 65:25:3.5:3.5
2nd Direction: SS III = butan-1-ol/acetic acid/water 80:20:20
The Arabic numbers refer to the metabolites, the Roman numbers refer to reference compounds

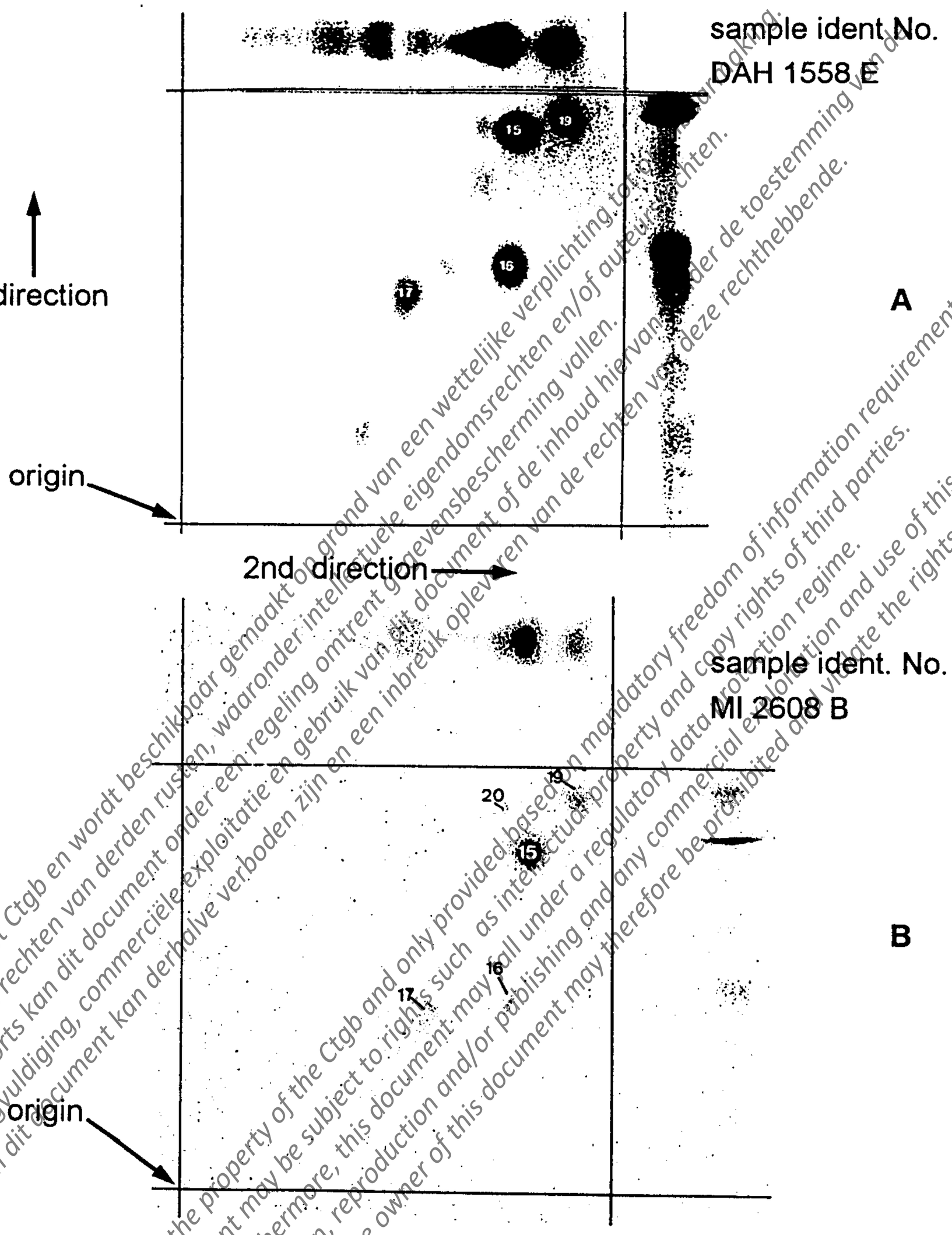


Figure 3:

Two-dimensional and one-dimensional TLC of the methanol/6N HCl reflux extract of seeds, A from the original study (1. analysis), B from the present study

1st Direction: SS IV = chloroform/methanol/acetic acid/water 65:25:3.5:3.5
2nd Direction: SS III = butan-1-ol/acetic acid/water 80:20:20
The Arabic numbers refer to the metabolites

sample ident. No.
MI 2624 A12

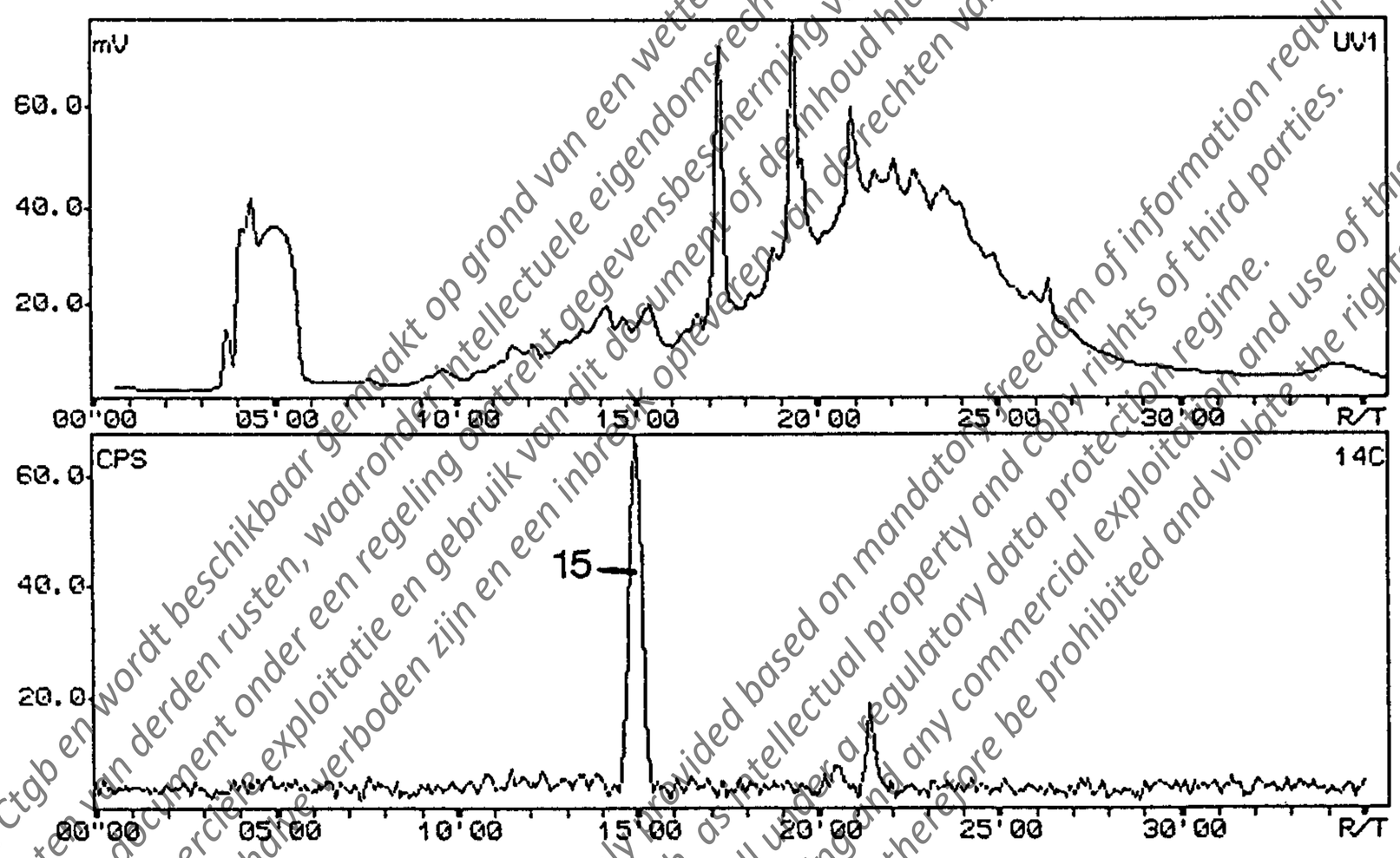


Figure 4:

HPLC chromatogram of the methanol/6N HCl reflux extract of seeds obtained during the isolation of metabolite 15

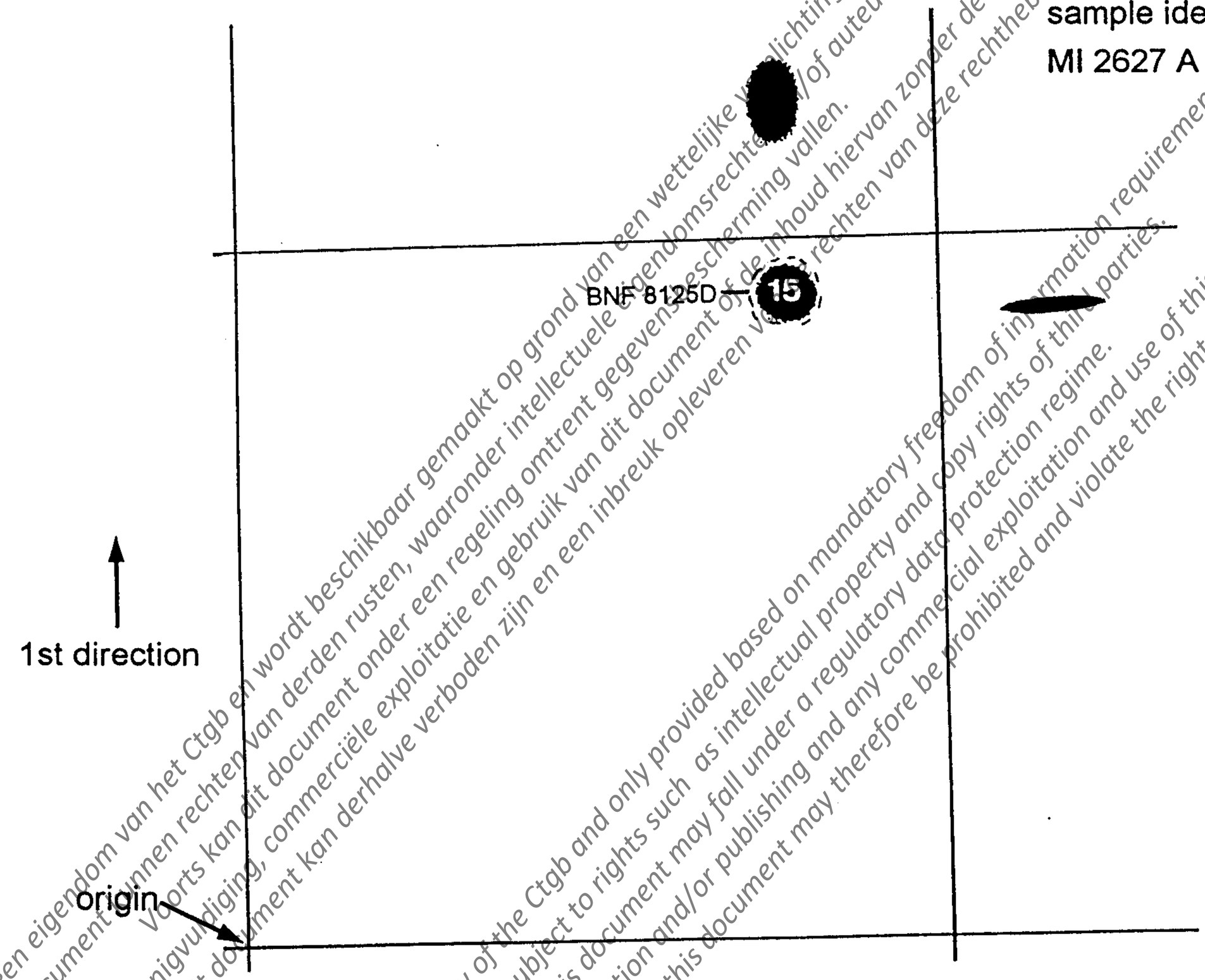


Figure 5:

Two-dimensional and one-dimensional TLC of metabolite 15, isolated using HPLC, spiked with the reference compound BNF 8125 D = 6-hydroxynicotinicacid methyl ester

1st Direction: SS IV = chloroform/methanol/acetic acid/water 65:25:3.5:3.5
2nd Direction: SS III = butan-1-ol/acetic acid/water 80:20:20

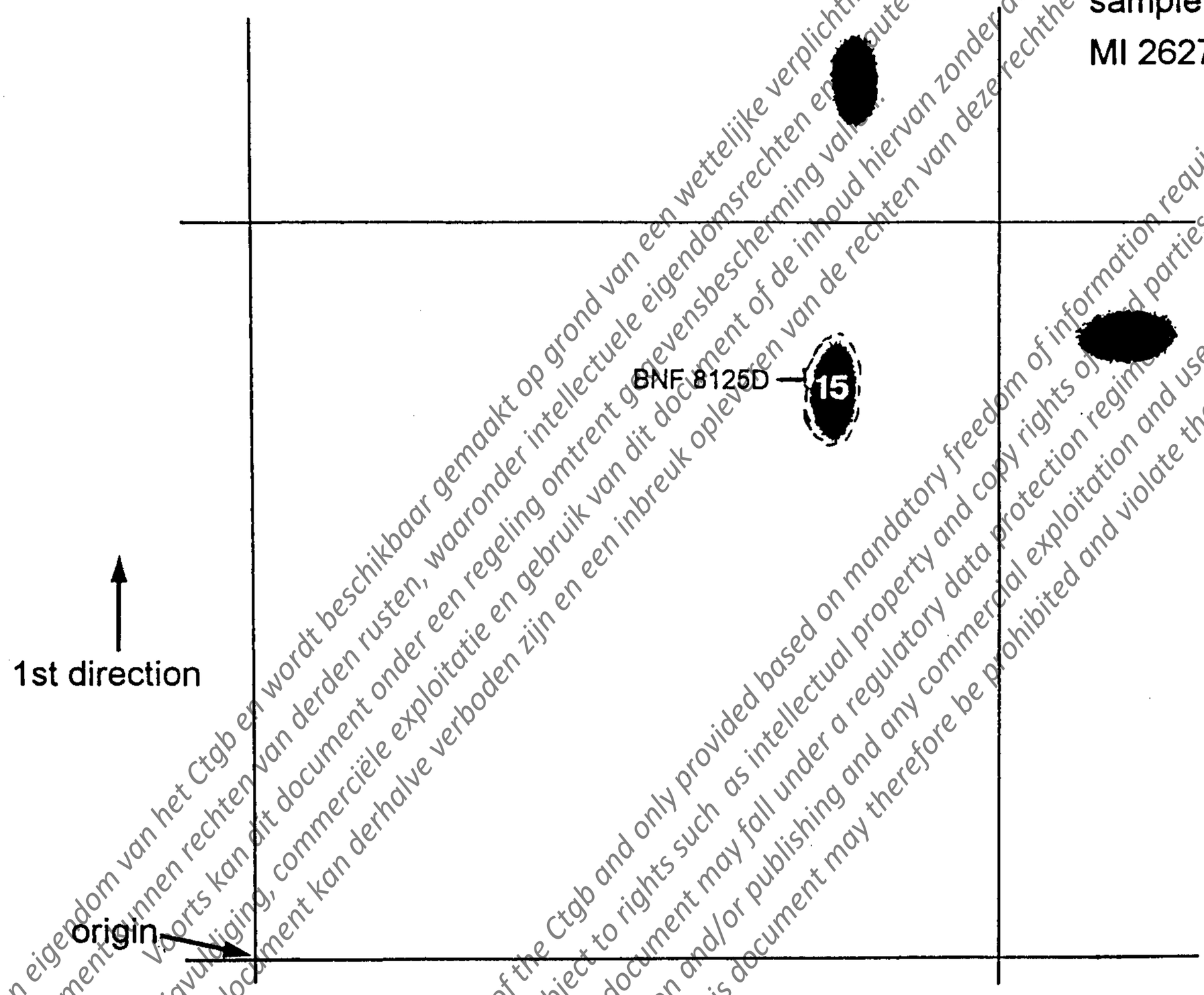
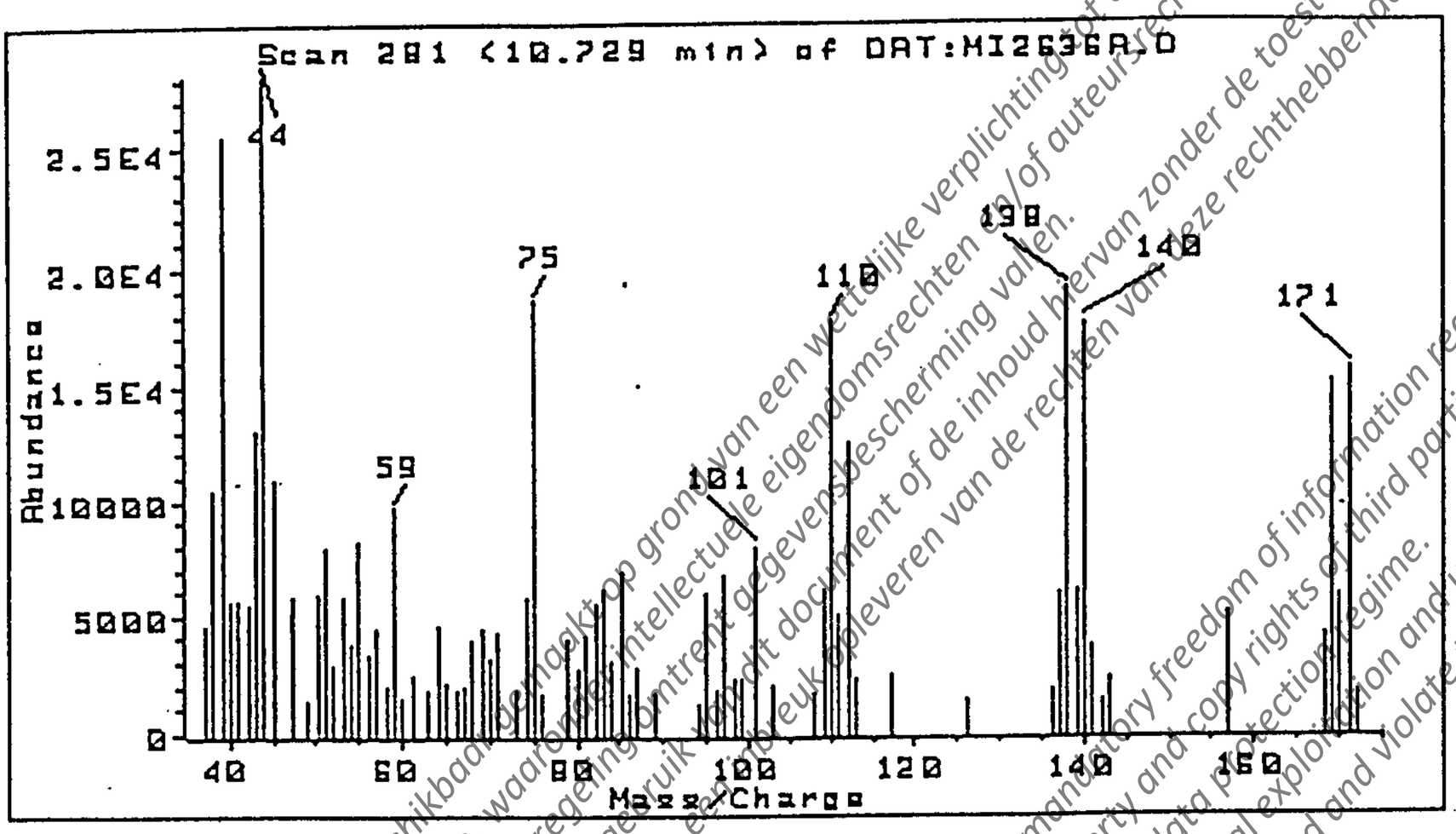


Figure 6:

Two-dimensional and one-dimensional TLC of metabolite 15, isolated using HPLC, spiked with the reference compound BNF 8125 D = 6-hydroxynicotinicacid methyl ester

1st Direction: SS II = ethyl acetate/toluene/methanol/acetic acid 80:20:20:1
2nd Direction: SS I = ethyl acetate/propan-2-ol/water 65:23:12



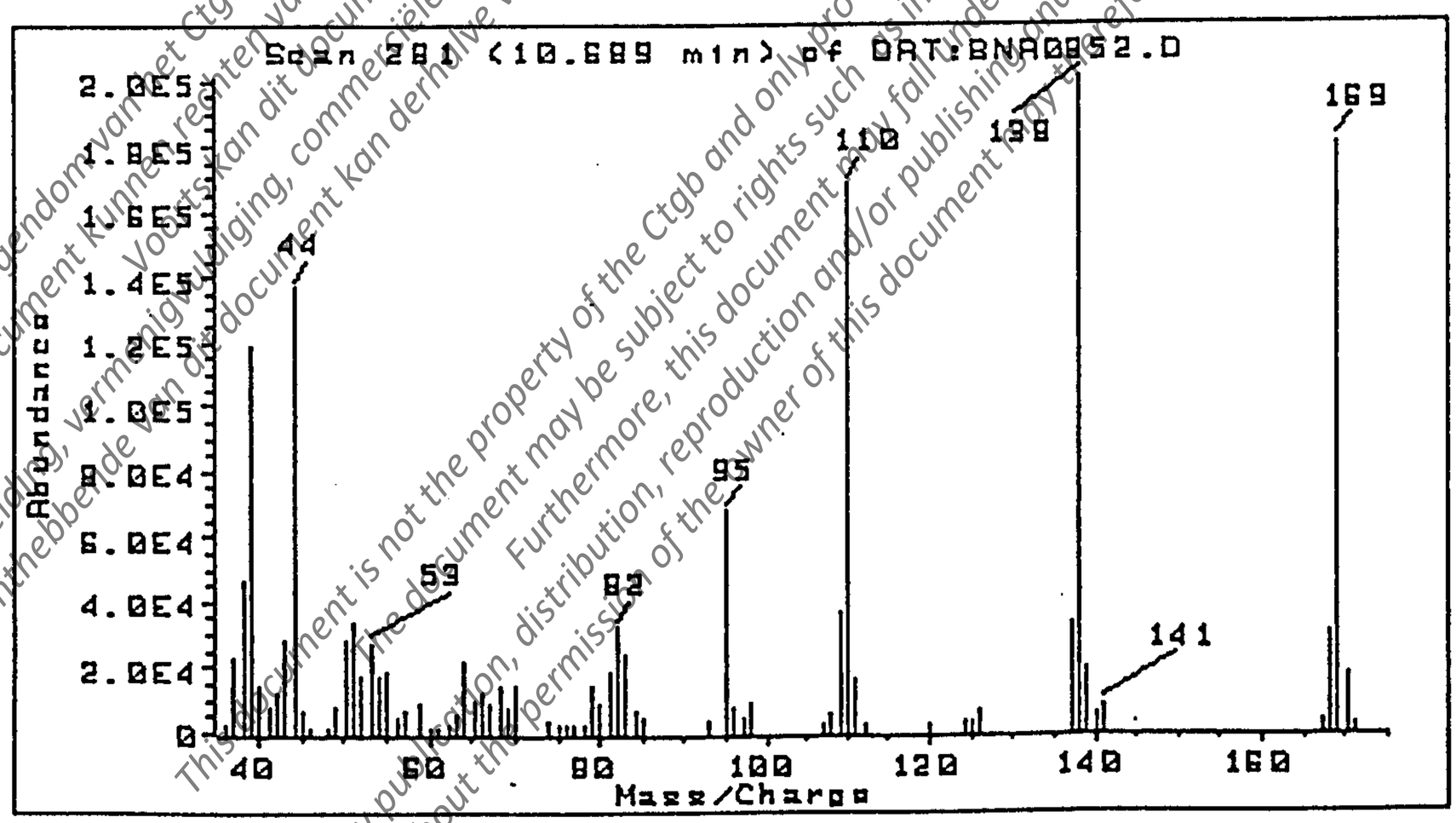


Figure 7:

Mass spectra of metabolite 15 (A) and of the reference compound BNF 8125 D (B), both after methylation with CD_2N_2

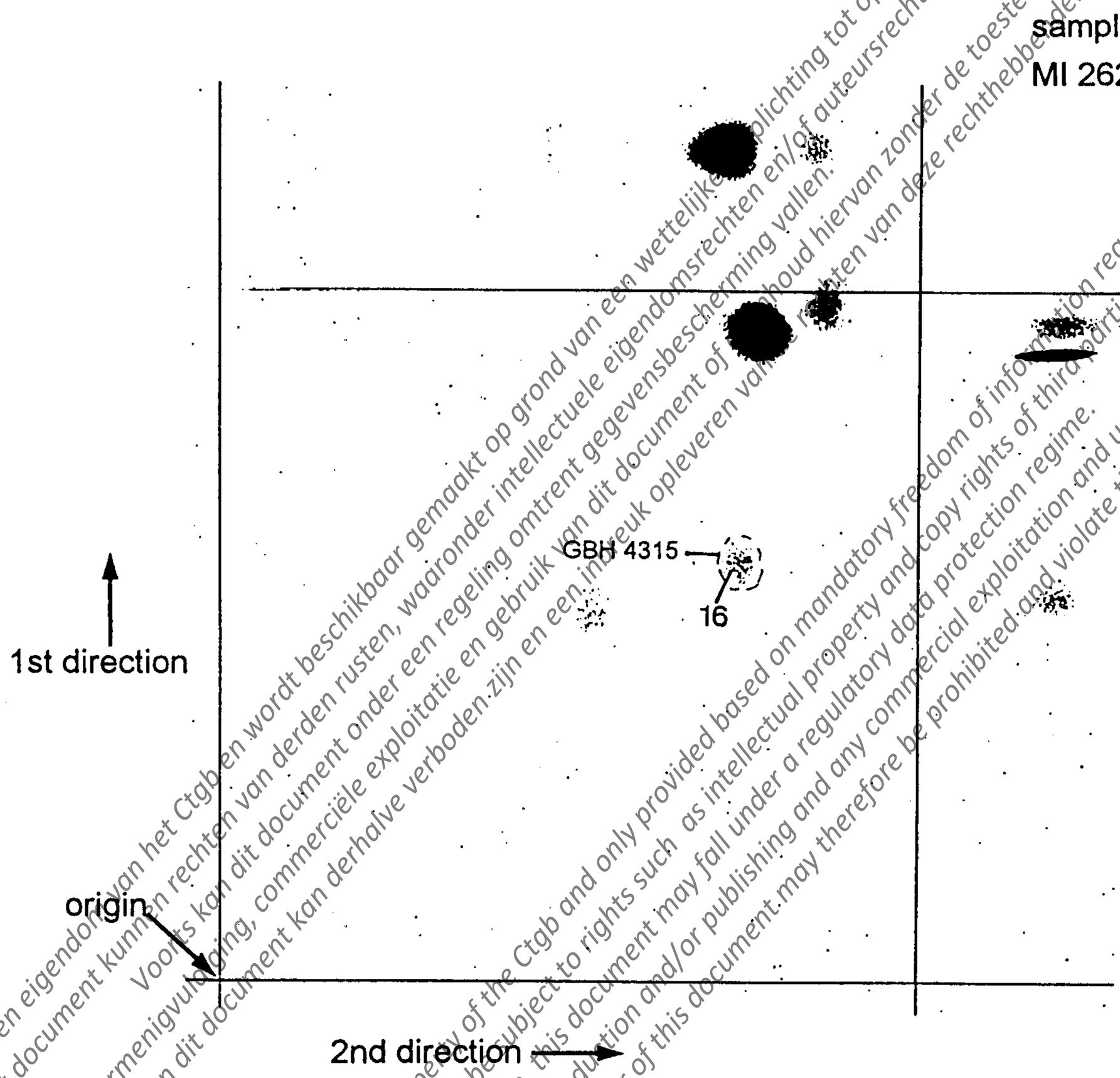


Figure 8:

Two-dimensional and one-dimensional TLC of the methanol/6N HCl reflux extract of seeds, containing metabolite 16, spiked with the reference compound GBH 4315 = 6-hydroxynicotinic acid

1st Direction: SS IV = chloroform/methanol/acetic acid/water 65:25:3.5:3.5
2nd Direction: SS III = butan-1-ol/acetic acid/water 80:20:20

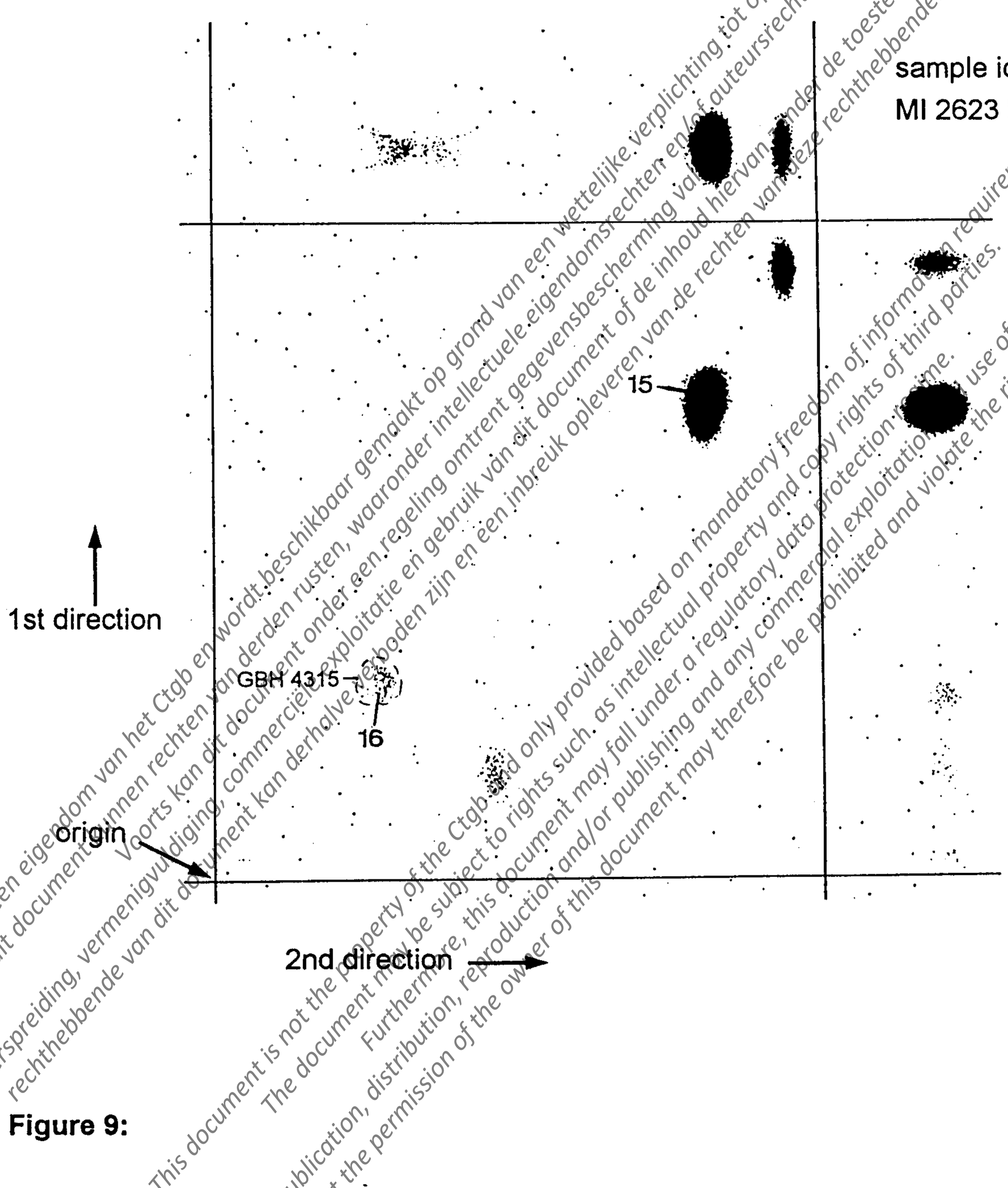


Figure 9:

Two-dimensional and one-dimensional TLC of the methanol/6N HCl reflux extract of seeds, containing metabolite 16, spiked with the reference compound GBH 4315 = 6-hydroxynicotinicacid

1st Direction: SS II = ethyl acetate/toluene/methanol/acetic acid 80:20:20:1
2nd Direction: SS I = ethyl acetate/propan-2-ol/water 65:23:12

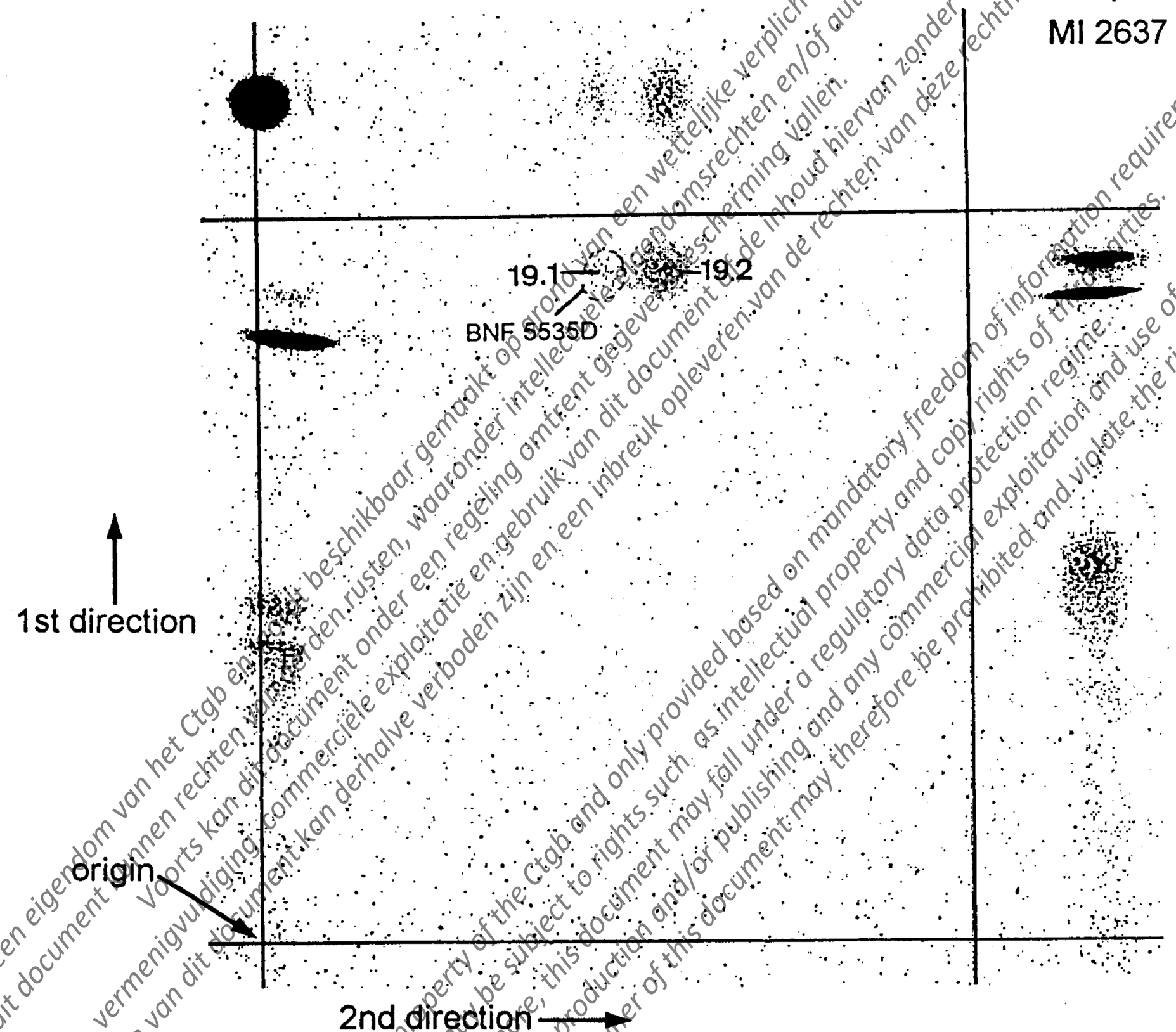


Figure 10:

Two-dimensional and one-dimensional TLC of the methanol/6N HCl reflux extract of seeds, containing metabolite 19.1, spiked with the reference compound BNF 5535 D = 6-CNA methyl ester

1st Direction: SS IV = chloroform/methanol/acetic acid/water 65:25:3.5:3.5
2nd Direction: SS V = n-hexane/ethyl acetate 80:20

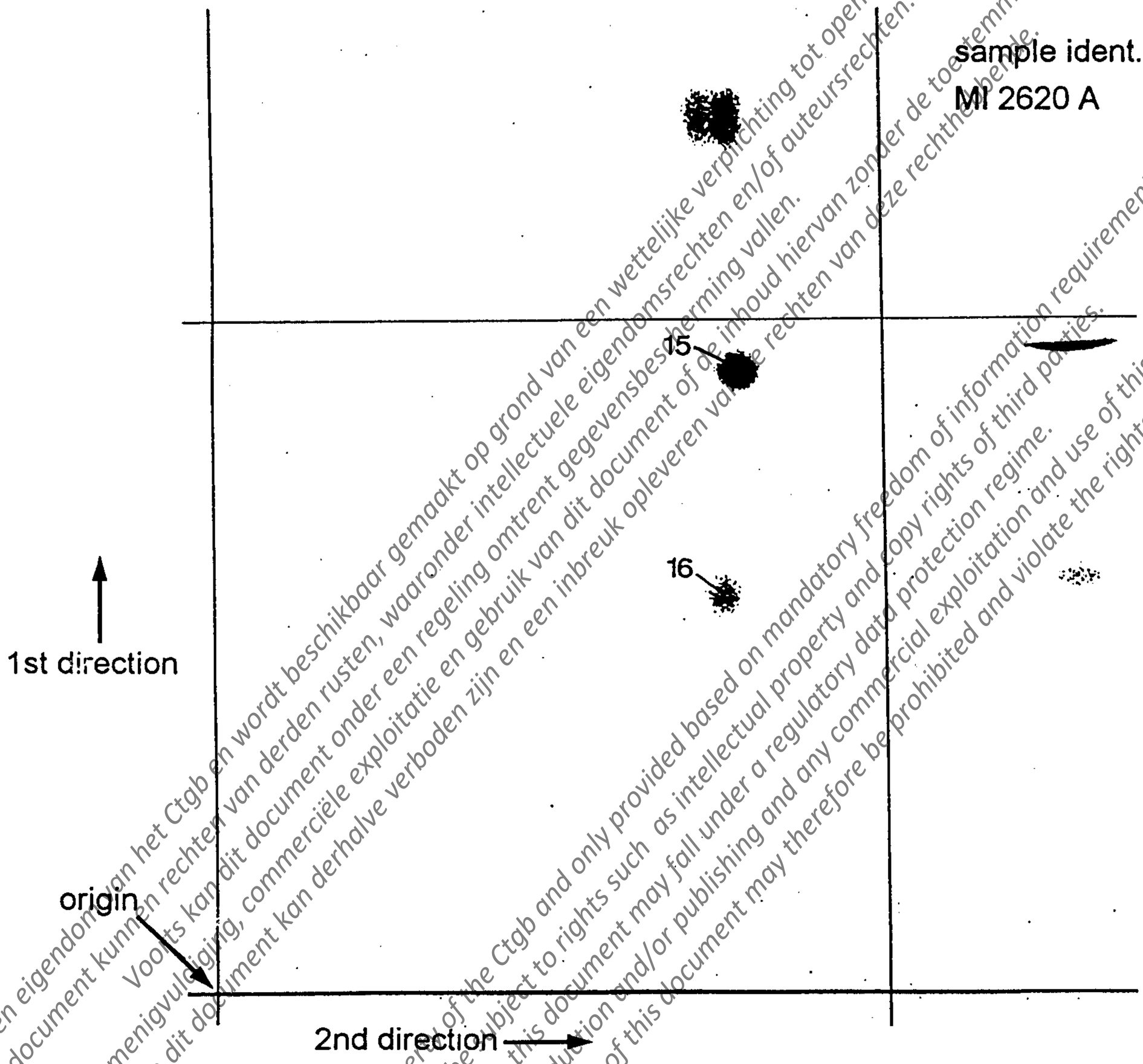


Figure 11:

Two-dimensional and one-dimensional TLC of the stability investigation of the metabolites in the methanol/water phase during reflux in methanol/6N HCl (1:1)

1st Direction: SS IV = chloroform/methanol/acetic acid/water 65:25:3.5:3.5
2nd Direction: SS III = butan-1-ol/acetic acid/water 80:20:20
The Arabic numbers refer to the metabolites

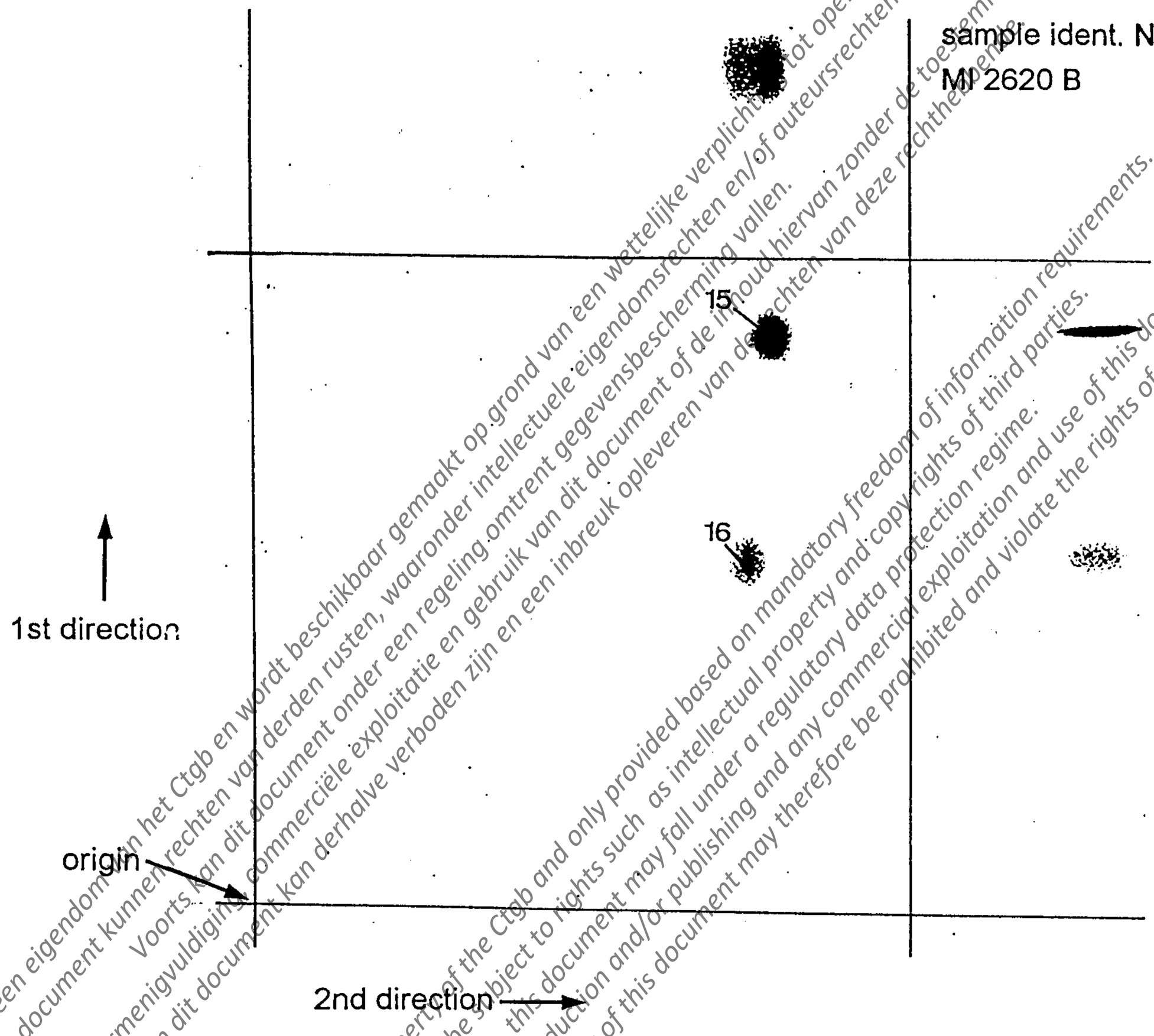


Figure 12:

Two-dimensional and one-dimensional TLC of the stability investigation of the metabolites in the methanol reflux extract during reflux in methanol/6N HCl (1:1)

1st Direction: SS IV = chloroform/methanol/acetic acid/water 65:25:3.5:3.5
2nd Direction: SS III = butan-1-ol/acetic acid/water 80:20:20
The Arabic numbers refer to the metabolites

WHEN REFERRING TO THIS REPORT
USE THIS NUMBER BAYER-PF REPORT
No. 3675
Date: August 5, 1992

Study Title

Metabolism of NTN 33893 in Cotton after Seed Treatment

Data Requirement

171-4 Nature of Residue (Metabolism) - Plants

Author

[redacted]

Completion Date

August 4, 1992

Performing Laboratory

Bayer AG
Crop Protection Research
Environmental Research
Institute for Metabolism Research
D-5090 Leverkusen-Bayerwerk
Germany

Laboratory Project ID

M 173 0311-6

[redacted]

TABLE OF CONTENTS

I. SUMMARY

The metabolism of the insecticide NTN 33893 was investigated in cotton after seed dressing with [pyridinyl-^{14}C-methyl] radiolabelled NTN 33893. The active ingredient was formulated as a 70 WS and applied at a rate equivalent to 460 g a.i./100 kg of seed. The cotton plants were grown in a greenhouse and harvested at maturity (day 211). The mature plants were separated into seed, lint and gin trash. Leaves which fell during the course of the experiment were also collected. The residues, expressed as active ingredient (a.i.) equivalents, were very low. In seed they amounted to 0.0049 mg/kg, in lint 0.0019 mg/kg, in gin trash 0.0050 mg/kg and in leaves 0.11 mg/kg. The oil fraction of the seed contained no radioactivity.

Due to the low residue levels metabolite identification was virtually impossible in seed, lint and gin trash. The only metabolite identified in seed, using two dimensional TLC, was 6-chloronicotinic acid (23 % of the radioactivity in the seed, i.e. 0.0012 mg/kg). No radioactivity could be extracted from lint (0.0019 mg/kg). In leaves the following metabolites were identified using two-dimensional TLC (amounts given are in per cent of the radioactivity in the leaves and in mg/kg a.i. equivalents):

Conjugate of 6-chloropicolyl alcohol		11.3 %	0.012 mg/kg
Guanidine compound	(II)	9.8 %	0.011 mg/kg
Glucoside of 6-chloropicolyl alcohol	(X)	6.3 %	0.007 mg/kg
Unchanged parent compound	(I)	2.9 %	0.003 mg/kg
6-Chloronicotinic acid	(XII)	2.2 %	0.002 mg/kg
6-Chloropicolyl alcohol	(XIII)	1.9 %	0.002 mg/kg
Olefine compound	(VI)	1.5 %	0.002 mg/kg
Nitrosimine compound	(VIII)	1.4 %	0.002 mg/kg

The model experiment, in which radiolabelled NTN 33893 was applied to soil at an exaggerated rate, confirmed the presence of 6-chloronicotinic acid in the seed.

II. INTRODUCTION

The compound NTN 33893 (proposed common name imidacloprid, chemical name 1-(6-chloro-3-pyridinyl)methyl-4,5-dihydro-N-nitro-1H-imidazol-2-amine, CA 105 827-78-9, 1987) is a systemic insecticide, the biological properties of which have been investigated after foliar and soil application.

In plant metabolism studies reported so far (cell culture: [redacted] 1989; tomatoes: [redacted], 1989; potatoes after granular treatment: [redacted] 1992 a; potatoes after spray application: [redacted] 1992; corn: [redacted] 992 b; rice: [redacted] 1991; egg plants: [redacted] 1991; apples: [redacted] 1992) the following metabolites were detected:- the monohydroxy compound (IV) and its conjugate, the dihydroxy compound (VII), the keto compound (XVI), the olefine compound (VI), the urea compound (III), the guanidine compound (II), the nitrosimine compound (VIII), the ring-opened guanidine compound (XV), 6-chloropicolyl alcohol (XIII) and its glucoside (X) and gentiobioside and 6-chloronicotinic acid (XII). After root uptake the proportion of the parent compound was lower than after spray application and was the main component in both.

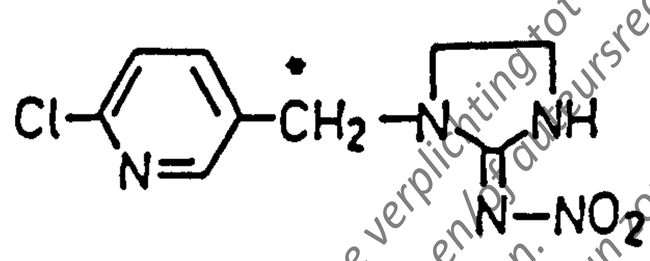
The objective of this study was to investigate the metabolism of NTN 33893 in cotton after seed dressing (experiment 1). This study was supported by a model experiment (experiment 2) where radiolabelled NTN 33893 was applied to soil at an exaggerated rate to obtain larger quantities of metabolites to help in their identification.

The study began with the seed treatment and planting on October 30, 1989 and finished on July 17, 1992. The raw data and original report will be archived in the central GLP Archive, building 6500.

III. MATERIALS

A. Test Compound

Structural formula:



(* denotes labelling position)

Chemical name	:	1-(6-chloro-3-pyridinyl)methyl-4,5-dihydro-N-nitro-1H-imadazole-2-amine (C.A.)
CAS number	:	105 827-78-9 (1987)
Molecular weight	:	255.7
Solubility	:	Water 580 mg/l (20°C), Acetone 46 g/l (20°C)
Specific radioactivity (a.i.)	:	Experiment 1 5.51 MBq/mg (149 µCi/mg) Experiment 2.1 5.53 MBq/mg (149.5 µCi/mg) Experiment 2.2 5.56 MBq/mg (150.2 µCi/mg) (see Appendices I to III, Laboratory Dr. Ecker)
Radiochemical purity	:	99.93 %

Formulation, Experiment 1:
The pyridinyl-^{14}C-methyl radiolabelled active ingredient was formulated as a WS 70 (Laboratory Dr. Ecker, Formulation ECW 6191/1). The radiolabelled active ingredient was finely ground with the formulation auxiliary mixture in a ball mill. The conditions needed to obtain a formulation equivalent to the commercial product were determined in pre-experiments. The content, purity and specific radioactivity of the active ingredient in the formulation were determined by HPLC and LS measurement following extraction from the formulation.

Formulation, Experiment 2:
The pyridinyl-^{14}C-methyl radiolabelled active ingredient was formulated as an SL 200 (Laboratory Dr. Ecker, Formulation ECW 6189/1 for Experiment 2.1 and ECW 6190/1 for Experiment 2.2). For this purpose, the radiolabelled active ingredient was dissolved in the blank formulation. The content, purity and specific radioactivity

of the active ingredient in the formulation were determined by HPLC and LS measurement following extraction from the formulation.

The identity of the active ingredient used for the formulations was confirmed by MS and ^{1}H-NMR spectrometry (see Appendices IV and V, Laboratory Dr. Brauner).

B. Reference Compounds

The structural formulae of the reference compounds, used for the identification of metabolites, and their chromatographic properties are given in Table I. The reference compounds were obtained from Dr. [redacted], Dr.[redacted]), Dr. [redacted]), Dr. [redacted], Dr.[redacted], Dr. [redacted](6-chloronicotinic acid), Dr. [redacted]), Dr. [redacted], Dr. [redacted] (all from Bayer AG) and from the Japanese company Nitokuno (NTN). The stability of the reference compounds was regularly checked during the course of the study by means of thin-layer chromatography.

C. Test Facilities

The study was carried out in the greenhouse (Building 6681) of the Institute for Metabolism Research, Bayer AG, Monheim. The study comprised a seed dressing experiment under normal practice conditions (Experiment 1) and a model experiment (soil drench treatment) using a considerable over-dose to produce larger quantities of metabolites for identification (Experiment 2; subdivided into Experiments 2.1 and 2.2 for reasons of radiation protection). A total of 18 35-litre plastic pots were used in Experiment 1 and two 5-litre plastic pots in Experiment 2. All pots were labelled with a serial number, the radioactivity symbol and the study number (M 173 0311-6). The plant pots were filled with a loamy silt soil (Frimmersdorf, see Appendix VI for composition). The right amount of water to obtain optimum growth of the plants was applied to the soil in the pots.

Fertilization and crop protection measures were carried out and documented and the data were stored with the raw data from the study (for details on plant growth, see Appendix VII).

IV. METHODS

A. Dressing of Cotton Seeds (Experiment 1)

The WS 70 formulation (actual content 60 %, 9.38 mg a.i. = 51.7 MBq) was placed in a 70-ml round-bottomed steel centrifuge bucket and suspended in 80 µl of a 0.2 % Dextrin solution. Eighteen cotton seeds (Coker 310 variety) were added to the solution, the bucket was sealed tight with a cap and then shaken for approx. 2 minutes by a laboratory vibrator in a fume cupboard. The uniformity of the seed coating could be monitored by following the colour of the formulation.

Of the 51.7 MBq radioactivity added, a total of 6.2 MBq was recovered from the centrifuge bucket which means that the total radioactivity applied to the seeds was 45.5 MBq. This corresponds to 2.53 MBq (68.3 µCi) or 0.46 mg a.i. per seed which is equivalent to approx. 460 g a.i./100 kg seed (seed weight approx. 100 mg).

The applied amount (460 g a.i./100 kg seed) is more or less equivalent to the proposed application rate for seed dressing (500 g a.i./ 100 kg).

To check the stability of the active ingredient at the time of application, the storage glass vial used for the WS 70 formulation and the centrifuge bucket in which the seed treatment was carried out were washed with methanol. A TLC investigation of the combined solutions revealed that the radioactivity was exclusively unchanged parent compound (Appendix VIII).

B. Planting and Sampling (Experiment 1)

The 18 seeds were each individually planted in 18 separate 35-l pots (October 30, 1989) immediately following dressing. From the 18 seeds only nine plants emerged. All leaves and flowers which fell from the plants during the period of the experiment were gathered. When the 9 cotton plants reached natural maturity (May 29, 1990 = 211 days after planting), they were harvested and separated into fruit (containing seeds and lint, 1001 g) and gin trash (568.6 g). Approximately half of the fruit was separated manually into seeds (279.4 g) and lint (211.6 g). To remove the lint from the seeds, concentrated sulphuric acid was poured onto the seeds (139.8 g) and stirred for several minutes and the seeds were then filtered through a Büchner funnel and neutralized with water. A total of 136.0 g of seeds was obtained. The sulphuric acid, which did not contain any radioactivity, was

discarded.

C. Planting, Application and Sampling (Experiment 2)

Cotton seeds (Coker 310 variety) were planted in separate 5-l pots (October 26, 1989). Two plant pots with emerged plants were used for the experiment. Increasing amounts of [^{14}C]NTN 33893 in an SL 200 formulation dissolved in water were applied to the soil of each plant pot as a drench treatment (Appendix IX). In total, approx. 30 mg a.i. was applied to each pot between 11 and 145 days after planting. The aim was to apply amounts which were large enough to produce significant amounts metabolites but which did not damage the plant. The right amount was determined in pre-experiments.

The stability of the active ingredient in the application solution was investigated by means of TLC for Experiments 2.1 and 2.2 before and after applications 1 and 5. The active ingredient was stable in the application solution (example for application 1 in Appendix X).

All leaves and flowers which fell from the cotton plants during the experiment period were gathered. As in Experiment 1, once the 2 cotton plants reached natural maturity (June 13, 1990 = 230 days after planting) they were harvested and separated. The respective plant parts of Experiments 2.1 and 2.2 were combined and the following amounts obtained: 82.1 g gin trash, 26.5 g lint and 33.5 g seeds, which were treated with sulphuric acid to remove lint.

D. Processing of the Plant Material

Aliquots of leaves, gin trash and seeds were homogenized in liquid nitrogen in a macerator (Polytron). The radioactivity of one aliquot of the homogenized samples, inclusive of lint, was determined by combustion. During processing, all extracts and solid plant material were stored at -20°C.

D.1. Extraction

The following procedures for extracting individual plant parts were applied in the following sequence (see Figure 1 for diagram and Appendix XI for amounts):

1. Soxhlet extraction (seeds only)

The homogenized seeds were treated with n-hexane and extracted for 5 hours in a Soxhlet. The solution was then reduced to an oily remainder using a rotary evaporator. The residue was dissolved in a few ml of n-hexane (n-hexane phase).

2. Methanol/water extraction (solid seed residue from Soxhlet extraction, gin trash and leaves)

These plant parts were macerated in the Polytron with methanol/water (1:1). The suspension was filtered through a Büchner funnel and the residue macerated further with methanol (2x). The filtrates were combined and, following the removal of an aliquot for radioactivity measurement, reduced to an aqueous remainder using a rotary evaporator (methanol/water phase).

3. Methanol extraction under reflux (seeds, gin trash and lint)

The solid residue of seeds and gin trash from the previous extraction process and the lint were treated with methanol and heated for 36 hours under reflux. Following filtration through a Büchner funnel, the solution was reduced to dryness and the residue dissolved in a few ml of methanol (methanol extract).

4. Methanol/hydrochloric acid extraction under reflux (seeds, gin trash and lint)

The respective solid residues from the previous extraction process were treated separately with methanol/6N hydrochloric acid (1:1) and heated for 6 hours under reflux. Following filtration through a Büchner funnel, the solution was reduced to dryness and dissolved in a few ml of methanol (methanol/ hydrochloric acid extract).

5. Methanol/sodium hydroxide extraction under reflux (seeds only)

The solid seed residue was treated with methanol/2N sodium hydroxide (3:2) and heated for 6 hours under reflux. Following filtration through a Büchner funnel, a solid residue (non-extractable residue) and an extract (methanol/ sodium hydroxide extract) were obtained. In the methanol/sodium hydroxide extract only the radioactivity was measured.

Raw data from the analysis of radioactivity and the material balance following extraction are given in Appendices XII-XV for Experiment 1 and Appendices XVI and XVII for Experiment 2. Of the radioactivity in the individual plant fractions, previously determined by combustion, between 85.1 and 107.2 % was recovered in Experiment 1 and 87.1 % (on day 21 of the storage stability investigation) and 82.5 % (on day 279) in Experiment 2. The sum of the radioactivity values of the respective extracts and of the solid residue was normalized to 100 %.

D.2. Purification of the methanol/water phase

The aqueous remainder of the methanol/water extract of seeds, gin trash and leaves was purified using an XAD 4 adsorber resin column (XAD 4 : 200 - 400 µm, Serva, Heidelberg, column: 50 cm long, 30 mm diameter).

The aqueous phase was added to the column, which had been pre-washed with methanol and water, and washed with 250 ml distilled water. The aqueous solution, which contained no radioactivity, was then discarded. The column was eluted with 250 ml methanol and the eluate reduced to dryness using a rotary evaporator. The residue was redissolved in methanol.

There was no significant loss of radioactivity during purification with the XAD 4 column.

D.3. Isolation of metabolite 6 from the methanol/water phase of leaves after clean-up by XAD 4

Metabolite 6 was isolated from the methanol eluate obtained after clean-up of the methanol/water phase of leaves by XAD 4. Initially, 200 µl of the methanol eluate (0.44 kBq) was applied to each of 20 aluminium sheets which were then developed with solvent system IV. The zones containing metabolite 6, located using a Linear Analyser, were cut from the sheets and metabolite 6 eluted with methanol. The 20 eluates were combined and further purified on 4 aluminium sheets developing this time with solvent system III. The 4 methanol eluates were combined and evaporated to a small volume (2 ml) which contained approximately 624 Bq.

E. Thin-Layer Chromatography

One- and two-dimensional TLC investigations of radioactive solutions were carried out using pre-coated silica gel glass plates or aluminium sheets (Kieselgel 60 F_{254}, Merck, Darmstadt) with the following solvent systems:

Solvent system SS I:	ethyl acetate/i-propanol/water 65:23:12
Solvent system SS II:	ethyl acetate/toluene/methanol/acetic acid 80:20:20:1
Solvent system SS III:	butanol/acetic acid/water 80:20:20
Solvent system SS IV:	chloroform/methanol/acetic acid/water 65:25:3.5:3.5
Solvent system SS V:	n-hexane/ethyl acetate 60:40

The radioactive compounds were detected either using a Linear Analyzer (Raytest TM 3000) or through exposure to X-ray film (Curix RP-1, Agfa-Gevaert). The co-chromatographed reference compounds were visualized by UV light (254 nm).

F. Enzymatic Hydrolysis

An aliquot (ca. 65 Bq) of the solution containing metabolite 6, obtained from section IV.D.3., was evaporated to dryness under a stream of nitrogen. To the dried residue 2 ml of a Cellulase solution (5 mg Cellulase from Aspergillus Niger, Serva, 1.3 U/mg dissolved in 2 ml acetate buffer pH 5) were added and stirred (24 h) at 37ºC. After cooling, the solution was partitioned with ethyl acetate (3 x 2 ml). The combined ethyl acetate phases were analysed by TLC.

A blank assay (same conditions as above but without enzyme) and a control assay (same conditions as above but in the presence of 4-nitrophenol-ß-D-glucopyranoside as a control substrate to check enzyme activity) were conducted in parallel to the main hydrolysis experiment. For these assays only ca. 32 Bq of the solution containing metabolite 6 were used.

G. Mass Spectrometry

The mass spectra were recorded on a mass selective detector (HP 5970) with GC HP 5880A (Hewlett-Packard). The capillary column used was a 15 m DB-1 (Fisons, inner diameter 0.25 mm, film thickness 0.25 µm). Splitless injection was done at 280° C. The oven temperature program was 80° C for one minute, heating rate 10°/min up to 280° C, the final temperature was kept constant for 20 minutes. The samples were silylated for 1 h at 70°C using MSTFA (N-methyl-N-trimethylsilyltrifluoroacetamide, PIERCE) in acetonitrile (1:1). Injection was done directly from the reaction mixture.

H. Measurement of Radioactivity

The ^{14}C-activity of liquid samples was measured by means of a liquid scintillation counter (PW 4700 from Philips/Raytest or LKB 1219 Rackbeta) and Instant-Scint-Gel (Packard). Details of scintillation measurement are given in Appendix XVIII.

Solid samples were combusted using an OX 300 oxidizer manufactured by the Harvey Instrument Corporation (Zinsser). The CO_2 produced by combustion was absorbed in a scintillation cocktail (8 ml Carbosorb + 10 ml Permafluor V, Packard) and the radioactivity in the solution measured by LSC (PW 4700, Philips).

The radioactivity on the one- and two-dimensional TLC plates was determined either by scraping off the silica gel from the area of the radioactive zones followed by suspension in a scintillation cocktail and measurement (quantitative determination of the metabolites) or by quantifying the radioactive zones by means of a linear analyzer (TM 3000, Raytest).

In the analysis of the two-dimensional TLC plates by scraping off the silica gel, only the results from the visible darkened zones were used for calculation. The results from the remaining zones, where there was no recognizable darkening and the radioactivity content was at the background level, were not taken into account in this calculation.

I. Storage Stability Investigation

The storage stability investigation was conducted on the seeds from the model experiment (Experiment 2), since the total residue from the seeds from the seed dressing experiment (Experiment 1) was too small for chromatographic investigation. The seeds were analysed 21 and 279 days after the beginning of the storage period. The plant samples were stored at -20°C in a freezer.

V. RESULTS AND DISCUSSION

A. Experiment 1 Following Seed Dressing

A.1. Total residues and distribution of radioactivity

The total residues in the individual cotton plant parts were determined by combustion and calculated as active ingredient equivalents. The levels were very low, amounting at maturity (211 days after planting) to 0.0049 mg/kg in the seeds, 0.0050 mg/kg in the gin trash, 0.0019 mg/kg in the lint and 0.11 mg/kg in the leaves (Table II). The raw data for the radioactivity measurements are given in Appendices XII to XV.

The up-take rate of the radioactivity applied to the whole cotton plant was 4.88 % (seeds 0.067 %, gin trash 0.068 %, lint 0.020 % and leaves 4.72 %).

The distribution of the radioactivity in the individual fractions following extraction is given in Table III. The n-hexane phase of the seed extract from the Soxhlet process, with which the plant oil was removed, contained no radioactivity. The proportion of radioactivity in the methanol/water phase, the methanol extract and the methanol/HCl extract of the seeds was more or less the same (33.4 %, 23.3 % and 28.9 % respectively). The methanol/NaOH extraction did not release any radioactivity. The remaining 14.4 % of the radioactivity was non-extractable. In gin trash 76.4 % of the radioactivity was extracted with methanol/water. The two subsequent extractions did not result in any further extraction of radioactivity. It was not possible to extract any radioactivity from the lint (methanol under reflux and methanol/HCl under reflux). A total of 73.2 % of the radioactivity in leaves was extracted with methanol/water.

A.2. Metabolism investigation

I. Seeds (0.0049 mg/kg)

Since the total residues, and therefore the ratio between amounts of radioactivity and plant matrix, were very low it was only possible to determine and identify the metabolites by TLC on a very limited scale. The results of the model experiment involving the drench treatment (Experiment 2) were used to support the results from Experiment 1.

In the methanol/water phase (33.4 % of radioactivity in seeds, 0.0016 mg/kg) traces of metabolite 2 = 6-chloronicotinic acid (XII) were detected by two-dimensional TLC (Figure 2). The area with an R_f-value of approximately 0.9, which is clearly visible in Figure 2, was attributed to natural plant constituents, since it did not contain any radioactivity. This was proven by scraping off the silica gel from this area and carrying out LS measurement. The methanol extract (23.3 %, 0.0012 mg/kg) contained 6-chloronicotinic acid only (Figure 3). Unchanged parent compound was not detected in any of the extracts. The radioactivity in the other seed extracts was too low to warrant chromatographic analysis.

The presence of 6-chloronicotinic acid (XII) as the main component in seeds (approx. 23 %, 0.0012 mg/kg) was confirmed by the model experiment (Experiment 2).

2. Gin trash (0.0050 mg/kg)

These extracts could not be chromatographed (TLC) due to the low levels of radioactivity. The gin trash from the model experiment was not investigated.

3. Lint (0.0019 mg/kg)

Since the radioactivity in this case was non-extractable, it was assumed that radioactivity was incorporated into the natural components of the lint. The lint from the model test was not investigated.

4. Leaves (0.11 mg/kg)

A large number of metabolites were detected (Table IV and Figure 4) in the methanol/water phase (73.2 %, 0.08 mg/kg) by two-dimensional TLC and identified by co-chromatography with reference compounds. Quantitative determination was carried out by scraping off the radioactive zones and carrying out LS measurement. The main identified or characterised metabolites were metabolite 6 = conjugate of 6-chloropicolyl alcohol (11.3 %, 0.012 mg/kg), metabolite 5 = guanidine compound (II) (9.8 %, 0.011 mg/kg) and metabolite 7 = glucoside of the 6-chloropicolyl alcohol (X) (6.3 %, 0.007 mg/kg). The other metabolites (between 1.4 % and 2.9 %, between 0.002 and 0.003 mg/kg, respectively) were: component 1 = unchanged parent compound (I), metabolite 2 = 6-chloronicotinic acid (XII), metabolite 9 = chloropicolyl alcohol (XIII), metabolite 11 = olefine compound (VI) and metabolite 10 = nitrosimine compound (VIII).

Metabolite 6 was isolated from the methanol/water phase of leaves (experiment 1) using TLC (see section IV.D.3., Figure 5) and subsequently characterised as a conjugate of 6-chloropicolyl alcohol. This was achieved by enzymatic cleavage of the conjugate with cellulase and the resulting aglycone identified as 6-chloropicolyl alcohol by cochromatography (TLC) with the reference compound (Figures 6 and 7) and by GC/MS. The electron impact mass spectrum of the trimethylsilyl derivative showed a molecular ion at m/z 215, loss of a methyl group yielded in the base peak m/z 200. The chloropicolyl fragment appeared at m/z 126. The spectrum was in good accordance to that of the reference compound DIJ 9805 (Figure 8). The slight differences in the relative intensities were caused by subtraction of the background spectrum due to the high complexity of the sample. The high intensity of the chlorine isotopic peaks was due to the high specific radioactivity of the active ingredient applied.

A further 4 unknown metabolites were detected and their amounts ranged between 0.8 and 4.4 %, 0.001 and 0.005 mg/kg, respectively. Part of the radioactivity consisted of polar material (25.5 %) which remained at the origin of the TLC plate and of compounds where distribution was diffuse. The non-extractable residue amounted to 26.8 % or 0.029 mg/kg.

B. Model Experiment (Experiment 2)

The aim of the model experiment, in which the active ingedient was poured onto the soil in an excess amount, was to support the results of the metabolism investigation following seed dressing (Experiment 1) and to carry out a storage stability investigation. Of the individual plant parts, only the seeds were examined in more detail.

B.1. Total residues and distribution of radioactivity

The total radioactivity in the seeds, which was determined by combustion, was 9.35 mg/kg (Table II). The distribution of radioactivity in the extracts was similar to that in Experiment 1 (Table V). Most of the radioactivity was present in the methanol/water phase (19.9 %), the methanol extract (44.5 %) and the methanol/hydrochloric acid extract (32.4 %).

Total residues amounted to 3.50 mg/kg in the gin trash and 0.72 mg/kg in the lint.

B.2. Metabolism in seeds

The results of the metabolism investigation are shown in Table VI (1st analysis). The seeds were analysed a second time (Table VI, 2nd analysis) for the storage stability study (see Section V.C.).

The methanol/water phase of seeds was analyzed by two-dimensional TLC in two solvent systems with co-chromatographed reference compounds. The following components were detected: component 1 = unchanged parent compound and metabolite 2 = chloronicotinic acid (XII) as the main constituents (Figures 9 and 10, Table VI). Also detected were a number of metabolites which did not correspond to reference compounds (Table I).

Two-dimensional TLC in 2 solvent systems indicated that the methanol extract contained mainly metabolite 2 = 6-chloronicotinic acid (XII) and small amounts of metabolite 3 = 6-chloronicotinic acid methyl ester (XVII, Figures 12 and 13). The identification of metabolite 3 was carried out for the methanol extract of the 1st and 2nd analysis (see section V.C.).

The methanol/hydrochloric acid extract mainly contained 4 metabolites which did not correspond to any of the reference compounds (Figure 15).

C. Storage Stability Investigation

The residue amounts in the plant material, in particular in the seeds from the seed dressing experiment (Experiment 1), were too low for metabolites to be determined. Therefore, the storage stability investigation was conducted on the seeds from the model experiment (Experiment 2). The 1st analysis (21 days after the beginning of the storage period) also involved the metabolism investigation. The 2nd analysis was carried out 279 days after the storage period began. The results are given in Table VI.

In all 3 extracts (methanol/water phase, methanol extract and methanol/ hydrochloric acid extract), the metabolite pattern was similar in the 1st and 2nd analyses. The respective two-dimensional TLC chromatograms from the 1st analysis are shown in Figures 9, 12 and 15, and Figures 11, 14 and 16 show the respective chromatograms from the 2nd analysis.

In the 2nd analysis of the methanol/water phase, a new metabolite (metabolite 4) was detected (3.8 %, approximately 0.36 mg/kg) while the concentration of metabolite 2 = 6-CNA (XII) had reduced by this amount. Metabolite 4 did not correspond to the 6-CNA methyl ester (XVII) (Figure 17). Treatment with 2N sodium hydroxide solution (for 10 min.) resulted in the disappearance of metabolite 4 and an increase in the proportion of 6-CNA (XII). For this reason, it was assumed that metabolite 4 was an ester formed by reaction of 6-CNA with a natural plant alcohol constituent.

In the methanol extract, the amount of metabolite 2 = 6-CNA (XII) decreased between the 1st and the 2nd analysis by approximately the same amount as metabolite 3 increased (Table VI). It was established by TLC that metabolite 3 corresponded to 6-CNA-methyl ester (XVII) (Figure 18). Hydrolysis of metabolite 3 with 2N sodium hydroxide produced 6-CNA and therefore metabolite 3 was considered to be an artifact formed by the esterification of 6-CNA with methanol.

In the methanol/hydrochloric acid extract, only unknown metabolites were detected. Their amount did not change very much between the 1st and the 2nd analyses (Figures 15 and 16).

To sum up, the results indicated that over the period of 258 days there was no appreciable degradation of the parent compound, 6-CNA and its esters or of unknown metabolites during storage at -20°C.

VI. CONCLUSIONS

Following seed dressing of cotton seeds with a WS 70 formulation (460 g a.i./100 kg) equivalent to the proposed application rate (500 g a.i./100 kg), the individual plant parts contained very low total residues at maturity, 211 days after planting. The seeds contained 0.0049 mg/kg, gin trash 0.0050 mg/kg, lint 0.0019 mg/kg total and leaves 0.11 mg/kg residues. There was no detectable radioactivity in the oil fraction of the seeds (n-hexane phase).

Due to the low amounts of total residues, metabolite determination was impossible, but it was possible to show in a model experiment that the active ingredient was virtually completely metabolized. The main metabolite in the seeds of the model experiment was 6-chloronicotinic acid (XII). Several unknown metabolites were also detected. There were indications of the presence of 6-chloronicotinic acid in the seeds of the seed dressing experiment.

None of the radioactivity contained in the lint of the seed dressing experiment could be extracted. In this case it was therefore assumed that radioactivity had been incorporated into natural plant constituents.

A large number of metabolites were detected in the cotton leaves from the seed dressing experiment, some of which were identified (see Table IV). The composition of metabolites was similar to that determined in a study of corn following seed dressing application [redacted] 1992 b) and a study of potatoes following granular application [redacted] 1992a).

Extrapolation of the results from the seeds of the model overdose experiment (about 60 times) to the seeds of the normal dose experiment suggest that, parallel to the presence of 6-chloronicotinic acid, one or several metabolites may exist which are derived from the dihydroimidazole ring after cleavage of the two rings. However, the total residues in seeds (0.0049 mg/kg) are below the 0.01 mg/kg level. This is also the case for gin trash (0.0050 mg/kg) and lint(0.0019 mg/kg). Although the total residues in leaves are higher (0.11 mg/kg) the amount of any metabolite derived only from the dihydroimidazole ring (based on 6-CNA and 6-chloropicolyl alcohol, free and conjugated, together 21.7 % of the total residue) would be only ca. 0.024 mg/kg.

In the storage stability investigation of seeds from the model experiment it was established that the metabolite 6-chloronicotinic acid (XII) formed esters in combination with alcohols, e.g. methanol. However, these were considered to be artifacts and were not produced by degradation since the basic structure of the 6-chloronicotinic acid remained intact.

A degradation pathway for NTN 33893 in cotton after seed dressing is shown in Figure 19.

VII. SIGNATURES

[redacted]

August 4 1992
Date

[redacted]opic

Aug 5 1992
Date

[redacted]ent

Aug. 5 1992
Date

Aug 5, 1992
Date

Acknowledgement:

We gratefully acknowledge the excellent technical assistance of [redacted]

VIII. Quality Assurance Statement

Referat GLP
Quality Assurance Statement
[redacted]

IX. REFERENCES

[redacted] (1992)
Study on the Metabolism of NTN 33893 in Potatoes after Spray Application.
Bayer AG, Internal PF-Report No. 3678, 8/5/92

[redacted] (1989)
Investigation of the Metabolism of NTN 33893 after Application to Tomatoes.
Bayer AG, Internal PF-Report No. 3257, 12/10/1989, revised 7/5/1992

[redacted] (1989)
Metabolism of [pyridinyl^{14}C-methyl]NTN 33893 in Potato, Wheat and Corn Cell Suspension Cultures.
Bayer AG, Internal PF-Report No. 3179, 16/5/1989

[redacted] (1991)
Metabolism of [pyridinyl^{14}C-methyl]NTN 33893 in Rice Plants (Nursery Box Application).
[redacted], Yuki Research Center, Japan, Report No. 1284 (ESR/ENG)

[redacted] (1992)
Metabolism of [^{14}C]NTN 33893 in Apples
Bayer AG, Internal PF-Report No. 3676, 27/2/1992

[redacted] (1992 a)
Investigation of the Metabolism of NTN 33893 in Potatoes after Granular Application.
Bayer AG, Internal PF-Report No. 3628, 19/7/1991, revised 4/5/1992

[redacted] (1992 b)
Metabolism of NTN 33893 in Corn after Seed Treatment.
Bayer AG, Internal PF-Report No. 3673, 14/1/1992

[redacted] (1991)
Metabolism of NTN 33893 in Eggplant.
[redacted] K.K., Yuki Research Center, Japan, Report No.1290
(ESR/ENG)

X. TABLES

Table I: Structures and R_f values of reference compounds

Solvent Systems
SS I : ethyl acetate/i-propanol/water 65:23:12
SS II : ethyl acetate/toluene/methanol/acetic acid 80:20:20:1
SS III : n-butanol/acetic acid/water 80:20:20
SS IV : chloroform/methanol/acetic acid/water 65:25:3.5:3.5

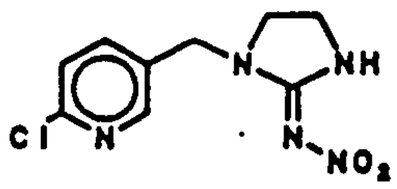
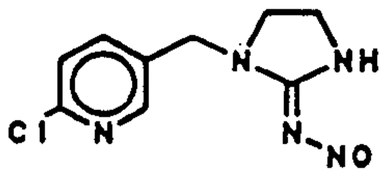
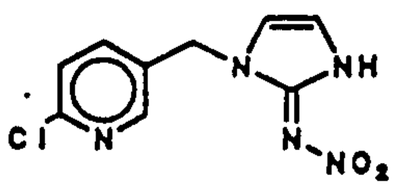
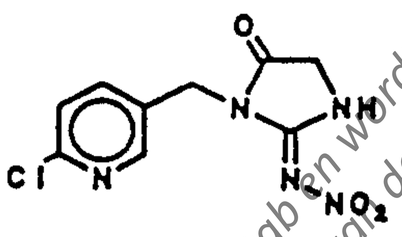
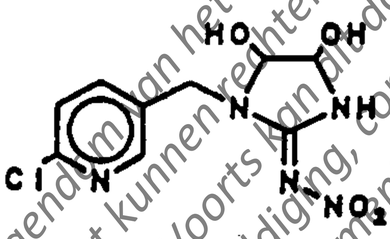
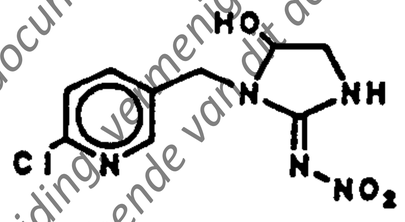
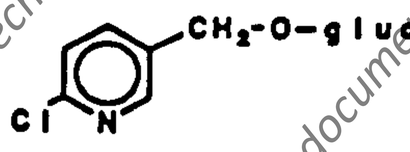
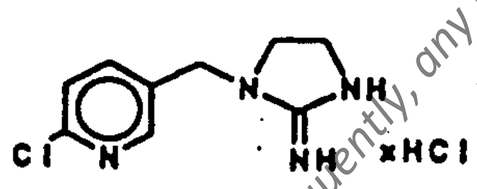
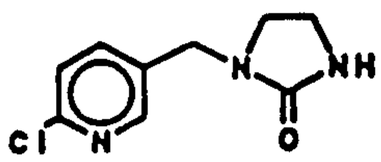
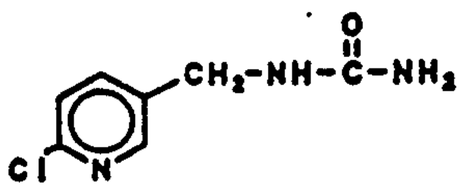
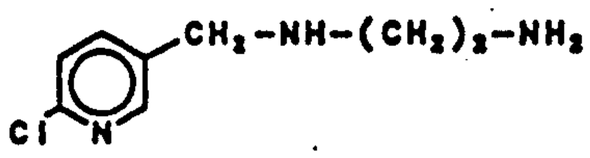
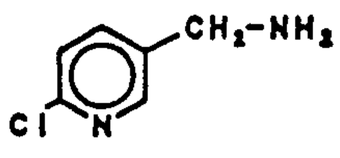
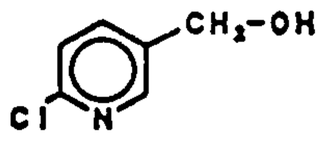
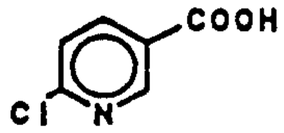
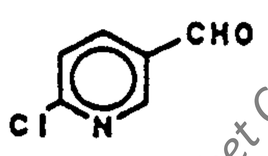
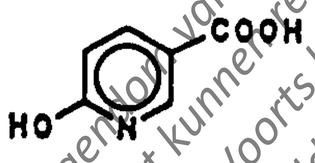
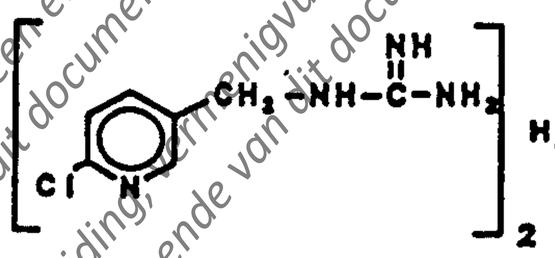
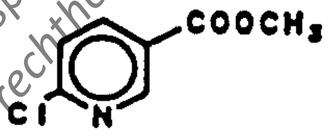
Compound		SS I	SS II	SS III	SS IV
	NTN 33893 (I) parent compound	0.76	0.65	0.60	0.93
	WAK 3839 (VIII) nitrosimine comp.	0.54	0.38	0.52	0.85
	NTN 35884 (VI) olefin compound	0.72	0.54	0.73	0.83
	WAK 3738 (XVI) 5-keto compound	0.93	0.85	0.78	0.93
	WAK 3772 (VII) dihydroxy compound	0.93	0.75	0.86	0.79
	WAK 4103 (IV) 5-hydroxy compound	0.89	0.73	0.74	0.89
	RBN 1114 (X) glucoside	0.60	0.27	0.61	0.46
	BEG 5322 (II) guanidine compound	Origin	Origin	0.35	0.39

Table I continued:

Compound		SS I	SS II	SS III	SS IV
	DIJ 9817 (III) urea compound	0.68	0.50	0.67	0.91
	NTN 36749	0.10	0.02	0.46	0.34
	DIJ 9646-2	Origin	Origin	0.15	0.14
	GSE 1478	0.15	0.05	0.47	0.35
	DIJ 9805 (XIII) 6-chloropicolyl alcohol	0.89	0.77	0.87	0.86
	6-chloro- (XII) nicotinic acid = 6-CNA	0.34	0.48	0.85	0.77
	MAT 10249-D	0.94	0.88	0.93	0.94
	GBH 4315	0.17	0.23	0.77	0.54
	WAK 4126 (XV) ring opened guanidine	0.05	Origin	0.42[1] 0.44	0.31
	BNF 5535D (XVII)[2] 6-CNA-metyl ester	nd[3]	nd[3]	nd[3]	nd[3]

1) Two spots were observed under UV light (254 nm)
2) BNF 5535D (XVII) was only used in SS V: n-hexane/ethyl acetate 60:40 (R_f-value 0.72)
3) nd = not determined

Table II:

Total ^{14}C-residues in the plant parts of cotton following treatment with [pyridinyl-^{14}C-methyl]NTN 33893

Experiment 1: Seed treatment
Experiment 2: Drench treatment (over dosage)

mg/kg values expressed as NTN 33893 equivalents

Experiment	Plant part	Total residues (mg/kg)
1	seed	0.0049
	gin trash	0.0050
	lint	0.0019
	leaves	0.11
2	seed	9.35
	gin trash	3.50
	lint	0.72

Table III

^{14}C-Residues in fractions after extraction of various plant parts of cotton following seed treatment (experiment 1) with [pyridinyl-^{14}C-methyl]NTN 33893

Radioactivity in the plant part = 100 %

mg/kg values expressed as NTN 33893 equivalents

Fraction	Seeds			Gin trash			Lint			Leaves		
	kBq	%	mg/kg	kBq	%	mg/kg	kBq	%	mg/kg	kBq	%	mg/kg
n-hexane phase	0	0	0	*	*	*	*	*	*	*	*	*
methanol/water phase	5.09	33.4	0.0016	11.89	76.4	0.0038	*	*	*	787.3	73.2	0.08
methanol extract, reflux	3.55	23.3	0.0012	0	0	0	0	0	*	*	*	*
methanol/6N HCl 1:1, reflux	4.40	28.9	0.0014	0	0	0	0	0	*	*	*	*
methanol/2N NaOH 3:2, reflux	0	0	0	*	*	*	*	*	*	*	*	*
non-extractable residue	2.20	14.4	0.0007	3.67	23.6	0.0012	4.51	100	0.0019	288.3	26.8	0.03
total	15.24	100	0.0049	15.56	100	0.005	4.51	100	0.0019	1076	100	0.11

* extraction was not performed

Table IV:

Distribution of the metabolites in the methanol/water phase of leaves, experiment 1

Radioactivity in the leaves = 100 %
mg/kg values expressed as NTN 33893 equivalents

Compound/Metabolite	%	mg/kg
component 1 = NTN 33893, parent compound (1)	2.9	0.003
metabolite 2 = 6-chloronicotinic acid (XII)	2.2	0.002
metabolite 5 = guanidine compound, BEG 5322 (II)	9.8	0.011
metabolite 6 = conjugate of 6-chloropicolyl alcohol	11.3	0.012
metabolite 7 = glucoside compound, RBN 1114 (X)	6.3	0.007
metabolite 9 = 6-chloropicolyl alcohol, DIJ 9805 (XIII)	1.9	0.002
metabolite 10 = nitrosimine compound, WAK 3839 (VIII)	1.4	0.002
metabolite 11 = olefine compound, NTN 35884 (VI)	1.5	0.002
total identified	37.3	0.041
metabolite 8 = unknown	0.9	0.001
metabolite 12 = unknown	0.8	0.001
metabolite 13 = unknown	4.4	0.005
metabolite 14 = unknown	4.3	0.005
origin and diffuse radioactivty	25.5	0.028
methanol/water phase	73.2	0.081
non-extractable residue	26.8	0.029
total	100	0.110

Table V:

^{14}C-Residues in fractions after extraction of cotton seeds following drench treatment of the soil with [pyridinyl-^{14}C-methyl]NTN 33893 (experiment 2)

Radioactivity in the seeds = 100 %
mg/kg values expressed as NTN 33893 equivalents

Fraction	kBq	%	mg/kg
n-hexane phase	10.41	0.6	0.05
methanol/water phase	345.39	19.9	1.86
methanol extract, reflux	772.36	44.5	4.16
methanol/6N HCl 1:1, reflux	562.35	32.4	3.03
methanol/2N NaOH 3:2, reflux	36.45	2.1	0.20
non-extractable residue	8.68	0.5	0.05
total	1735.64	100	9.35

Table VI:

Results of the storage stability study of parent compound (I) and metabolites in the seeds of the model experiment

Compound/Metabolite	1. Analysis Extraction date 4/7/90(day 21)		2. Analysis Extraction date 19/3/91(day 279)	
	%	mg/kg	%	mg/kg
n-hexane phase	0.6	0.05	0.7	0.06
methanol/water phase	(19.9)	(1.86)	(19.2)	(1.80)
component 1 = parent compound (I)	0.8	0.08	1.0	0.09
metabolite 2 = 6-chloronicotinic acid (XII)	7.4	0.69	3.7	0.35
metabolite 4 = unknown ester of 6-CNA	0	0	3.8	0.36
unknown components and diffuse radioactivity	11.7	1.09	10.7	1.00
methanol extract, reflux	(44.5)	(4.16)	(40.1)	(3.75)
metabolite 2 = 6-chloronicotinic acid (XII)	33.5	3.13	25.5	2.38
metabolite 3 = 6-CNA methyl ester (XVII)	2.6	0.24	6.8	0.64
minor unknown metabolites	8.4	0.79	7.8	0.73
methanol/6N HCl 1:1 reflux	(32.4)	(3.03)	(37.2)	(3.48)
metabolite 19 = unknown	3.6	0.33	2.6	0.24
metabolite 15 = unknown	17.3	1.62	16.7	1.56
metabolite 16 = unknown	4.7	0.44	6.7	0.63
metabolite 17 = unknown	1.9	0.18	2.8	0.26
metabolite 18 = unknown	0	0	2.5	0.24
minor unknown components	4.9	0.46	5.9	0.55
methanol/2N NaOH 3:2 reflux	2.1	0.20	*	*
non-extractable residue	0.5	0.05	2.8	0.26
total	100	9.35	100	9.35

* extraction was not performed

XI. FIGURES

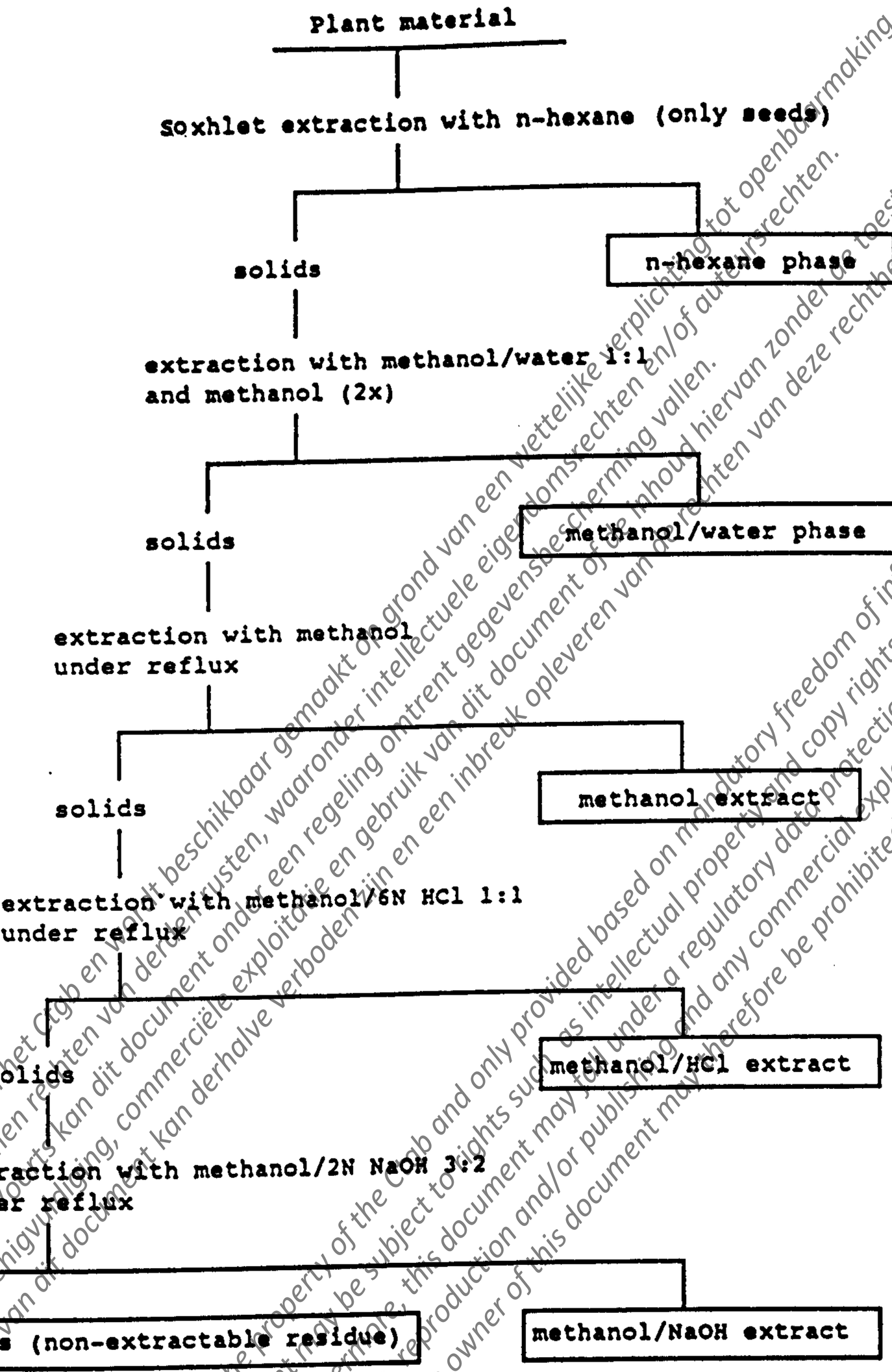


Figure 1:

Scheme of the extraction procedure for NTN 33893 treated cotton plant material

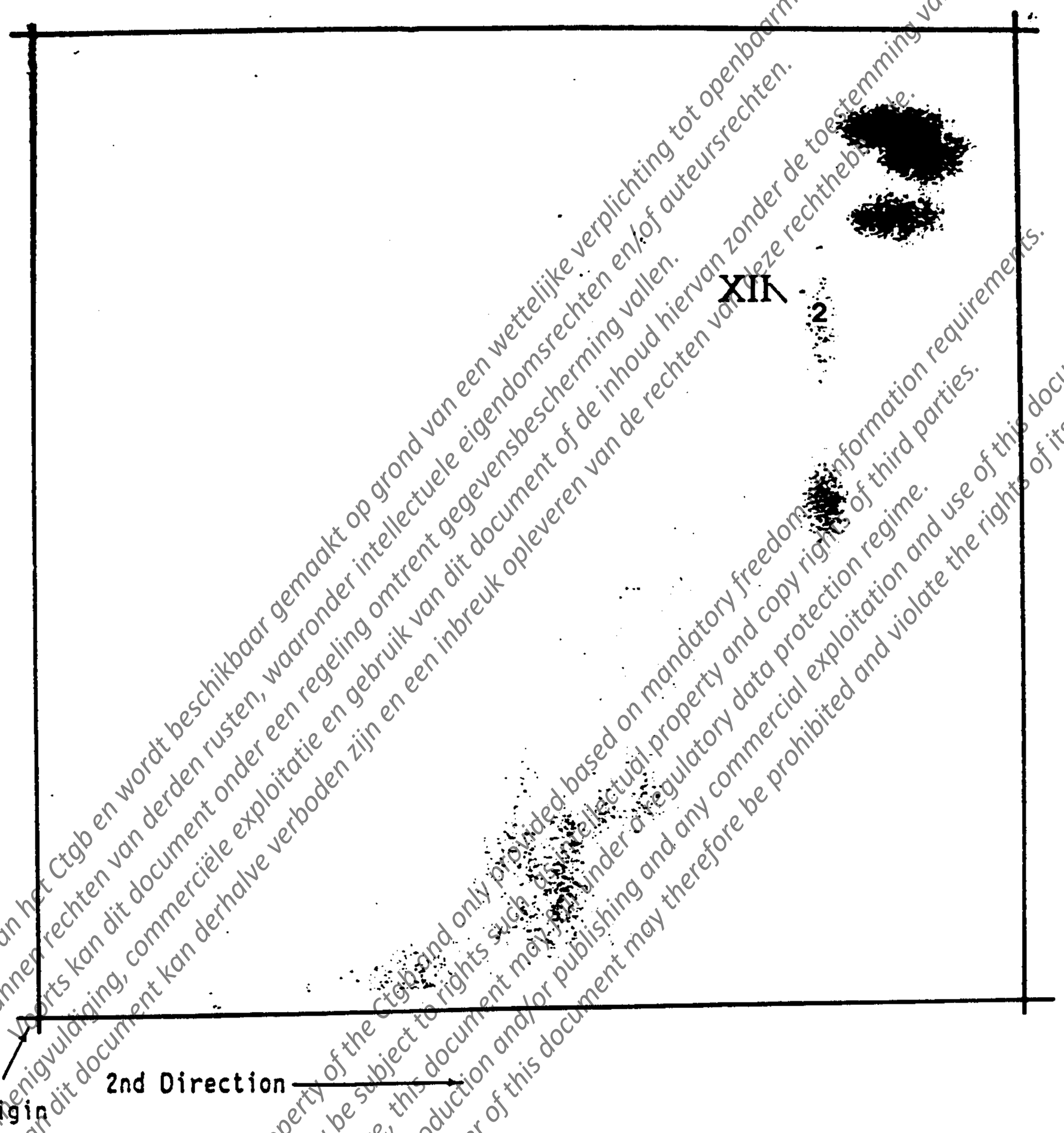


Figure 2:

Autoradiogram of two-dimensional TLC of the methanol/water phase of seeds, experiment 1

1st Direction: SS IV = chloroform/methanol/acetic acid/water 65:25:3.5:3.5
2nd Direction: SS III = n-butanol/acetic acid/water 80:20:20
The Arabic numbers refer to the metabolites, the Roman numbers refer to reference compounds.

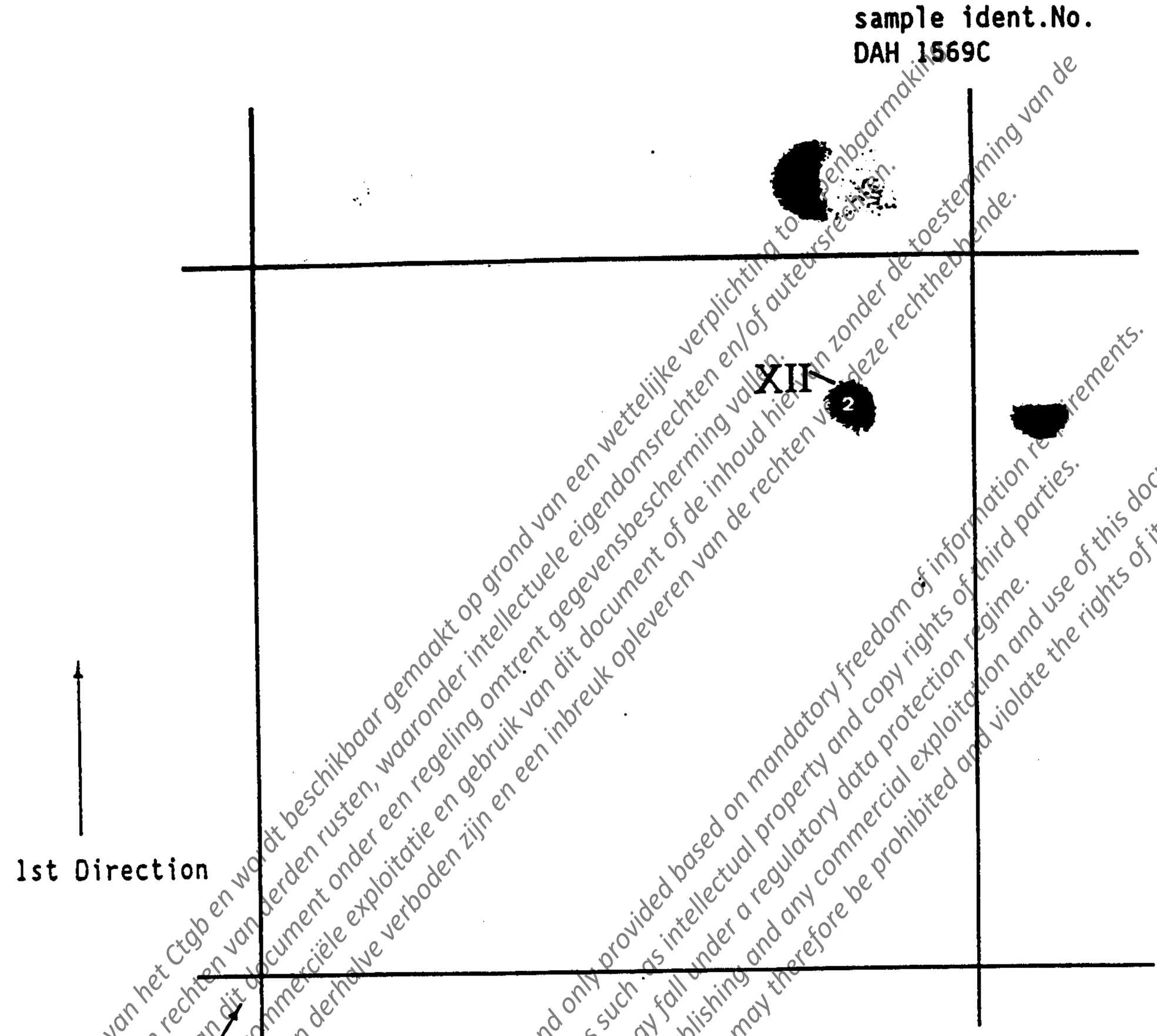


Figure 3:

Autoradiogram of two-dimensional and one-dimensional TLC of the methanol extract of seeds, experiment 1

1st Direction: SS IV = chloroform/methanol/acetic acid/water 65:25:3.5:3.5
2nd Direction: SS III = n-butanol/acetic acid/water 80:20:20
The Arabic numbers refer to the metabolites, the Roman numbers refer to reference compounds.

sample ident.No.
DAH 1577A

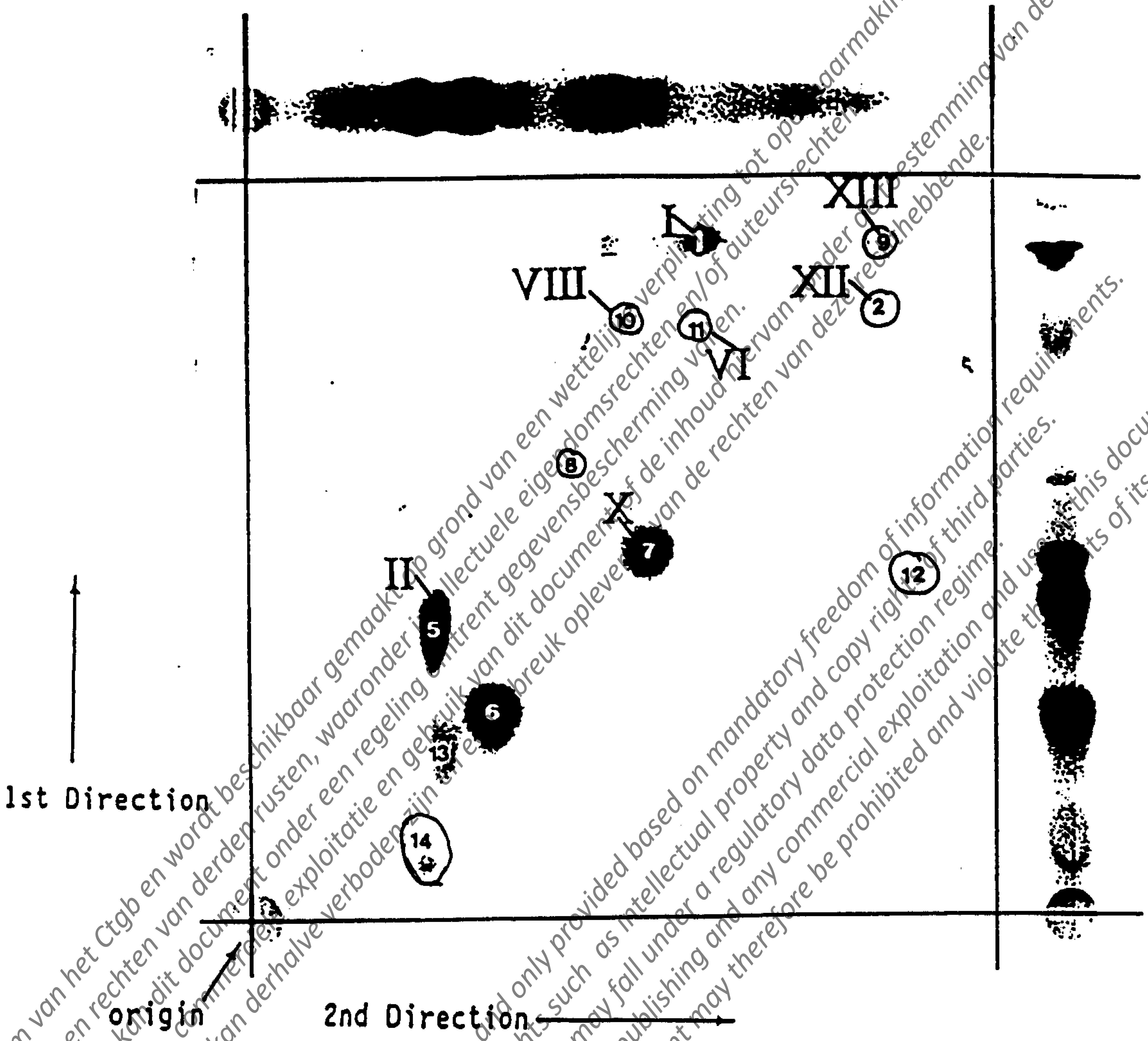


Figure 4:

Autoradiogram of two-dimensional and one-dimensional TLC of the methanol/water phase of leaves, experiment 1

1st Direction: SS IV = chloroform/methanol/acetic acid/water 65:25:3.5:3.5
2nd Direction: SS III = n-butanol/acetic acid/water 80:20:20
The Arabic numbers refer to the metabolites, the Roman numbers refer to reference compounds.

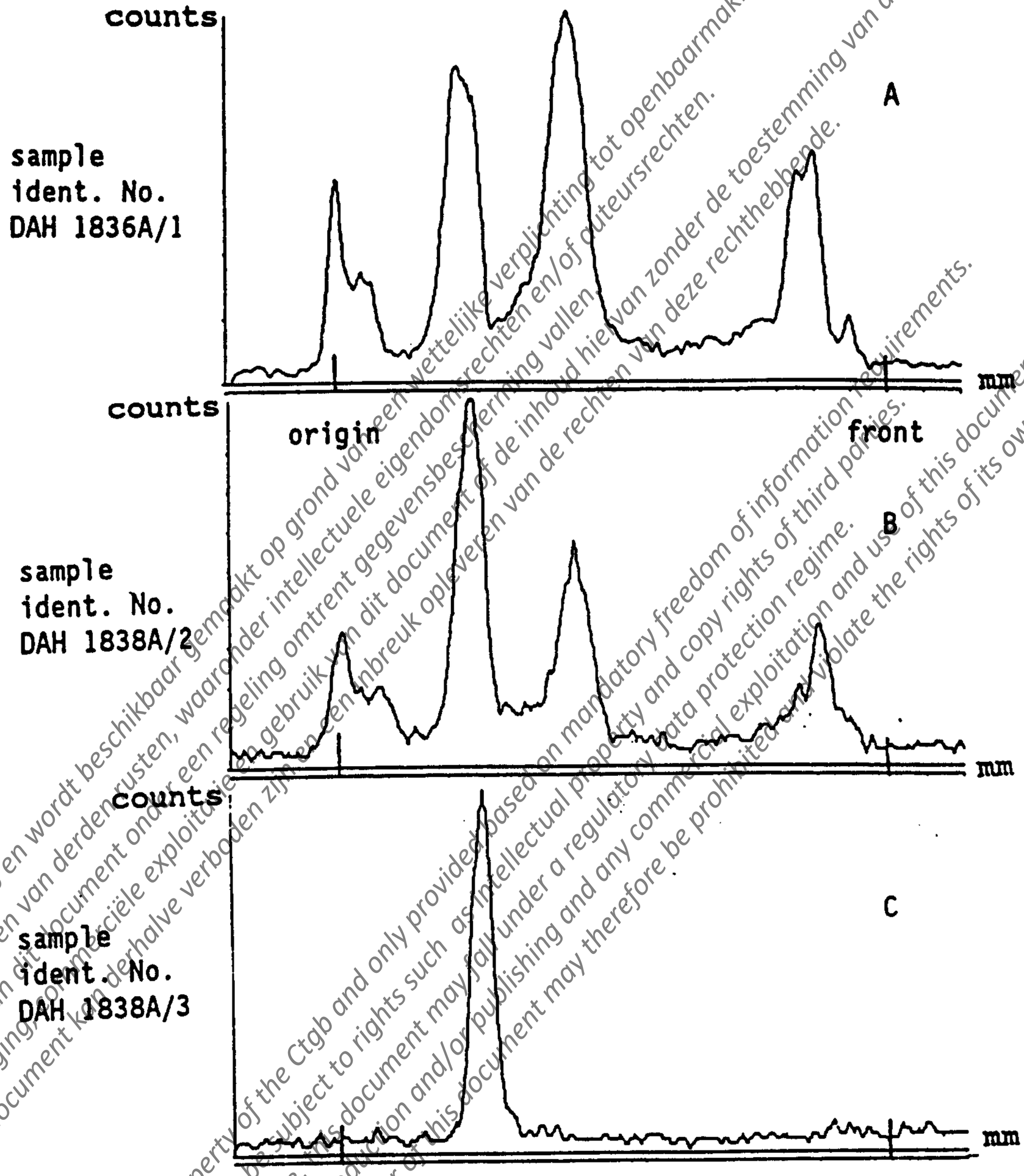


Figure 5:

TLC chromatograms of the methanol/water phase of leaves, experiment 1 (A), of the isolated metabolite 6 (C) and of a mixture of A and C (B).

Solvent system IV: chloroform/methanol/acetic acid/water 65:25:3.5:3.5

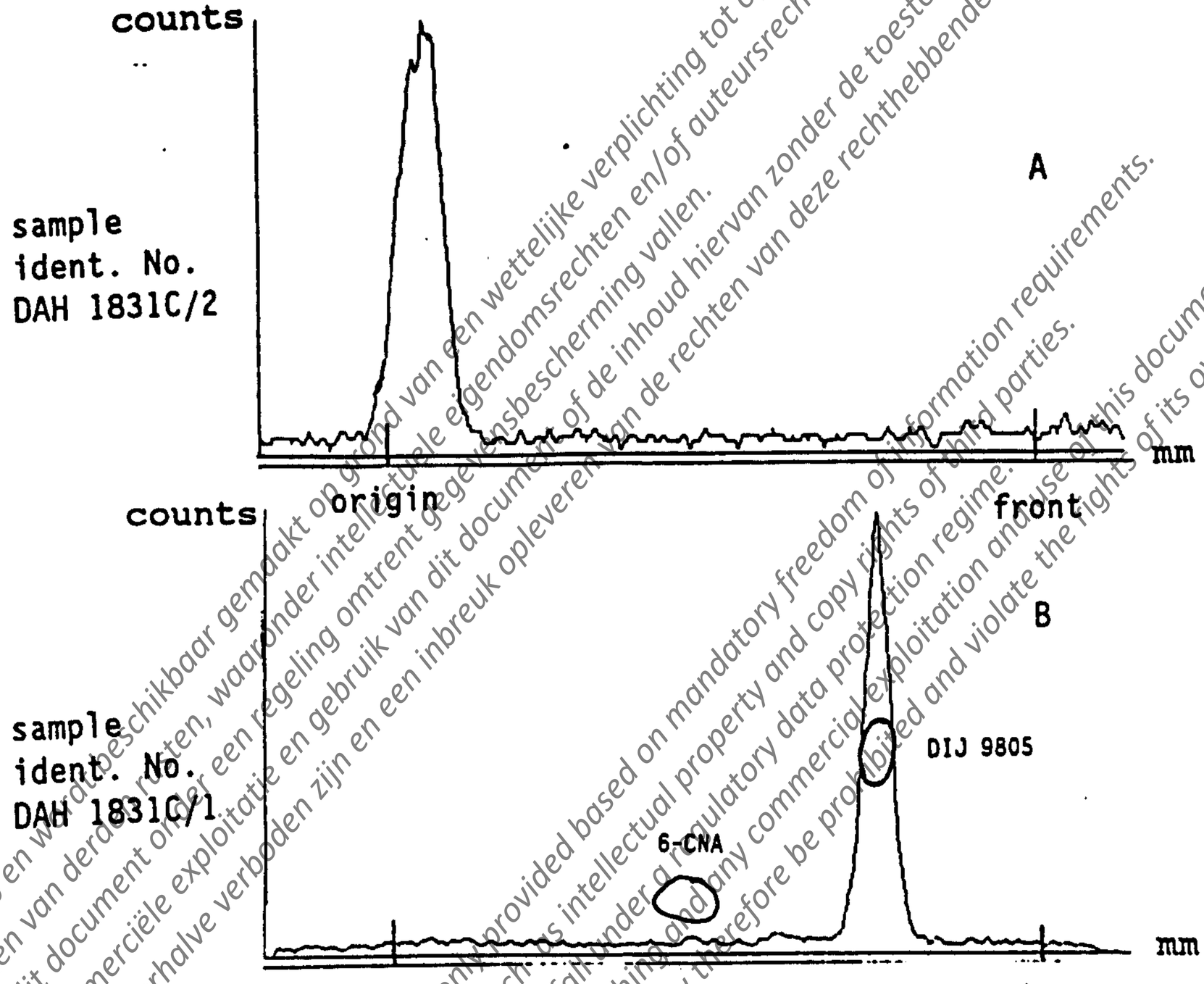


Figure 6:

TLC chromatograms of the isolated metabolite 6 before hydrolysis with cellulase (A) and after hydrolysis (B)

Solvent system II: ethyl acetate/toluene/methanol/acetic acid 80:20:20:1

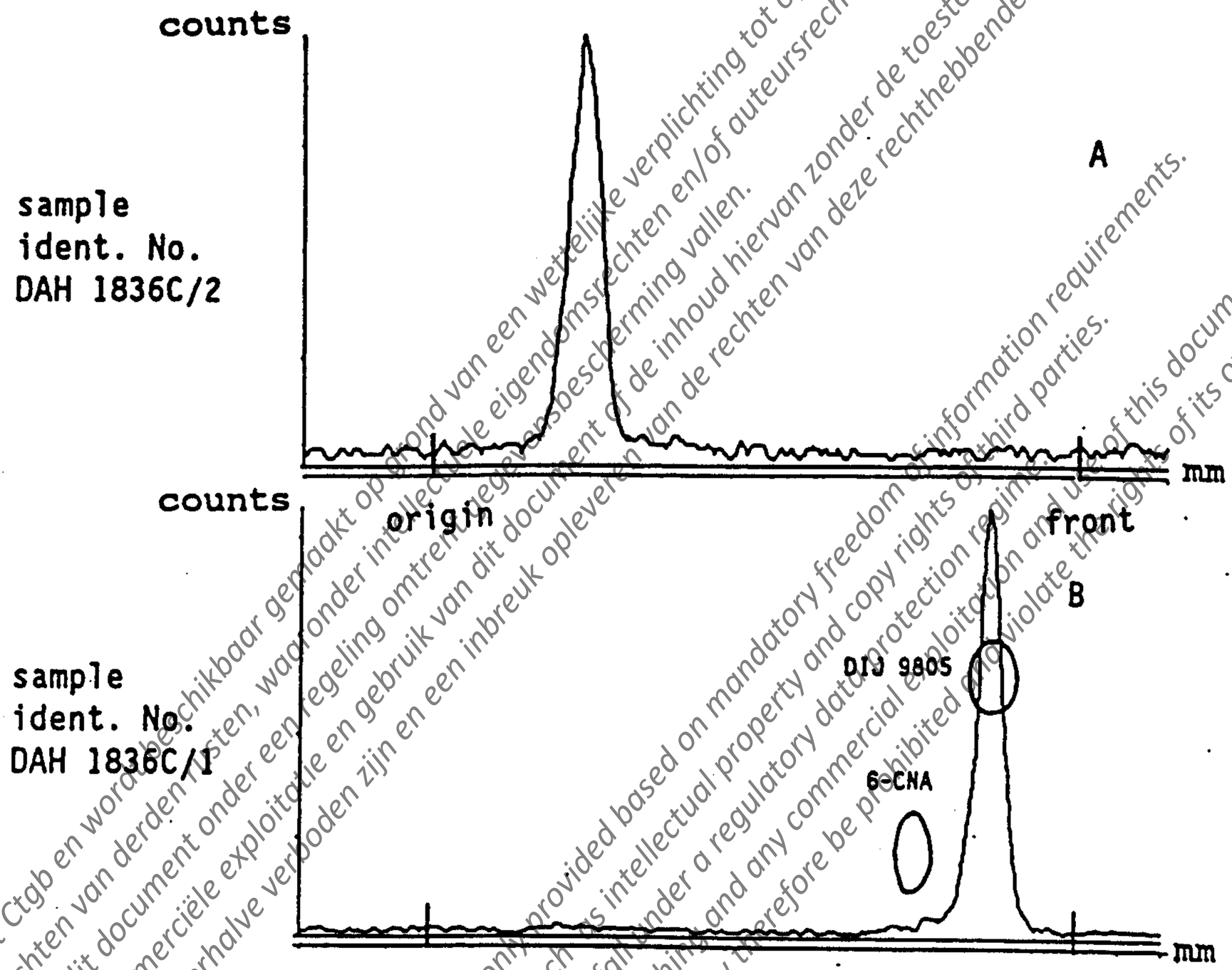
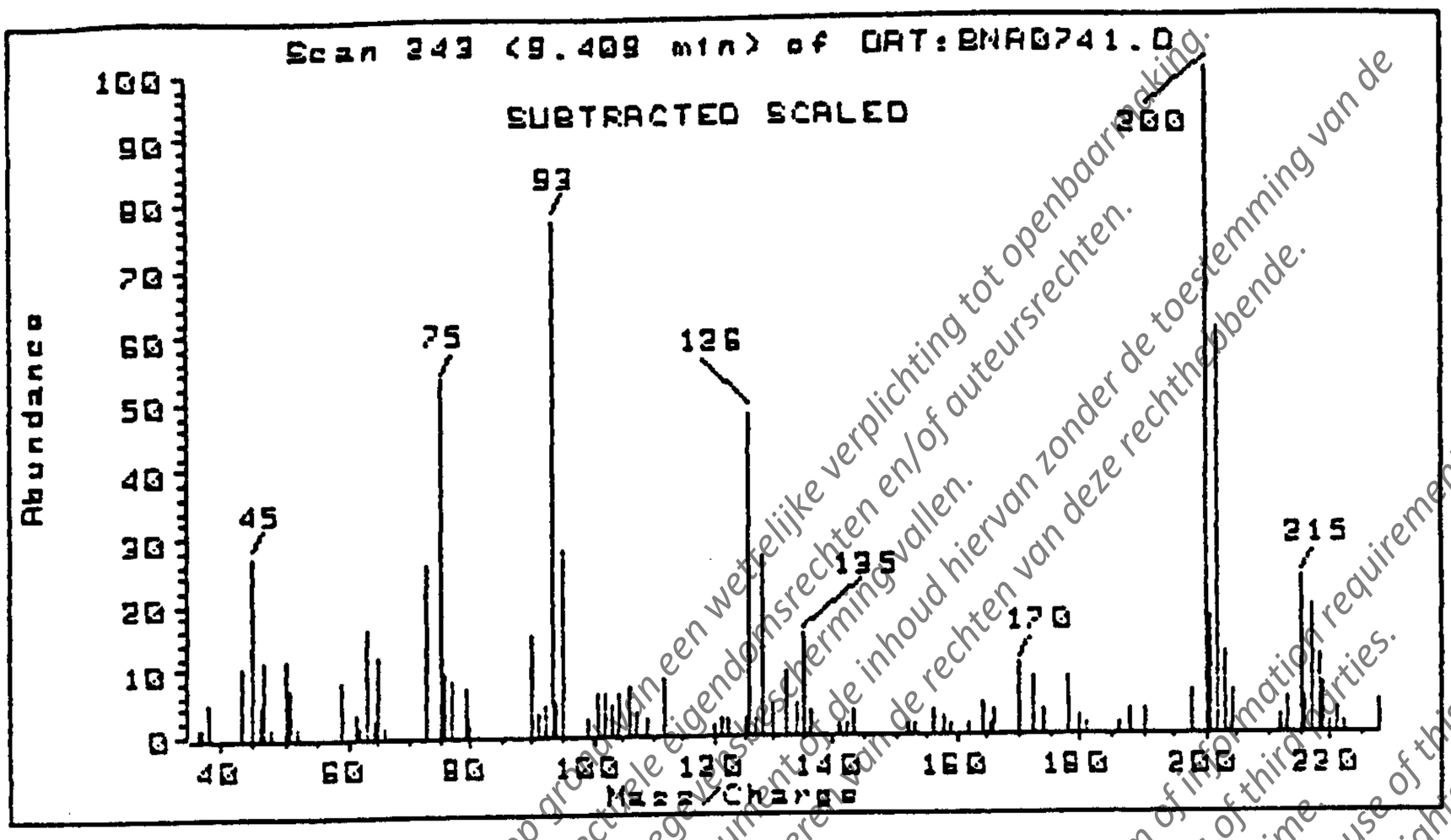


Figure 7:

TLC chromatograms of the isolated metabolite 6 before hydrolysis with cellulase (A) and after hydrolysis (B).

Solvent system IV: chloroform/methanol/acetic acid/water 65:25:3.5:3.5



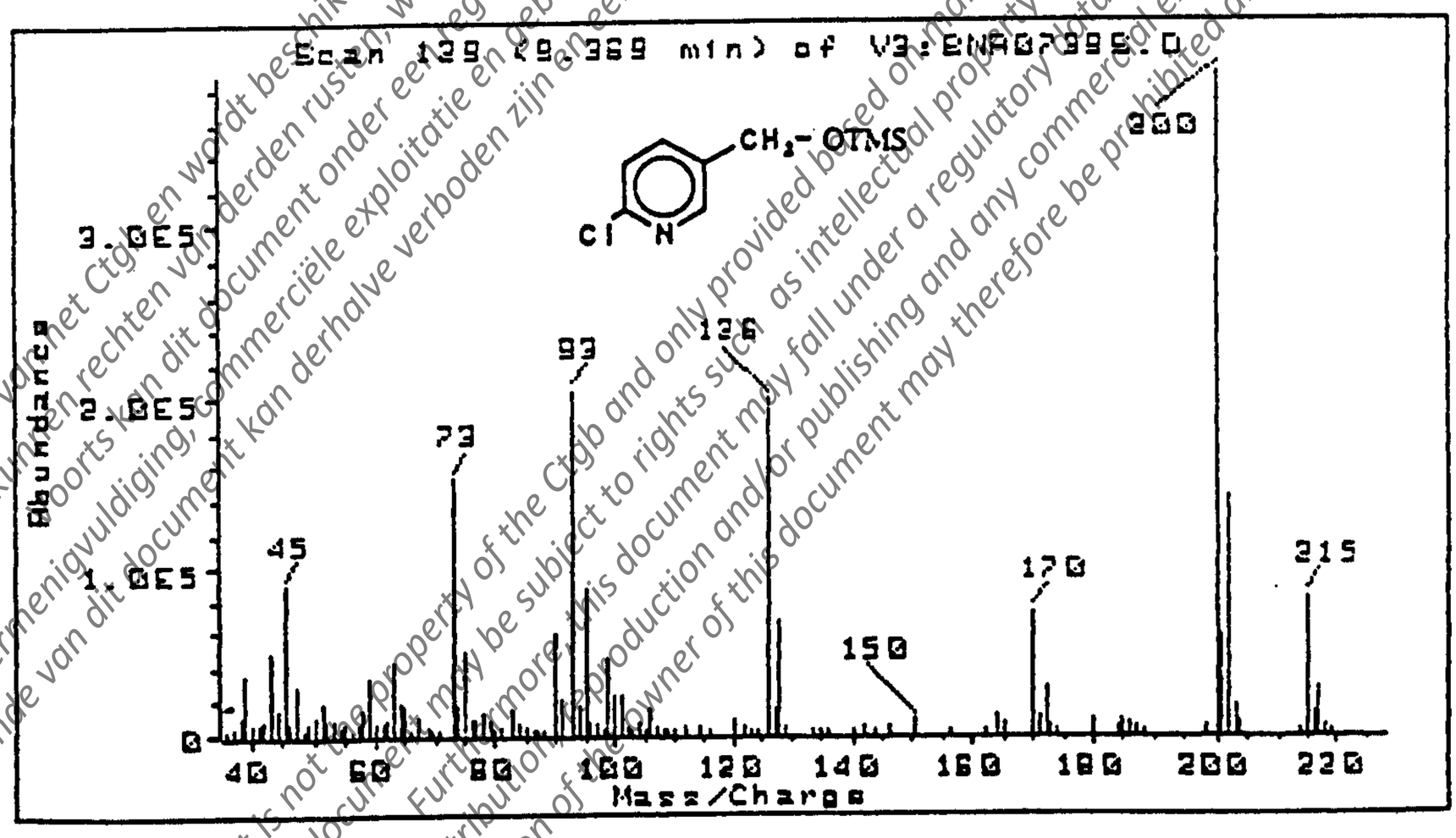


Figure 8:

Mass spectra of metabolite 6 as TMS-derivate (A) and of the reference compound DIJ 9805 as TMS-derivate (B).

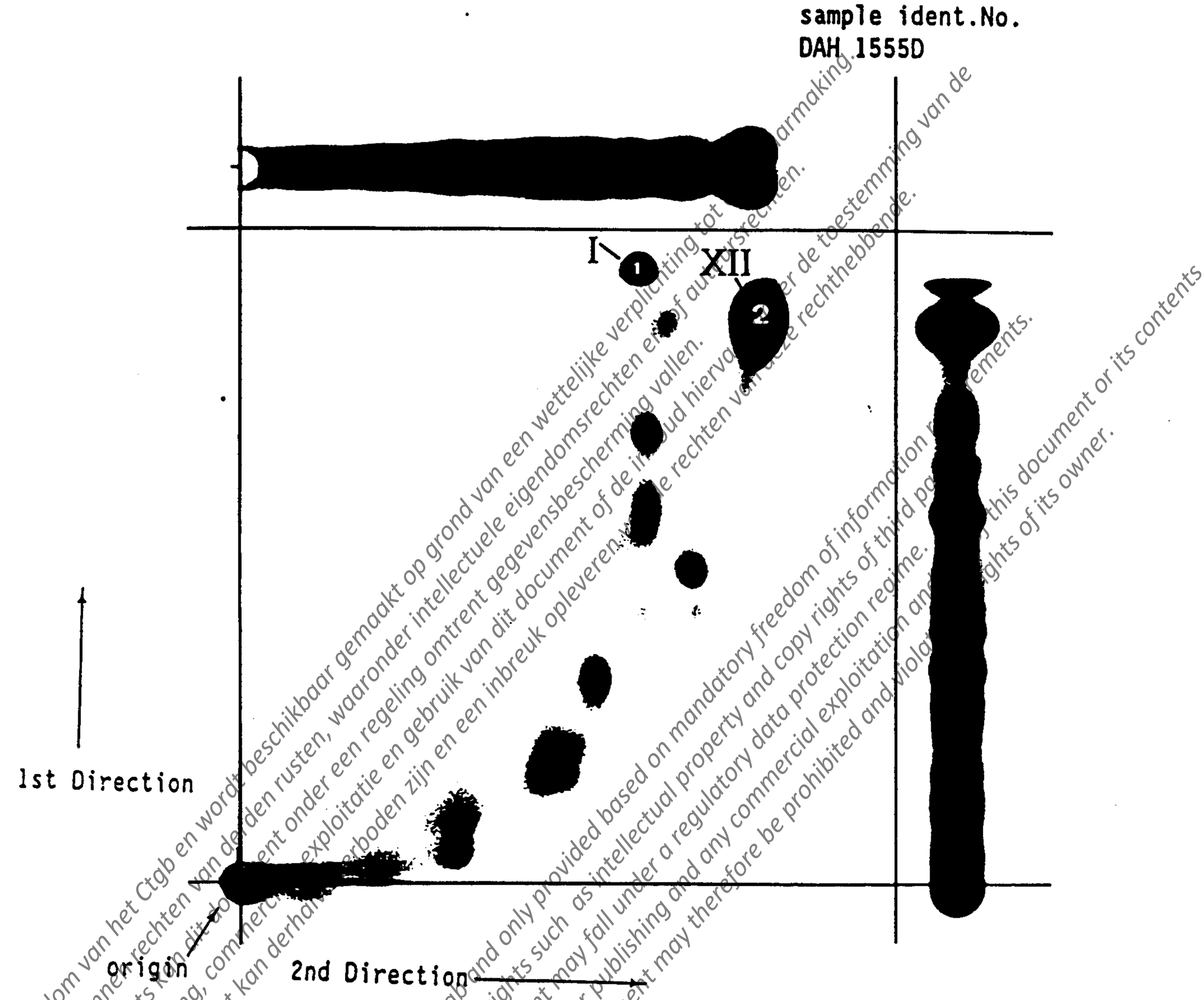


Figure 9:

Autoradiogram of two-dimensional and one-dimensional TLC of the methanol/water phase of seeds, experiment 2

1st Direction: SS IV = chloroform/methanol/acetic acid/water 65:25:3.5:3.5
2nd Direction: SS III = n-butanol/acetic acid/water 80:20:20
The Arabic numbers refer to the metabolites, the Roman numbers refer to reference compounds.

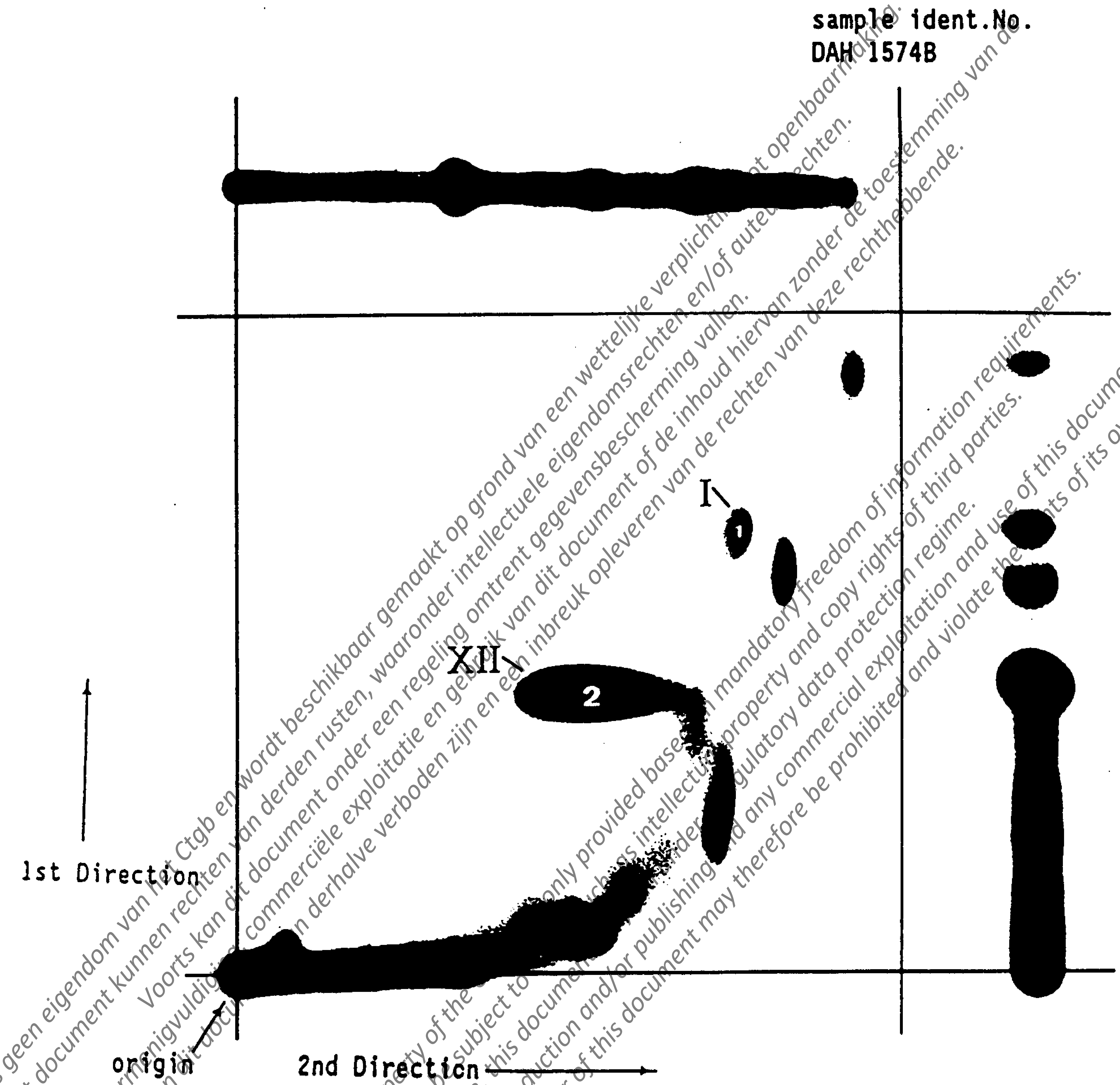


Figure 10:

Autoradiogram of two-dimensional and one-dimensional TLC of the methanol/water phase of seeds, experiment 2

1st Direction: SS II = ethyl acetate/toluene/methanol/acetic acid 80:20:20:1
2nd Direction: SS I = ethyl acetate/i-propanol/water 65:23:12
The Arabic numbers refer to the metabolites, the Roman numbers refer to reference compounds.

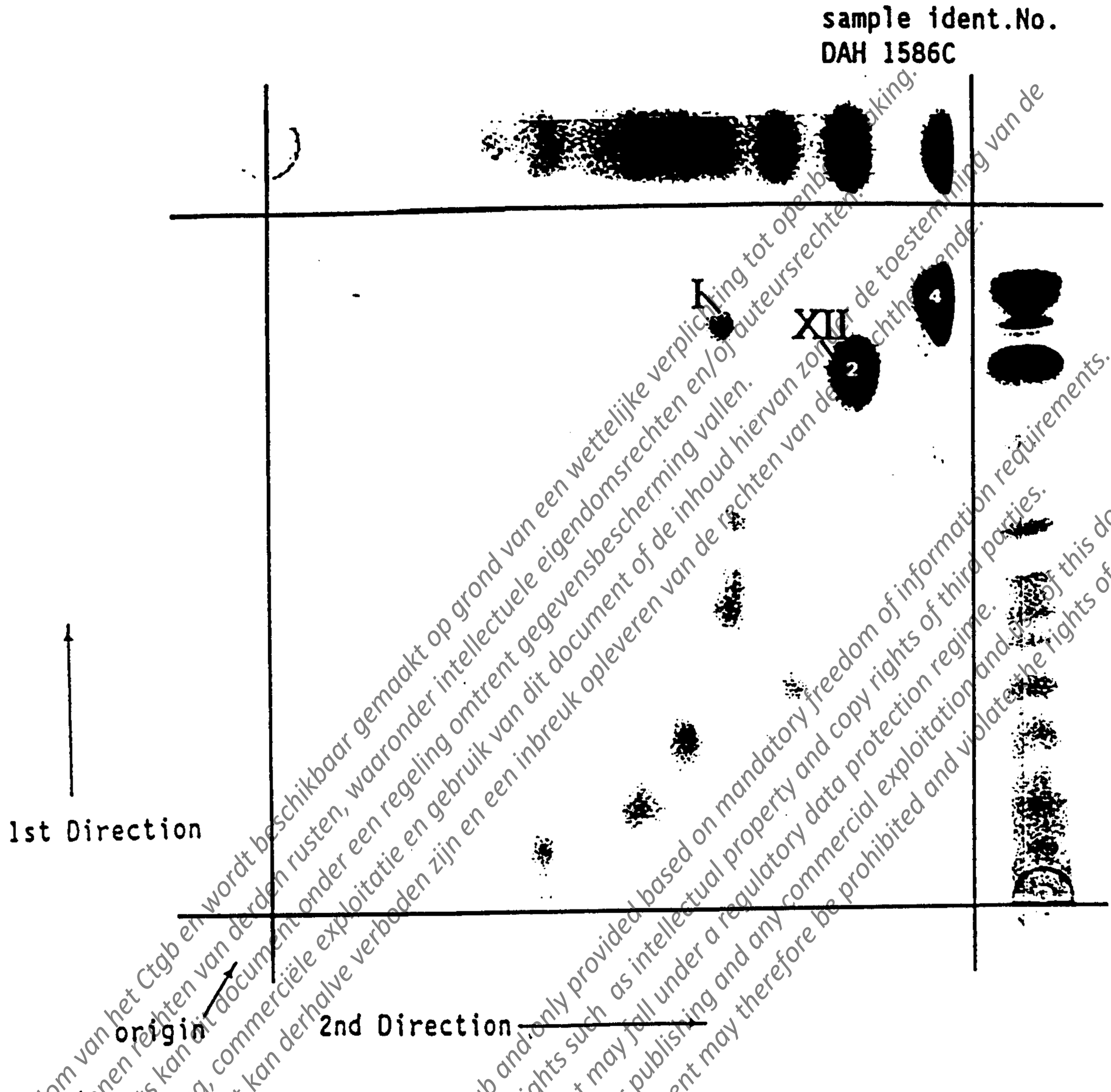


Figure 11:

Autoradiogram of two-dimensional and one-dimensional TLC of the methanol/water phase of seeds for the storage stability study (2. analysis, day 279), experiment 2

1st Direction: SS IV = chloroform/methanol/acetic acid/water 65:25:3.5:3.5
2nd Direction: SS III = n-butanol/acetic acid/water 80:20:20
The Arabic numbers refer to the metabolites, the Roman numbers refer to reference compounds.

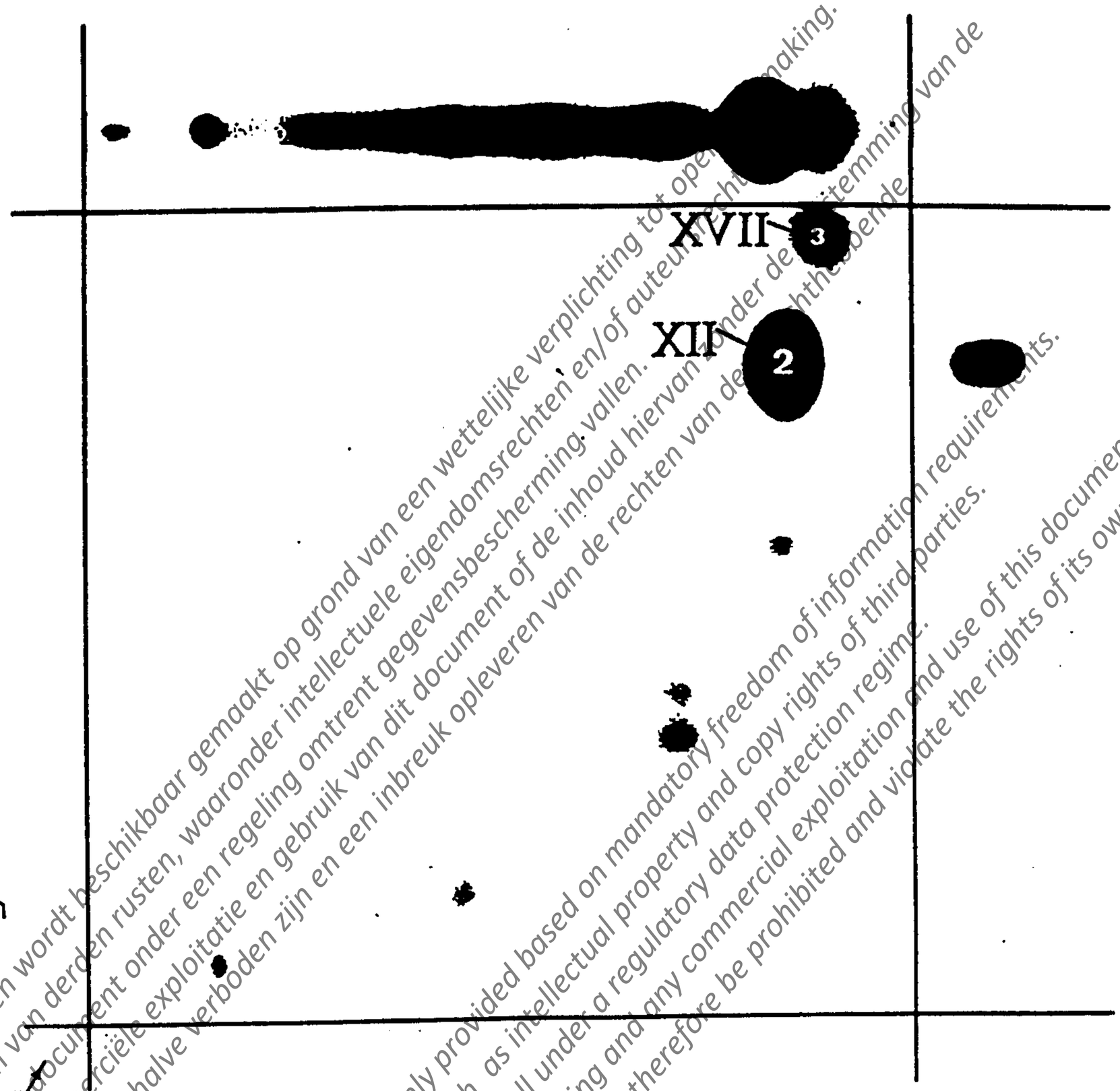


Figure 12:

Autoradiogram of two-dimensional and one-dimensional TLC of the methanol extract of seeds, experiment 2

1st Direction: SS IV = chloroform/methanol/acetic acid/water 65:25:3.5:3.5
2nd Direction: SS III = n-butanol/acetic acid/water 80:20:20
The Arabic numbers refer to the metabolites, the Roman numbers refer to reference compounds.

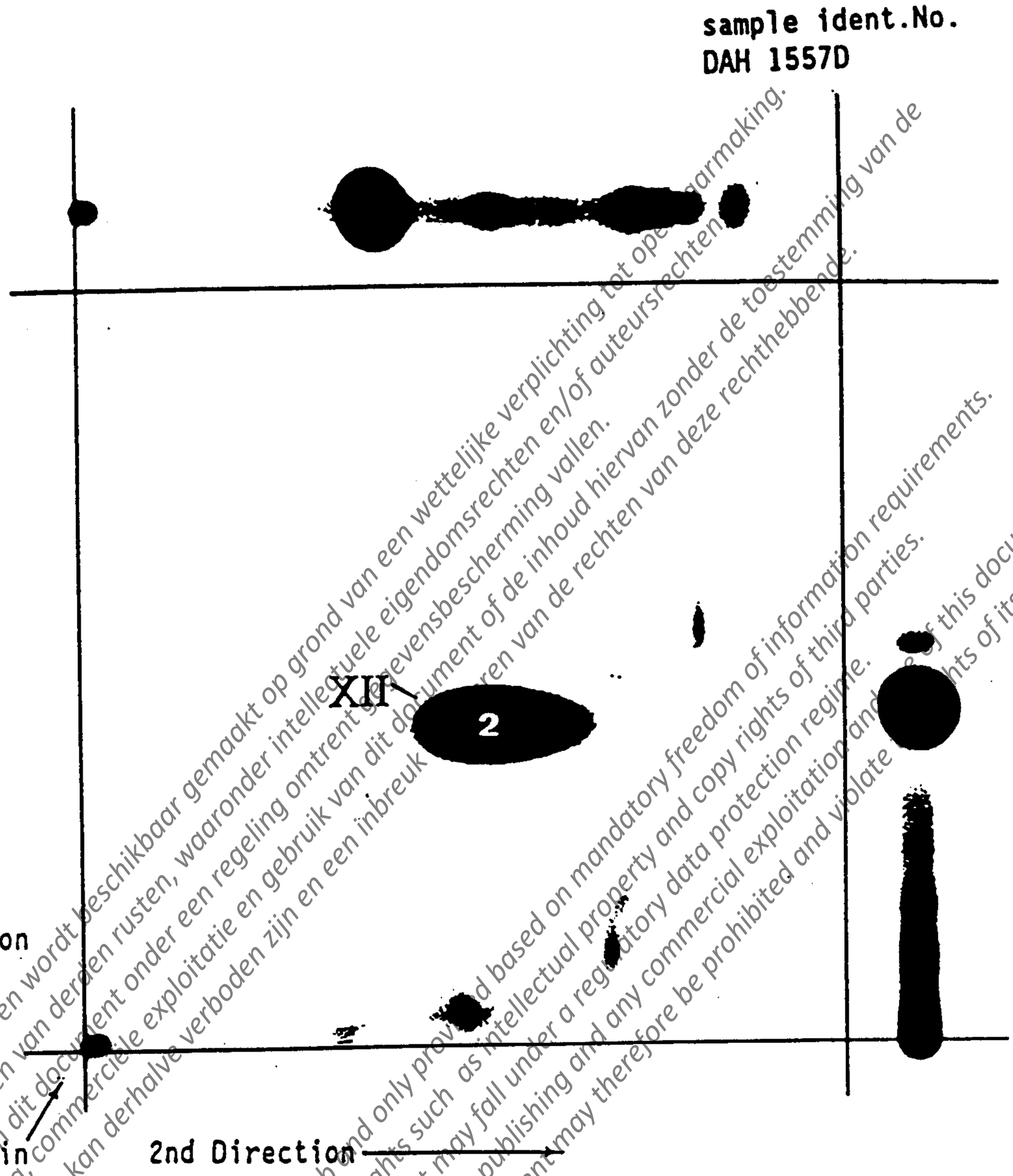


Figure 13:

Autoradiogram of two-dimensional and one-dimensional TLC of the methanol extract of seeds,experiment 2

1st Direction: SS II = ethyl acetate/toluene/methanol/acetic acid 80:20:20:1
2nd Direction: SS I = ethyl acetate/i-propanol/water 65:23:12
The Arabic numbers refer to the metabolites, the Roman numbers refer to reference compounds.

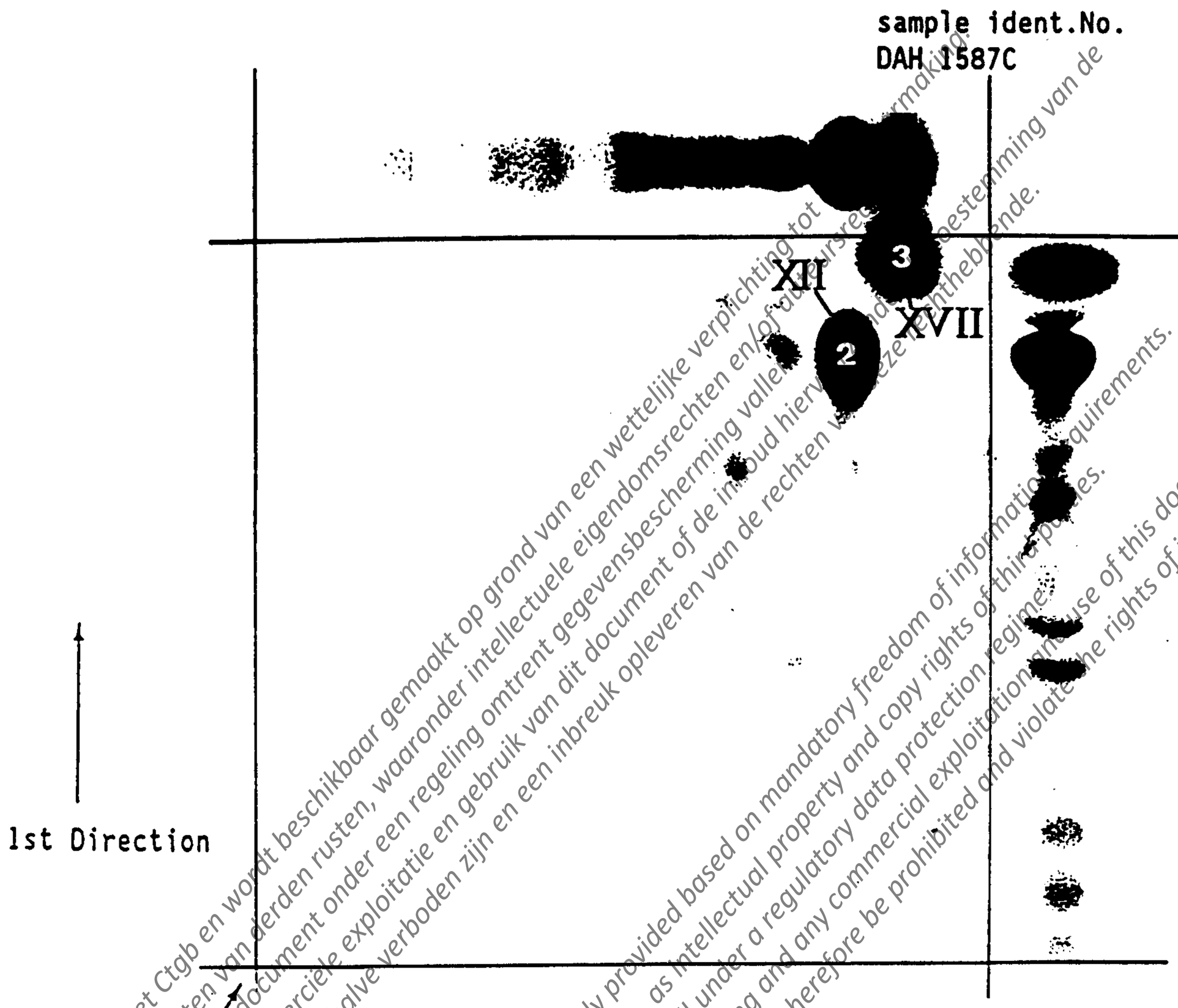


Figure 14:

Autoradiogram of two-dimensional and one-dimensional TLC of the methanol extract of seeds for the storage stability study (2. analysis, day 279), experiment 2

1st Direction: SS IV = chloroform/methanol/acetic acid/water 65:25:3.5:3.5
2nd Direction: SS III = n-butanol/acetic acid/water 80:20:20
The Arabic numbers refer to the metabolites, the Roman numbers refer to reference compounds.

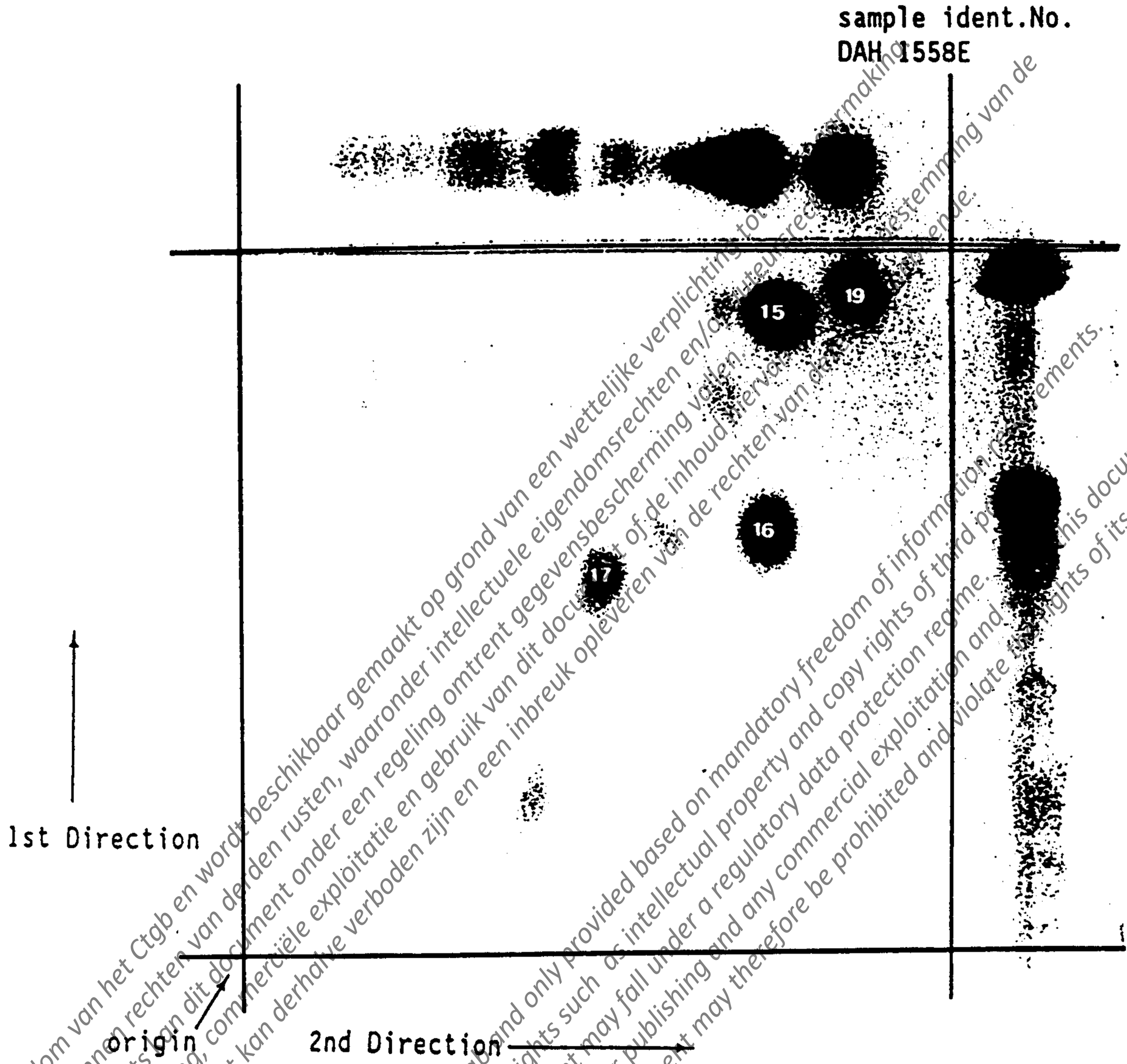


Figure 15:

Autoradiogram of two-dimensional and one-dimensional TLC of the methanol/6N HCl extract of seeds, experiment 2

1st Direction: SS IV = chloroform/methanol/acetic acid/water 65:25:3.5:3.5
2nd Direction: SS III = n-butanol/acetic acid/water 80:20:20
The Arabic numbers refer to the metabolites.

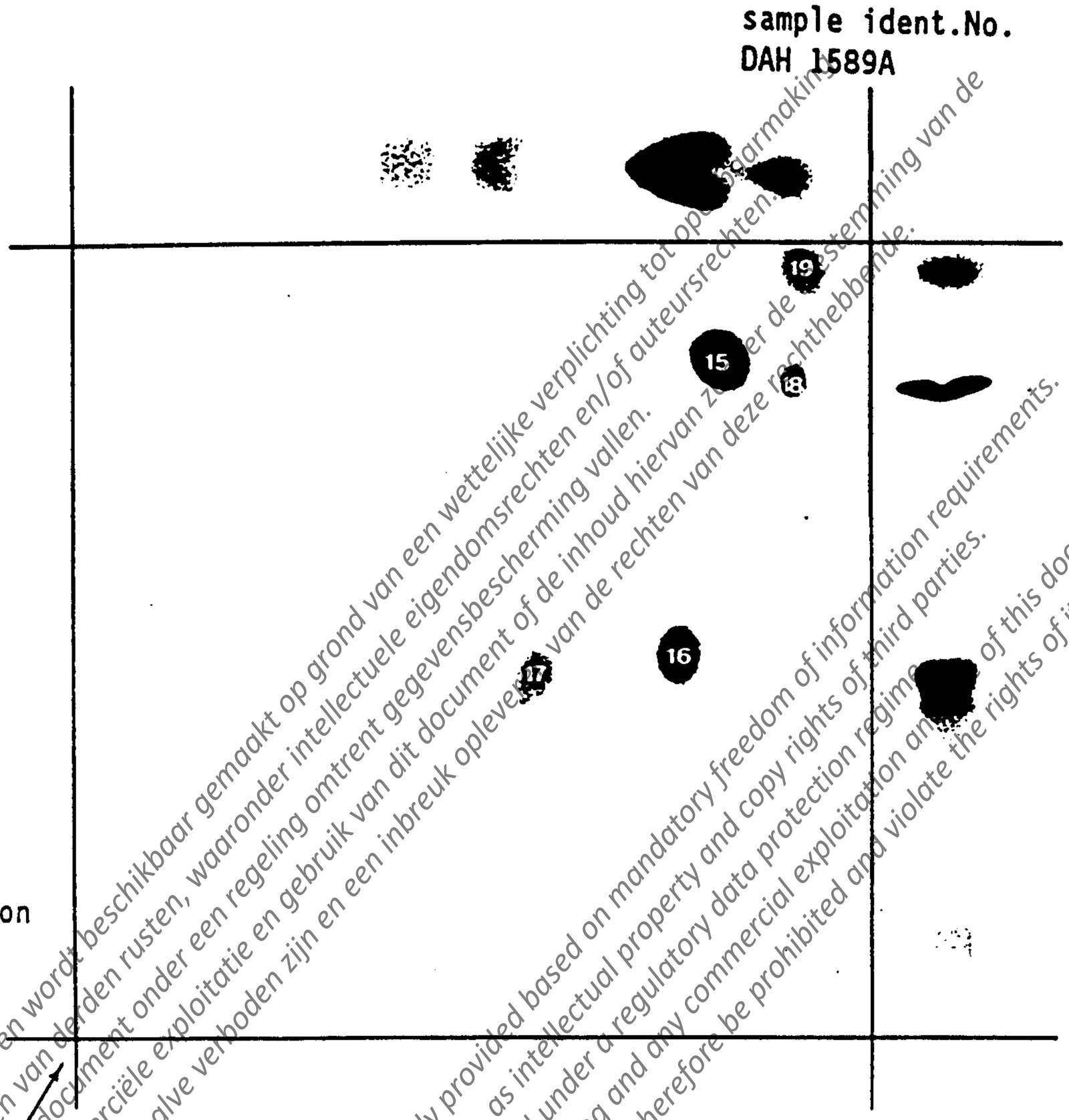


Figure 16:

Autoradiogram of two-dimensional and one-dimensional TLC of the methanol/6N HCl extract of seeds for the storage stability study (2. analysis, day 279), experiment 2

1st Direction: SS IV = chloroform/methanol/acetic acid/water 65:25:3.5:3.5
2nd Direction: SS III = n-butanol/acetic acid/water 80:20:20
The Arabic numbers refer to the metabolites.

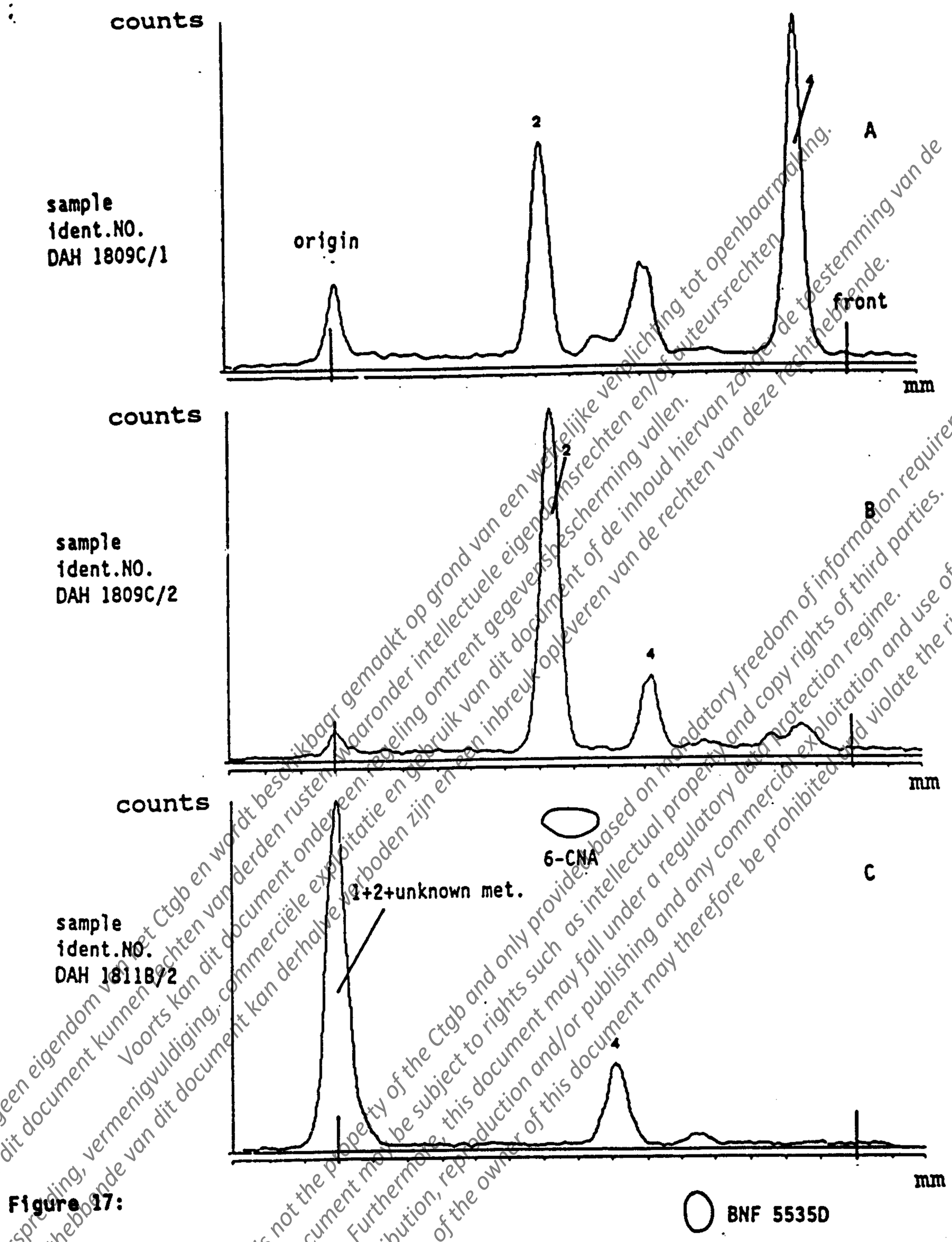


Figure 17:

Radio-TLC of the methanol/water phase of seeds for the storage stability study (2. analysis, day 279), experiment 2

A: before hydrolysis with 2N NaOH, SS II
B: after hydrolysis with 2N NaOH, SS II
C: before hydrolysis with 2N NaOH, SS V

The Arabic numbers refer to the metabolites.

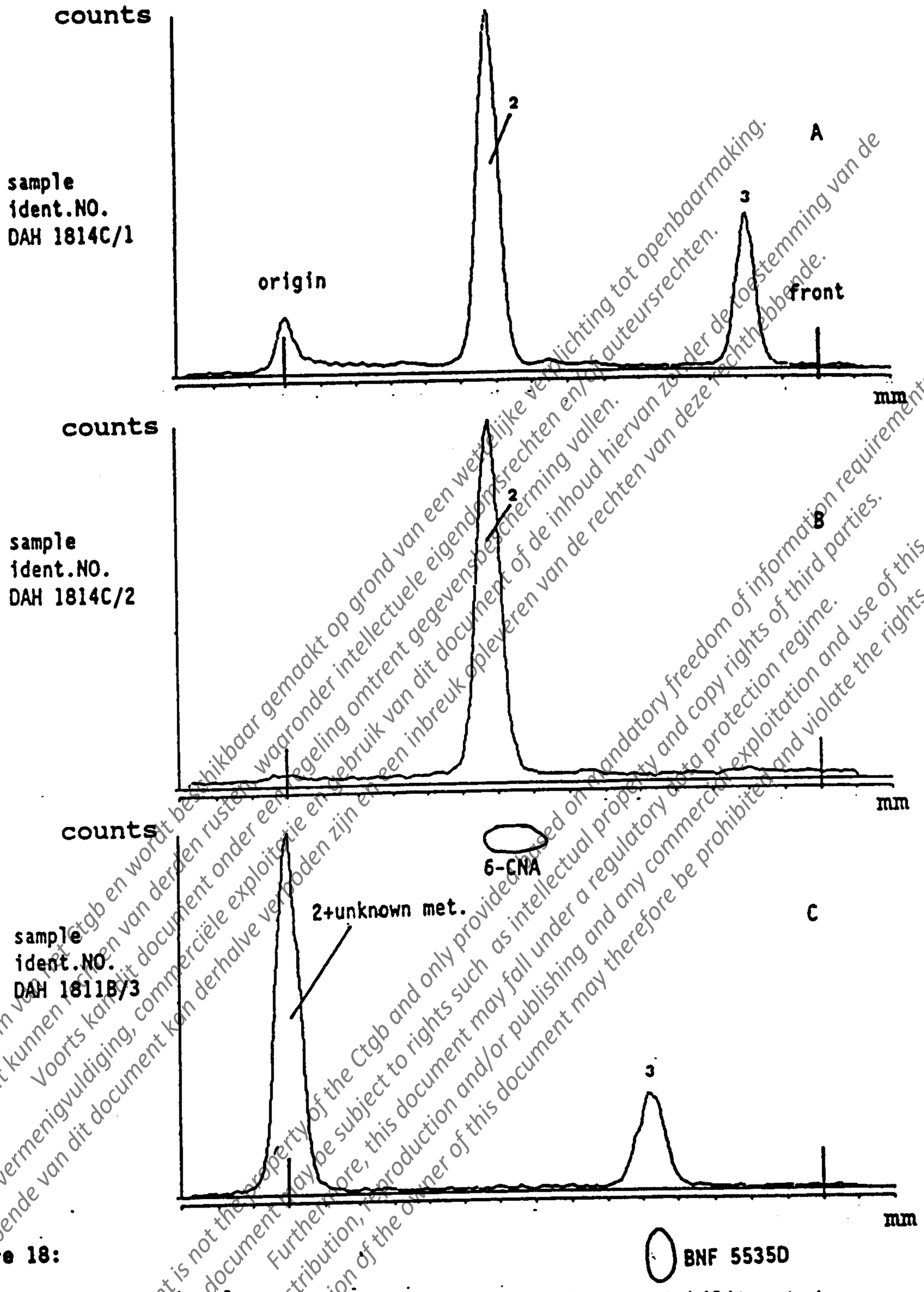


Figure 18:

Radio-TLC of the methanol extract of seeds for the storage stability study (2. analysis, day 279), experiment 2

A: before hydrolysis with 2N NaOH, SS II
B: after hydrolysis with 2N NaOH, SS II
C: before hydrolysis with 2N NaOH, SS V

The Arabic numbers refer to the metabolites.

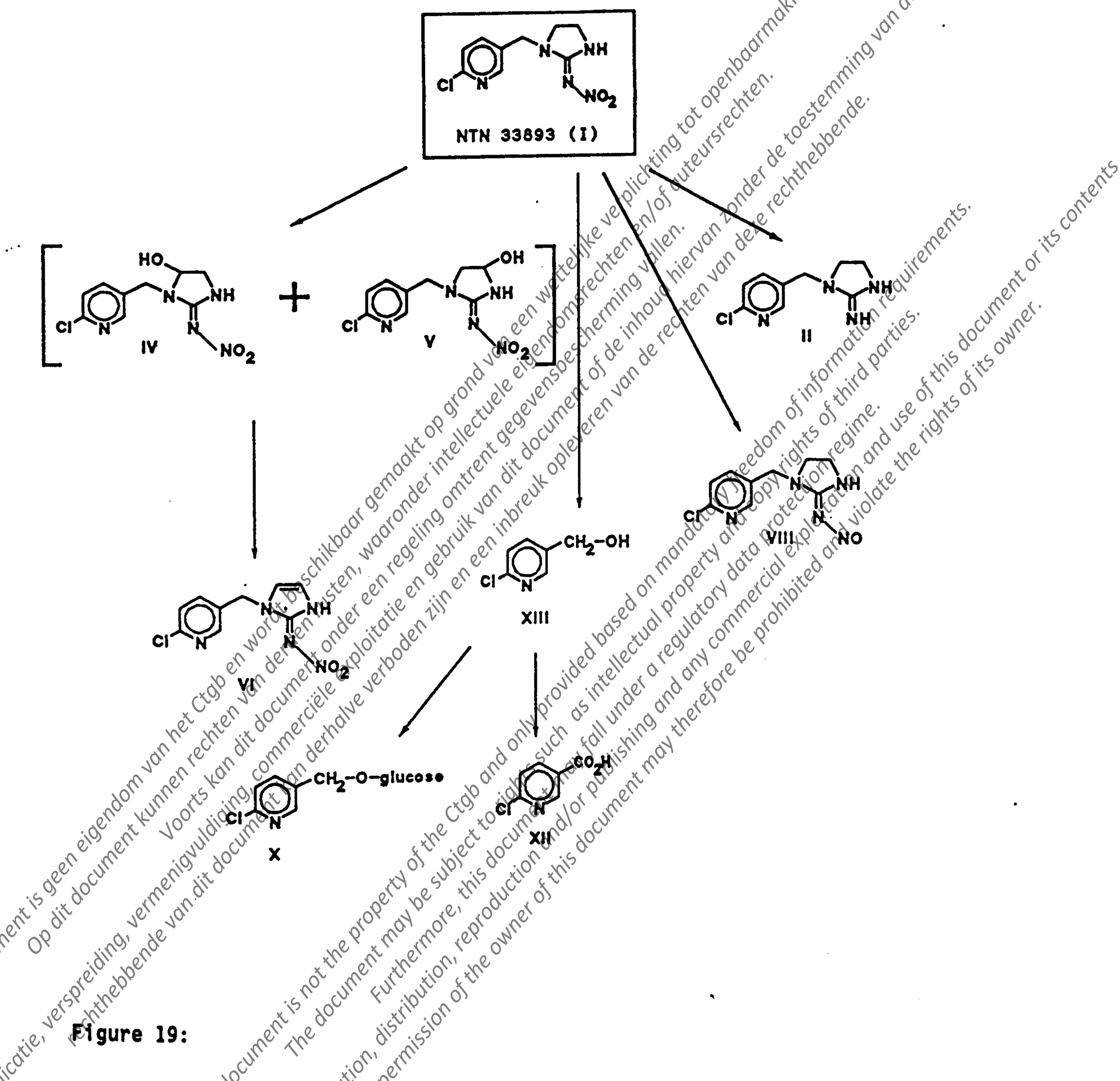


Figure 19:

Proposed metabolic pathway of NTN 33893 in cotton plants following seed dressing [formulae in brackets = not detected]

XII. APPENDICES

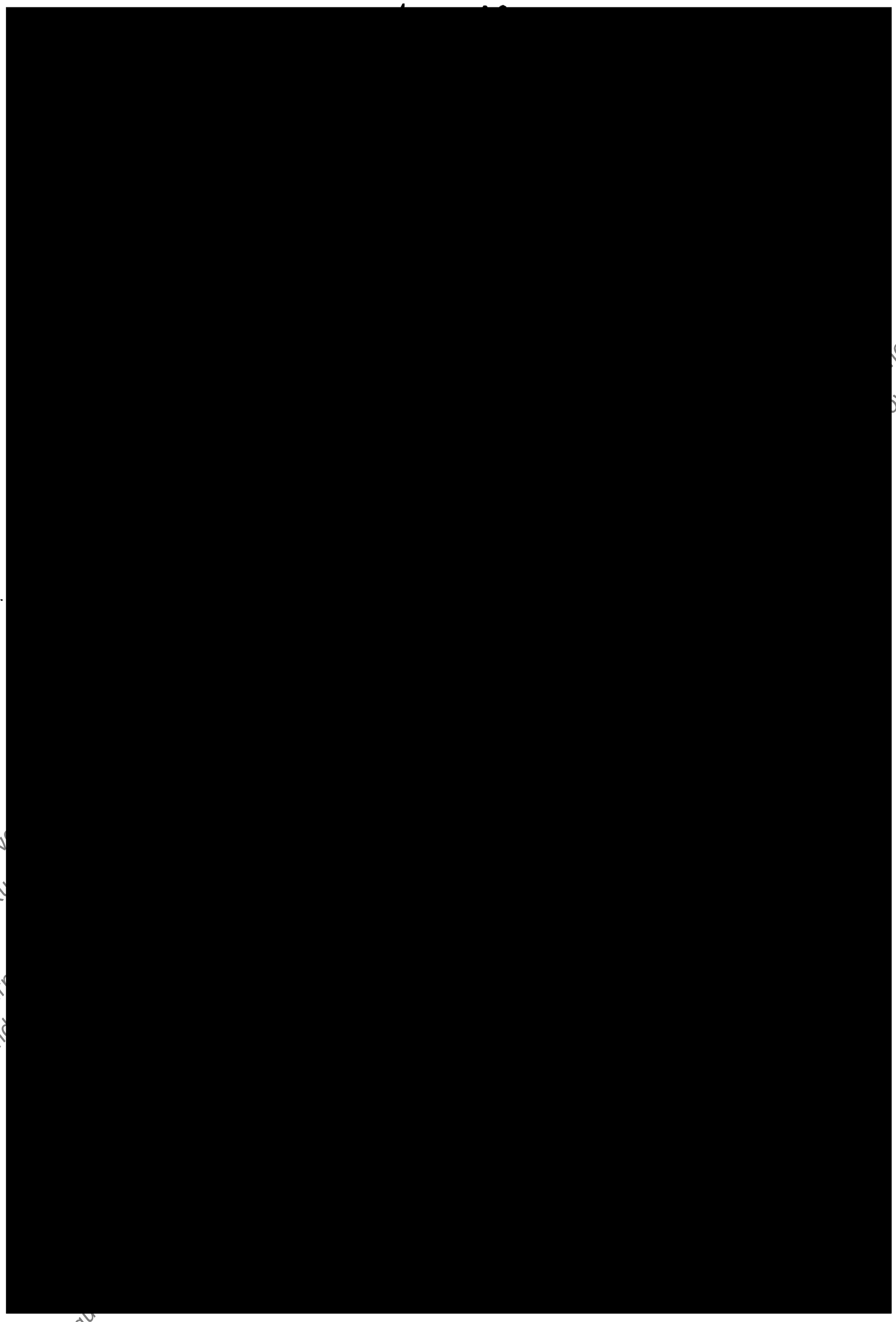
SAMPLE CERTIFICATION

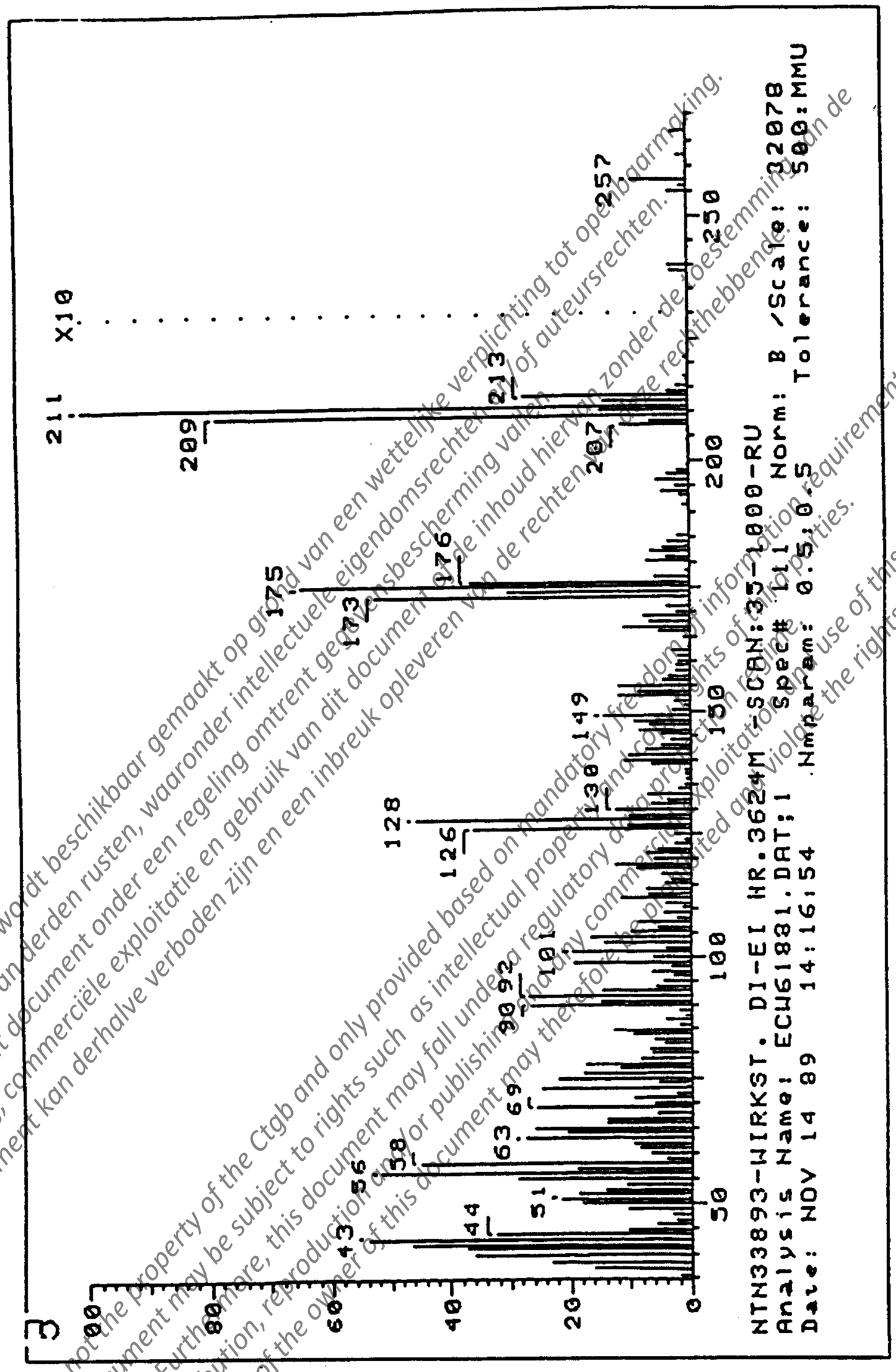
[redacted]

SAMPLE CERTIFICATION

[redacted]

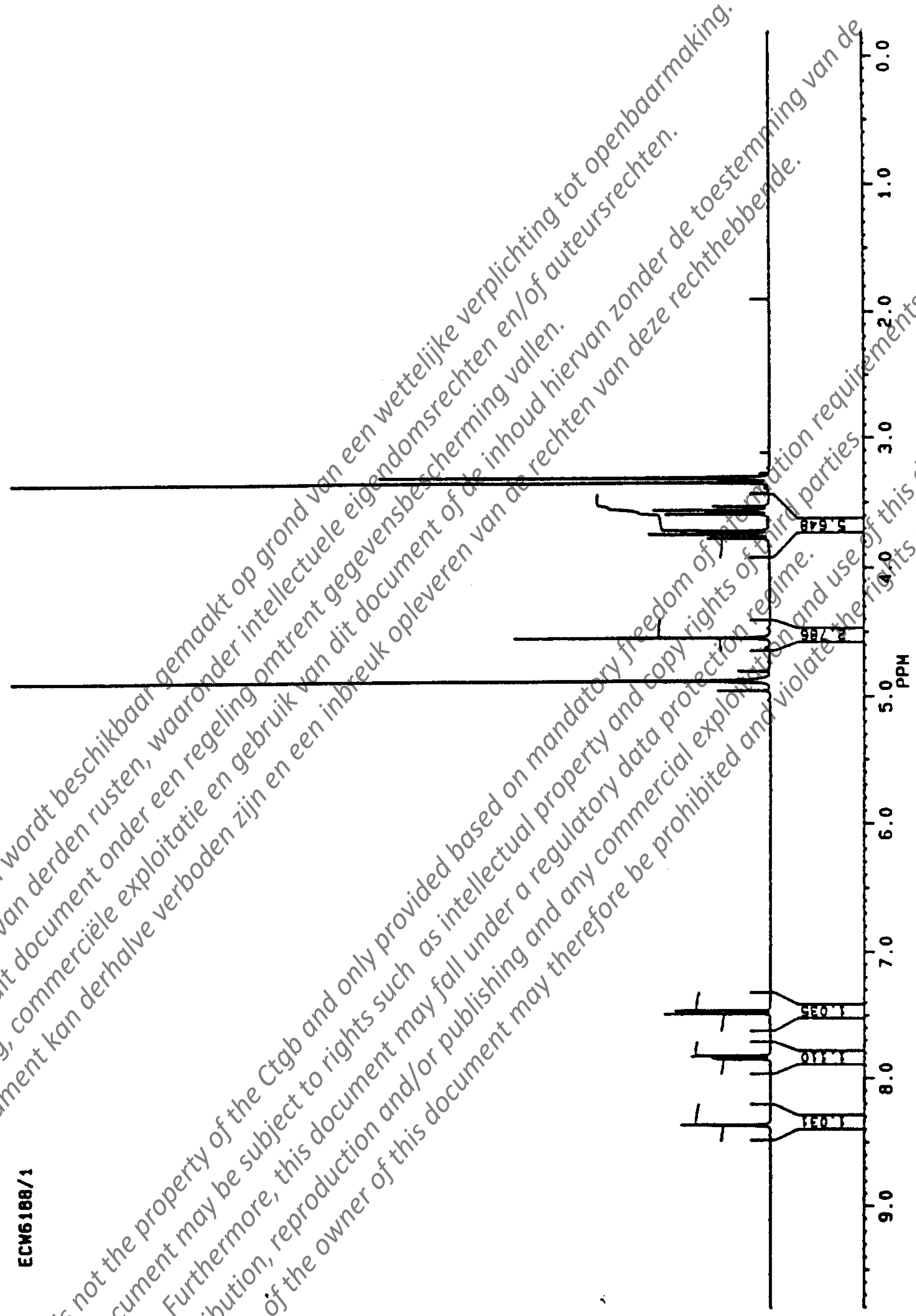
SAMPLE CERTIFICATION





Appendix IV:

EI-MS-spectrum of the test substance



Appendix V:

^{1}H-NMR-spectrum of the test substance

Organic carbon%	1.14
Microbial carbon/DS soil [mg/kg]	497
pH (KCl)	6.63
Cation exchange capacity [meq/100g]	15
Particle density [g/ml]	2.35

Texture analysis according to DIN 19682 diagram;

Sand	%	17.1
Silt	%	71.8 = loamy silt
Clay	%	11.8

Texture analysis according to USDA soil diagram;

Sand	%	17.1
Silt	%	71.8 = silty loam
Clay	%	11.1

Appendix VI:

Soil analysis (Frimmersdorf)

Conducted by Dr. [redacted] Institute for Environmental Biology

(Report No. 12485, November 21, 1985) and LUFA Speyer (July 1989)

Crop	: Cotton	
Variety	: Coker 310	
Soil type	: Frimmersdorf (see appendix VI)	
Filling date	: 25/8/1989	
Container size	: Experiment 1, 35 l with ca. 36 kg soil	
	Experiment 2, 5 l with ca. 4.5 kg soil	
Sowing date	: Experiment 1: 30/10/1989	
	Experiment 2: 26/10/1989	
Treatment dates (experiment 2)	: 6/11/89, 12/12/89, 16/1/90, 16/2/90, 20/3/90	
Fertilisation measures	: 29/8/89 400 kg/ha Nitrophoska special	
	From 13/11/1989 for all containers one application a week alternating between Wuchsal and Fertisal both at 2.5 g/l, ca. 500 ml	

Plant protection measures (only experiment 1)

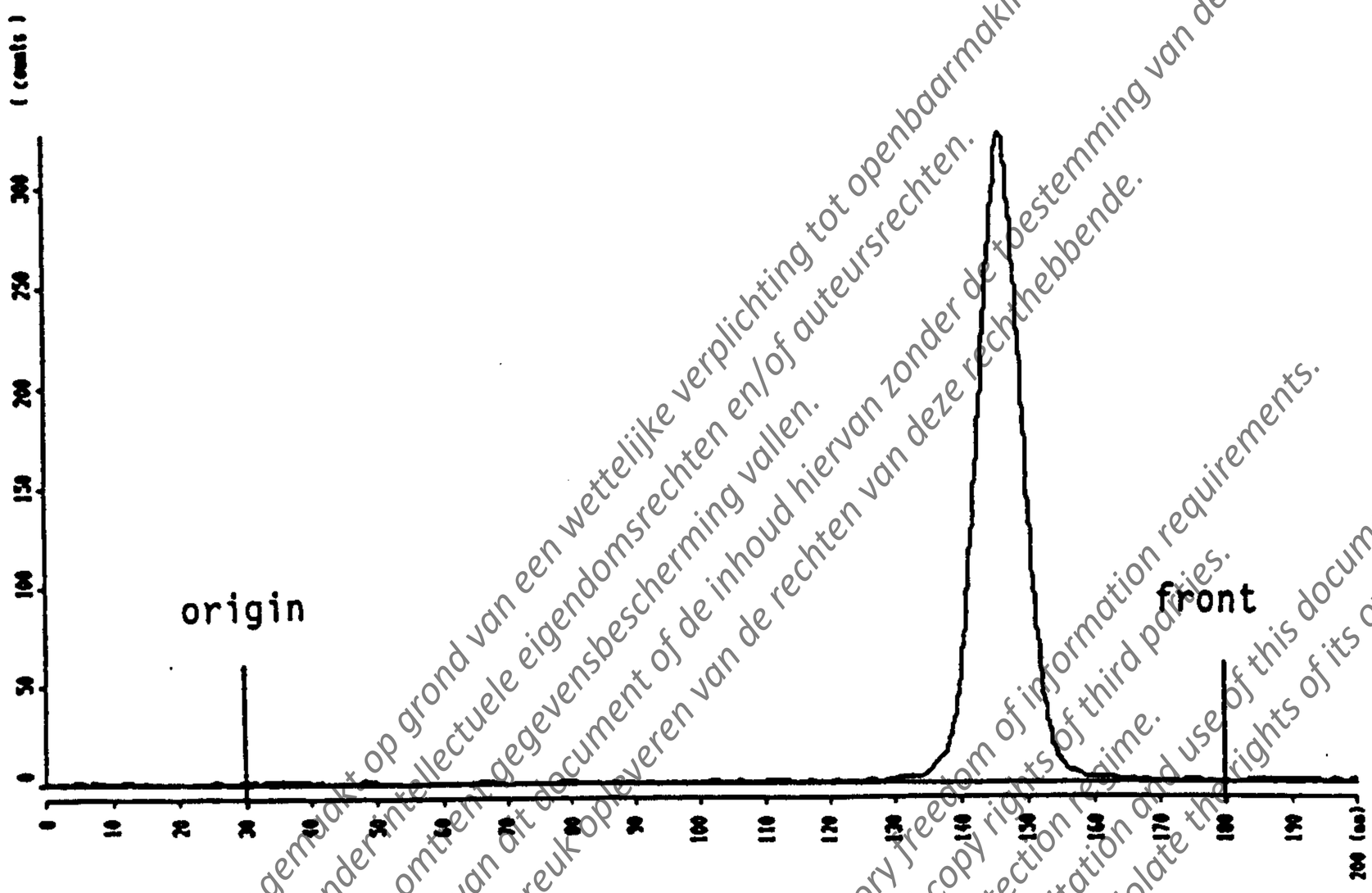
Date	Chemical	% a.i.
24/11/89	Mesurol	4 g/m^2
10/1/90	Torque	0.05 %
26/1/90	Pentac	0.1 %
7/2/90	E 605 forte	0.05 %
4/3/90	Pentac	0.1%

Temperature	: Day 22 °C
	Night (from 20.00 until 07.00) 18°C
Humidity	: 60 %
Light	: 35 kLx (from 08.00 until 20.00)

Appendix VII:

Growth and environmental conditions for cotton after seed dressing (experiment 1) and drench treatment (experiment 2) with [pyridinyl-^{14}C-methyl]NTN 33893

sample
ident.NO.
DAH 1505C



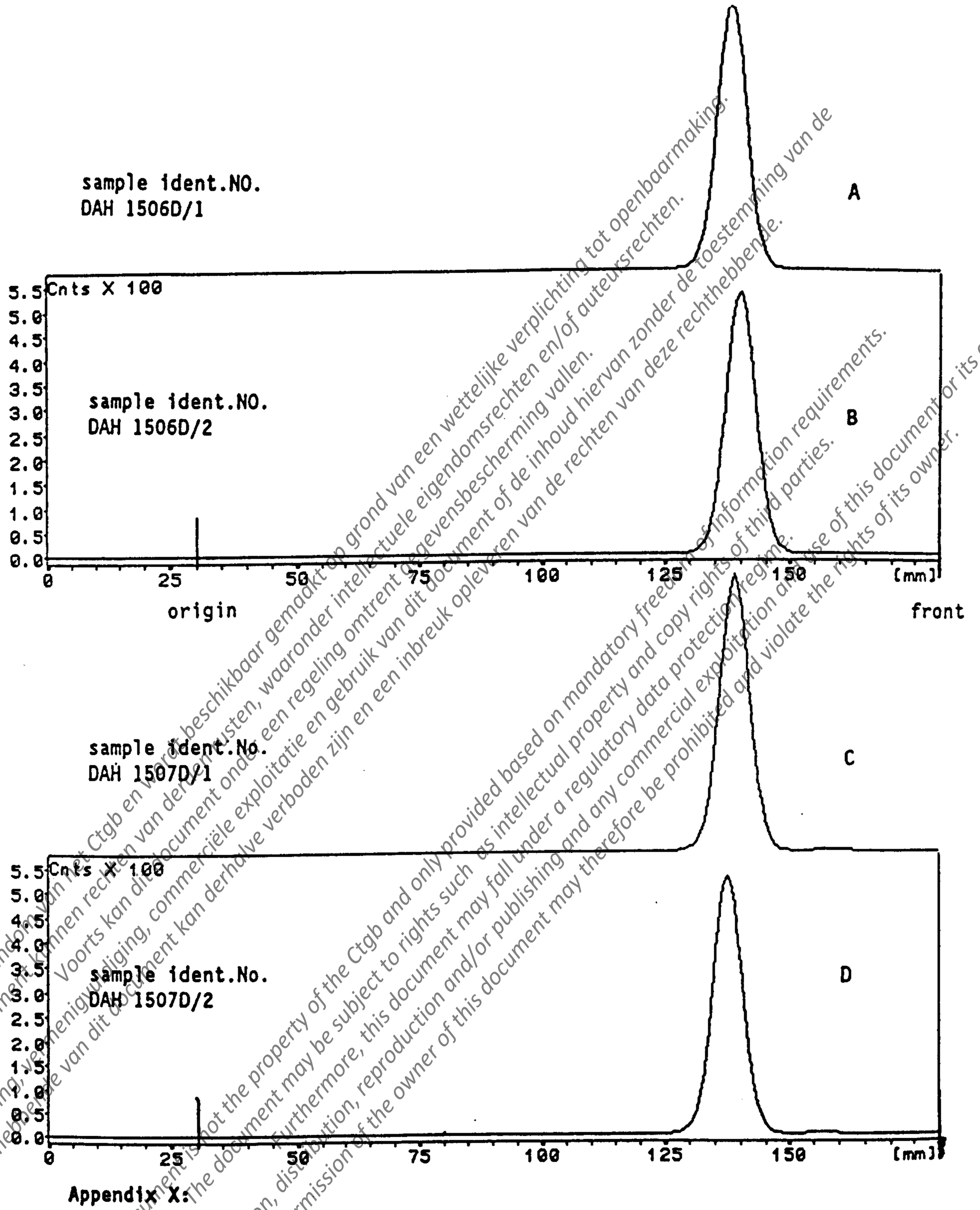
Appendix VIII:

Radio-TLC stability check of the radioactive ingredient in the 70 WS formulation
Solvent system I: ethyl acetate /i-propanol/water 65:23:12

Experiment	Application days after planting	Applied amount formulation mg	a.i. mg	MBq	Amount of water ml
2.1	11	2.7	0.52	2.87	50
	47	9.1	1.75	9.68	150
	82	21.4	4.11	22.73	400
	113	41.6	8.00	44.22	800
	145	92.7	17.82	98.53	1500
	total	167.5	32.20	178.03	
2.2	11	3.4	0.57	3.17	50
	47	11.8	1.97	10.95	150
	82	26.9	4.49	24.96	400
	113	49.8	8.32	46.26	800
	145	86.3	14.41	80.12	1500
	total	178.2	29.76	165.46	

Appendix IX:

Amount of [^{14}C]NTN 33893 applied to each cotton plant by drench treatment (experiments 2.1 and 2.2).
For formulation details see Appendices II and III



Appendix X:

Radio-TLC stability check of the radioactive ingredient in the 200 SL-formulation, experiment 2, 1st application
A: experiment 2.1, before pouring
B: experiment 2.1, after pouring
C: experiment 2.2, before pouring
D: experiment 2.2, after pouring
Solvent system I: ethyl acetate/i-propanol/water 65:23:12

Plant part	Weight at harvest	Weight of subsample used for extraction	Volume of solvents					
			n-hexane	methanol/ water 1:1	methanol	methanol reflux	methanol/ 6N HCl reflux	methanol/ 2N NaOH reflux
	g	g	ml	ml	ml	ml	ml	ml
experiment 1								
seed	569.6	100	600	300	2x150	200	200	200
gin trash	568.6	100	*	800	2x400	750	800	*
lint	431.4	25	*	*	*	1500	1500	*
leaves	1718.2	100	*	200	2x200	*	*	*
experiment 2								
seed	33.5	6	150	200	2x200	100	200	100

* extraction was not performed

Appendix XI:

Weights of cotton samples and subsamples and volumes of solvent used for extraction

Extract	Analysis sample	Total sample	% Recovered
n-hexane phase	0	0	0
methanol/water phase	0.64 Bq/500µl	0.76 kBq/595 ml	28.4
methanol extract, reflux	8.89 Bq/100µl	0.53 kBq/ 6 ml	19.8
methanol/6N HCl, reflux	0.85 Bq/100µl	0.66 kBq/ 78 ml	24.6
methanol/2N NaOH, reflux	0	0	0
non extractable residue	10.72 Bq/g	0.33 kBq/30.85 g	12.3
total		2.28 kBq	85.1
seeds, combustion value	26.76 Bq/g	2.68 kBq/100 g	100

Appendix XII:

Raw data from the analysis of radioactivity in the seeds and material balance following extraction, experiment 1

Extract	Analysis sample	Total sample	% Recovered
methanol/water phase	1.33 Bq/ml	1.94 kBq/1455 ml	70.8
methanol extract, reflux	0	0	0
methanol/6N HCl, reflux	0	0	0
non extractable residue	7.08 Bq/g	0.60 kBq/84.99 g	21.9
total		2.54 kBq	92.7
gin trash, combustion value	27.36 Bq/g	2.74 kBq/100 g	100

Appendix XIII:

Raw data from the analysis of radioactivity in gin trash and material balance following extraction, experiment I

Extract	Analysis sample	Total sample	% Recovered
methanol extract,reflux	0	0	0
methanol/6N HCl, reflux	0	0	0
non-extractable residue	10.91 Bq/g	0.27 kBq/25 g	103.9
total		0.27 kBq	103.9
lint, combustion value	10.46 Bq/g	0.26 kBq/25 g	100

Appendix XIV:

Raw data from the analysis of radioactivity in lint and material balance following extraction, experiment 1

Extract	Analysis sample	Total sample	% Recovered
methanol water extract	40.95 Bq/500 µl	49.14 kBq/600 ml	78.5
non-extractable residue	625.05 Bq/g	17.94 kBq/28.7 g	28.7
total		67.08 kBq	107.2
leaves, combustion value	626 Bq/g	62.6 kBq/100 g	100

Appendix XV:

Raw data from the analysis of radioactivity in leaves and material balance following extraction, experiment 1

Extract	Analysis sample	Total sample	% Recovered
n-hexane phase	22.88 Bq/100 µl	1.72 kBq/7.5 ml	0.5
methanol/water phase	79.01 Bq/ml	53.73 kBq/680 ml	17.3
methanol extract, reflux	826.8 Bq/50 µl	120.61 kBq/7.2 ml*	38.8
methanol/6N HCl, reflux	166.12 Bq/500 µl	88 kBq/265 ml	28.3
methanol/2N NaOH, reflux	17.18 Bq/500 µl	5.60 kBq/163 ml	1.8
non-extractable residue	1.48 kBq/g	1.18 kBq/0.79 g	0.4
total		270.84 kBq	87.1
seeds, combustion value	51.81 kBq/g	310.86 kBq/6 g	100

* determined by summation of the calculated value (119.06 kBq/7.2 ml) and values obtained from samples previously taken to follow the progress of the extraction (1.55 kBq)

Appendix XVI:

Raw data from the analysis of radioactivity in the seeds and material balance following extraction, experiment 2

Extraction date 4/7/90 (day 21 for storage stability investigation)

Extract	Analysis sample	Total sample	% Recovered
n-hexane phase	23.31 Bq/100 µl	1.72 kBq/7.4 ml	0.6
methanol/water phase	81.44 Bq/ml	47.23 kBq/580 ml	15.8
methanol extract, reflux	1180.74 Bq/100 µl	99.18 kBq/8.4 ml	33.1
methanol/6N HCl, reflux	244.6 Bq/500 µl	91.97 kBq/188 ml	30.7
methanol/2N NaOH, reflux	*	*	*
non-extractable residue	7594.02 Bq/g	6.83 kBq/0.9 g	2.3
total		246.93 kBq	82.5
seeds, combustion value	51.81 kBq/g	299.46 kBq/5.78 g	100

* extraction not conducted

Appendix XVII:

Raw data from the analysis of radioactivity in the seeds and material balance following extraction, experiment 2

Extraction date 19/3/91 (day 279 for storage stability investigation)

Liquid samples:

Number of aliquots : 3
Amount per aliquot : 0.1 - 7.0 ml
Instruments : 1. PW 4700 (Philips/Raytest)
2. Rackbeta 1219 Spectral (LKB)
Quench correction : External standard

Solid samples:

Number of aliquots : 3-5 (normally 3)
Amount per aliquot : 30-250mg (normally 50-100mg)
Instruments : Oxidizer OX 300 (Harvey)

Statistics:

Reproducibility : + 1 - 2% (standard deviation of the mean value)
Comparability : + 1 - 2% (1 sample measured with different instruments)

Background radioactivity of the instrument (automatically subtracted from the measurement results):

1. Instant Scint Gel (7ml)(Packard) : 20 - 30 cpm
2. Carbosorb (8ml)/Permafluor (10ml)(Packard) : 19 - 37 cpm (guaranteed <40 cpm)

Appendix XVIII:
Measurement of Radioactivity

Measuring time of samples:

Generally between 10 sec. and 40 min. depending on the amount of radioactivity in the sample.
The measurements are stopped after reaching a 2-sigma error of 0.7%. If this error is not reached within 10 min. the measurement is stopped and the 2-sigma error of the cpm-value (PW 4700, Philips/Raytest) or the error of the dpm-value (Rackbeta 1219 Spectral, LKB) reached at that time is printed out. The error of the dpm-value is calculated from the 1-sigma error of the cpm-value and the error of the quench correction curve.

Detection limit:

2 x background

Counting efficiency:

Instrument: 1. PW 4700 (Philips) = 84-93%
2. Rackbeta 1219 (LKB) = 48-96%