NR 1284 (ESR/ENG)

Date: February 12, 1991
1. Revision: March 4, 1991
2. Revision: August 23, 1991
3. Revision: January 28, 1992

STUDY TITLE

Metabolism of [pyridyl-^{14}C-methyl] NTN33893 in Rice Plants (Nursery Box Application)

DATA REQUIREMENT

EPA Guideline [Subdivision O, Section 171-4(a)]

AUTHOR

[redacted]

STUDY COMPLETION DATE

Jan. 4, 1991

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LABORATORY PROJECT ID

No. 89053/ESR

Translated by: [redacted] Date: 4/1/1991

Approved by: [redacted] Date: 12 Feb. 1991

101320

Data Confidentiality Statement

[redacted]

Good Laboratory Practice Statement

[redacted]

Study director: [redacted] Date: Jan. 4, 1991

Facility manager: [redacted] Date: Jan. 7, 1991

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Quality Assurance Statement

A quality assurance statement prepared by the performing laboratory is presented on page 6 of this report.

Statement of Authenticity

Subsequent pages of this report are presented as received from the performing laboratory, except for:

1. Addition of Mobay Report Number
2. Data Confidentiality Statement, page 2
3. Statement of Authenticity, page 4

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Signature: [redacted] Date: 7/23/91

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NR 1284 (ESR/ENG)

Test substance: NTN33893

Study initiation date: June 5, 1989

Experimental start date: June 14, 1989

Experimental termination date: March 26, 1990

Signature: [redacted]

Jan. 4, 1991
Date:

Jan. 4, 1991
Date:

Jan. 4, 1991
Date:

Dec. 25, 1990
Date:

Jan. 7, 1991
Date:

Archives: GLP Archives (Room No. 2206) in the ESR Building

Quality Assurance Statement

101320

(NIHON TOKUSHU NOYAKU SEIZO K.K./ Yuki Research Center/Environmental Science Research/GLP)

[redacted]

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Statement of amendments

Following points of the report described below were corrected.

Report No. :NR 1284(ESR/ENG)(English version translation)

Project No. :89053

Tittle :Metabolism of [pyridyl-^{14}C-methyl] NTN33893 in Rice Plants (Nursery Box Application)

Corrected points:

i) p8, 3rd line form the bottom: 79.4% ==> 79.2%

ii) p8, 3rd line from the bottom: 73.9% ==> 73.8%

iii) p25, 27th line from the bottom: 79.4% ==> 79.2%

iv) p25, 28th line from the bottom: 73.9% ==> 73.8%

v) p35, Table IX, column "Fractions": Unextaractable ==> Unextractable

vi) p42, Table XVI, column "Group 1--Conv.":
1.2(WAK3839) ==> 1.1, 0.3(NTN35884) ==> 0.2,
0.4(M-3) ==> 0.2, 59.2(Total) ==> 58.9

vii) p42, Table XVI, column"Group 1--Conv.+Exha.":
1.2(WAK3839) ==> 1.1, 0.3(NTN35884) ==> 0.2,
79.4(Total) ==> 79.2

viii)p42, Table XVI, column "Group 1--mg/kg":
0.001(NTN35884) ==> <0.001,
0.001(M-1) ==> <0.001, 0.005(M-3) ==> <0.001,
0.012(M-6) ==> 0.005, 0.024(M-7) ==> 0.009,
0.342(Total) ==> 0.312

ix) p42, Table XVI, column "Group 2--Conv.":
0.2(NTN35884) ==> 0.1, 53.3(Total) ==> 53.2

x) p42, Table XVI, column "Group 2--Conv.+Exha."
0.2(NTN35884) ==> 0.1, 73.9(Total) ==> 73.8

xi) p42, Table XVI, column "Group 2--mg/kg"
0.003(NTN35884) ==> 0.001, 1.053(Total) ==> 1.051

Reasons :i)-iv), vi)-xi): miscalculations, v): mistyping

Signatures :

[redacted]

TABLE OF CONTENTS

[ABSTRACT]

The absorption, translocation and metabolism of [pyridinyl-^{14}C-methyl] NTN33893 in rice plants were investigated in a laboratory study. The total terminal residues in rice grain and straw were also characterized. The application rates were normal (0.32kg a.i./ha) and exaggerated four-fold (1.26kg AI/ha). The normal dose corresponds to the maximum application rate by nursery box treatment.

Approximately 4% of applied dose was translocated to immature rice shoot within 65 days posttreatment. The level of translocation did not increase appreciably afterwards and only 4.4% of applied dose was found in the aerial part harvested at 124 days posttreatment. Rice grain contained trace amounts of radioactive residues, while 98% of the radioactive residues in the aerial part remained in straw. The total terminal residues in grain were 0.014 ppm (normal dose) and 0.064 ppm (exaggerated dose) ^{14}C-NTN33893 equivalents.

In the shoot and straw, 7 compounds were identified including unchanged NTN33893. The metabolites were NTN38014, WAK3839, WAK4103, NTN35884, NTN33519 and CNA (6-chloronicotinic acid). NTN38014 was the major component in both shoot and straw, accounting for 53% and 46% of the total radioactivities, respectively, while the quantity of NTN33893 was 9%. Of the other metabolites, WAK3839, WAK4103 and NTN35884 were less than 2% respectively. NTN33519 (11 - 12%) and CNA (4 - 6%) were primarily found in the unextractable fraction by stringent extraction. NTN33519 released from the unextractable fraction was considered to be an artifact.

NTN33893 was the major component in the extractable fraction from grain, accounting for 12% of the total terminal residues. Metabolites in the extractable fraction included WAK4103 (3.5%), NTN35884 (2.0%) and trace amounts of NTN38014, CNA and WAK3839. About 70% of the radioactivity in grain was unextractable bound residues. The crude starch contained 67% (48% of total ^{14}C) of the bound residues. The glucose obtained by glycolysis of the starch was revealed to be radiolabeled with a constant specific radioactivity, suggesting that ^{14}C-carbon dioxide derived from ^{14}C-NTN33893 was incorporated into natural constituents.

The percentages of metabolites identified in shoot, straw and grain were 79.2%, 73.8% and 83.8%, respectively. The metabolic pathway of NTN33893 in rice plants was proposed on the basis of metabolites identified in this study.

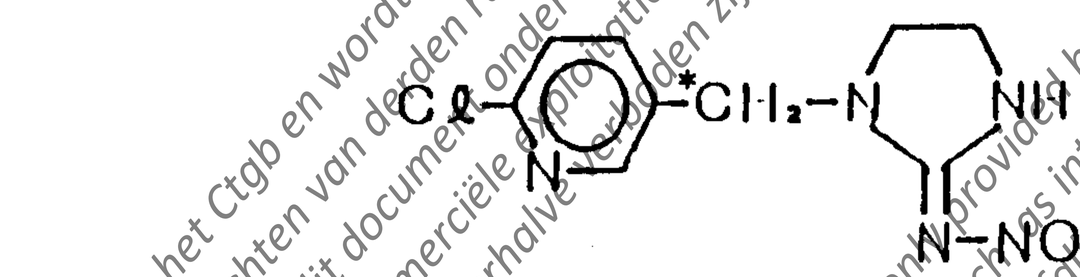
[INTRODUCTION]

NTN33893 (imidacloprid, ISO proposed), 1-[(6-chloro-3-pyridinyl)methyl]-4, 5-dihydro-N-nitro-1H-imidazole 2-amine (CAS Reg. No. 105827-78-9), is a promising imidazolidine type insecticide having excellent insecticidal activity with low dosage and relatively long residual effectiveness. In Japan, it has been developed under the testing code name 6331, mainly for controlling rice planthoppers and leafhoppers by nursery box treatment with granular formulation. Also in upland crop protection, planting hole treatment of granular formulation is being tested against aphids and slips of fruit vegetables. The present report describes the absorption, translocation, metabolism and total terminal residues of NTN33893 in rice plants by a nursery box treatment. The nursery box treatment is analogous to the seed treatment from a viewpoint that the active ingredient exists initially in soil. Therefore, it is considered that the metabolic profiles of NTN33893 obtained by a nursery box treatment would include those by a seed treatment. This study was performed under GLP standards.

[MATERIALS AND METHODS]

1. Test substance

(1) Radiolabeled compound



[pyridinyl-^{14}C-methyl] NTN33893

Spec. radioactivity: 5.58 MBq/mg (150.68μCi/mg, 38.5mCi/mmole)
Radiochemical purity: >99% (TLC, HPLC)
ESR vial No.: 45-nit-1
Source: Bayer AG [redacted]

(2) Non-radiolabeled substance

NTN33893 2% GR
Formulation No.: FLN05
A.I. content: 2.56% (HPLC-External standard method)
Recipe: see Appendix 1
Source: Formulation Research Department of Nitokuno

2. Reference substances

The structures and code names of reference compounds used in this study are shown in Table I. Those compounds are obtained from Bayer AG and Synthesis laboratory of Nitokuno.

3. Plant

Species: Rice plant (*Oryza sativa L.*)
Variety: Koshihikari
Date seeded: 23/May/1989
Date obtained: 13/June/1989
Stage: Around 3-leaf stage at transplanting
Origin: Biological Field Research of Nitokuno

4. Soil

Alluvial soil collected from Nakamura paddy field of Yuki Research Center (Soil No. 89-004) was used in this study. The soil was stored in a dark under non-flooded conditions. Before packing into planting vessels, the soil was passed through a 5mm-sieve. The texture and physico-chemical properties (Table II) were analyzed by Palynosurvery company Ltd.

5. Planting vessels

(1) Wagner pot (radiolabeled NTN33893 treatment, Group 1, 2 and 3)

Are-sized: 1/2000 a
Surface area: 500 cm^2
Height: 30 cm

(2) Plant box (non-radiolabeled NTN33893 treatment, Group 4)

Are-sized: 1/400 a
Surface area: 2500 cm^2
Height: 38 cm

6. Experimental groups

Following four groups were arranged according to the objectives of experiments. Table III shows in detail each experimental group.

Exp. group 1: The qualitative and quantitative analyses of residues of immature shoot
Exp. group 2: The qualitative and quantitative analyses of total terminal residues of rice grain and straw
Exp. group 3: Exaggerated dose experiment (4-fold)
Exp. group 4: The enrichment of metabolites for identification purpose, with non-radiolabeled NTN33893

7. Treatment solution

It was considered to be difficult to apply ^{14}C-NTN33893 quantitatively to rice plants with a granular formulation in accordance with intact nursery box treatment. Therefore, in this study, the definite quantity of ^{14}C-NTN33893 was dissolved in a solvent followed by applying it to soil. The maximum application

rate in the expected registration rate, described below, is defined as the "normal application rate" in this study.

Normal application rate
= 2% GR 80g/box x 200 boxes/ha
= 0.32kg a.i./ha = 1.6mg a.i./pot (1/2000 a)

The treatment solution was prepared as follows. ^{14}C-NTN33893 (106.9MBq, 2.889mCi, 19.16mg) was dissolved in 9 ml of acetone by ultrasonication for 10 min. Four 750 μl portions of this solution were pipetted separately into 10 ml test tubes (each 1.6mg AI, normal dose group). The rest of the solution was subdivided into two 3 ml portions (each 6.3 mg AI, exaggerated dose group). Acetone was removed under gentle stream of nitrogen. ^{14}C-NTN33893 in the test tubes was left at ambient temperature after dissolving again into 700 μl of acetone. No decomposition of ^{14}C-NTN33893 in acetone was observed. The radiochemical purity of ^{14}C-NTN33893 was more than 99% at 5 hours after preparation.

8. Soil conditioning, treatment of test substances and transplanting of rice plants

Exp. group 1, 2, 3: Six pots for cultivation of rice plants were prepared as follows (2 pots per group). Ten kg of fresh soil and 200g of Bark manure were placed in individual pot and mixed thoroughly. The soil was further enriched by adding 1.8g of chemical fertilizer in the top 10 cm layer of each pot. Then, the soil was flooded to keep 1-2 cm depth of surface water and allowed to stand 3 days for stabilization to approximate paddy conditions. Additional 1 kg of fresh soil was added and thoroughly mixed on the next day, since the soil core in the pot caved in about 2 cm. After three days, the surface water was removed completely by unstopping the draining channel installed at the bottom of pot. A glass tube (5 cm diameter x 5 cm length) was buried in the soil to a 5 cm depth, in the center of each pot. The soil core inside of the glass tube was removed using a spatula. The draining channel was stoppered tightly after water in the glass was diminished. About 30g of fresh soil was placed in the tube to make about a 2 cm layer and subsequently the acetone solution of ^{14}C-NTN33893 described above was added dropwise to the fresh soil using an Eppendorf applicator. The test tube was washed with three 100 μl portions of acetone. The acetone washing was added to the soil, followed by mixing with soil by using a spatula. The pot was allowed to stand 1 hr in green house to evaporate acetone. Three rice seedlings were transplanted into the tube and fixed by introducing wet soil in it. After that, each pot was flooded to a 2 cm depth by adding water. The glass tubes were removed from the soil after a week. The exact radioactivities applied were 238 μCi in the normal dose groups and 952 μCi in the exaggerated dose group.

Exp. group 4: About 98 g of NTN33893 2% GR equivalent to 2.5g a.i. was added to 5.6kg of soil (dry weight basis) and blended

1 hr on a rocking mixer (RM-30, Aichi Electric Ltd.) to gain a concentration of 446.4mg a.i./kg. The whole premix containing NTN33893 was further mixed with 44.5kg of soil (dry weight basis) in the plant box prior to flooding to a 2 cm water depth. The final concentration of NTN33893 was 50mg AI/kg. Eight bundles of rice seedlings (each 3 seedlings) were transplanted keeping an appropriate distance between the rice seedlings.

9. Cultivation of rice plants

The rice plants were grown to maturity in the green house of ESR building, in which the temperature was controlled at 30°C and the relative humidity was maintained at 60%. Although the actual temperature varied 16-30°C and the actual humidity varied 36-79%, there was no significant influence on the normal growth of rice plants, except spindly growth was observed at the tillering stage. In order to avoid the spindly growth, artificial light was supplied (28/June - 24/July, 9:00 -17:00/day) and the growing rice plants were supported by props. Water was supplied timely to keep appropriate depth of surface water according to conventional agricultural practice. Weeds were also controlled timely by proper manner.

10. Sampling and sample processing

Immature shoots were sampled 65 days posttreatment (before heading stage, 18/August/1989) from experimental group 1 and a part of group 4. Mature rice was harvested 124 days posttreatment (16/October/1989) from experimental group 2, 3 and the rest of group 4. At each sampling, the aerial part was collected by cutting about 3 cm above the soil surface. Sample processing was accomplished in the following manner.

(1) Immature shoot

The shoot samples were cut into 0.5 - 1 cm pieces using scissors and freeze-crushed by adding liquid nitrogen. There was no significant variation of sample weights during the sampling and this sample processing. The powdery samples were stocked in a -20°C freezer until analysis

(2) Mature rice

Rice ears were separated from straw using scissors. The straw was dried a week in the green house. Rice ears were separated into grain and rachis by forceps and were hulled by a roller huller. Hulled rice and chaff were separated by passing through a sieve for grain. All samples were finely ground by a vibrating sample mill (TI-100, Heiko Seisakusho). Rachis and straw were cut into 0.5 - 1cm pieces before grinding. Straw from experimental group 4 was ground by a Retsch grinder (Mitamura Riken). There was no significant variation of sample weights during the sampling, separation, cutting and grinding processes. The powdery samples were stored at -20°C until they were analyzed.

11. Extraction and fractionation

(1) Shoot (Exp. group 1) (Fig. 1)

Fig. 1 shows the scheme for extraction and fractionation. Duplicates of shoot subsample 50g were extracted by using a Polytron® extractor 4 times with 80% aqueous methanol and once with methanol/ethyl acetate mixture (2/1), (solvent volume was 300 ml at each extraction). The extractable (combined extracts) and unextractable (fibrous residue) fractions were further fractionated as follows.

The extractable fraction was concentrated *in vacuo* on a rotary evaporator. The remained aqueous solution was partitioned three times each with 150 ml portions of n-hexane (discarded) and dichloromethane. The remaining aqueous phase in 100 ml was passed through an XAD-4 column, followed by washing it with 100 ml of water and then 500 ml of methanol. The dichloromethane phase and the methanol eluate were subjected to TLC and HPLC for metabolite analyses.

The unextractable fraction (ca. 12g by dry weight, equivalent to 50g of fresh shoot) was first refluxed 48 hours with 200 ml of methanol. The residue was extracted 3 times for 30 minutes with 100 ml of dioxane/water mixture (9/1) by shaking and further extracted once for an hour with 100 ml of dioxane/2M hydrochloric acid (9/1) by stirring. After each extraction step, the extract was centrifuged (9000 rpm, 10 minutes). The acidic dioxane extract was adjusted to pH 7 and was combined with the neutral dioxane extract. Following evaporation of dioxane, the residual solution stood overnight at 4°C after addition of ice to precipitate lignin. The lignin was collected by centrifugation (3000 rpm, 10 minutes). The supernatant was concentrated to about 50 ml, and then applied to XAD-4 column which was washed with 50 ml of water and 300 ml of methanol. The residue which remained, 2g by dry weight, was stirred a hour with 30 ml of 2M sodium hydroxide/methanol mixture (2/3). The temperature was maintained at 60°C during the extraction. The extraction mixture was filtered off to separate alkaline extract and residue. The extract was neutralized by adding conc. hydrochloric acid, evaporated and applied to XAD-4 column. The column was washed with 100 ml of water and eluted with 300 ml of methanol. The methanol extract obtained by reflux and the methanol eluates from lignin-free supernatant and the alkaline methanol extract were surveyed by TLC and HPLC.

(2) Straw (Exp. group 2 and 3)

Twenty g of straw were extracted in duplicate. Prior to homogenization by Polytron® extractor, the straw was soaked overnight at 0°C in 80% acetonitrile for extraction efficiency. The extraction was repeated 3 times with 300ml

of 80% acetonitrile, and then with acetonitrile/ethyl acetate mixture (2/1).

The extractable and unextractable fractions were further subdivided, on the whole, in accordance with the method for shoot. The unextractable fraction from experimental group 2 (ca. 34g on dry weight, equivalent to 40g of straw) was extracted in a similar manner as shoot. The methanol extract obtained by reflux extraction was partitioned between 50 ml of dichloromethane and 50 ml of water. The water phase was subjected to XAD-4 column. The column was washed with 50 ml of water and then eluted with 400 ml of methanol.

The unextractable fraction from experimental group 3 was fractionated by an alternate method. Two g of fibrous solid were extracted an hour at 60°C with 30 ml of alkaline solution/methanol mixture (2/3). The alkaline solutions used were 1M sodium acetate, 1M potassium hydrogen phosphate, 1M sodium carbonate and 2M sodium hydroxide. Each extract was neutralized with hydrochloric acid, evaporated just to dryness and dissolved in 5 ml of water. Twenty ml of ethanol were added to the aqueous solution for desalting. The turbid solution was centrifuged to analyze bound metabolites present in the supernatant by HPLC.

(3) Grain (Exp. group 2 and 3) (Fig. 2)

The extraction of hulled grain followed the scheme given in Fig. 2. The extractable and unextractable fractions of grain were gained in a similar manner as straw. Two 30g portions of grain were each extracted with 300 ml solvent.

The cereal oily substance in dichloromethane phase was removed by partitioning it between acetonitrile saturated with n-hexane and n-hexane saturated with acetonitrile (20 ml/20 ml). The acetonitrile phase was analyzed for metabolites by TLC and HPLC.

The unextractable fraction of grain from experimental group 3 was subdivided by the method of J. Rouchaud et al. (1979) modified. About 50g of the grain residue was first refluxed with methanol. The remaining residue was homogenized with three 200 ml portions of 12M hydrochloric acid/methanol mixture (3/97). The combined extract (crude amino acid fraction) was neutralized with potassium hydroxide. The precipitated KCl was removed by centrifugation (3000 rpm, 10 minutes). The resulting supernatant was referred to as the crude amino acid fraction. The remaining residue was further homogenized with four 200 ml portions of 0.03M sodium hydroxide solution. The homogenized mixture was centrifuged (3000 rpm, 10 minutes) at each extraction step. The combined supernatant was referred to as the crude protein fraction. The final residue was the crude starch fraction. To the crude protein solution, conc. hydrochloric acid was added dropwise for adjusting to pH 6. The white precipitate (= rice glutelin) was collected by centrifugation (3000

rpm, 10 min.). The glutelin, powdered by freeze-drying, was purified by dissolving again in 0.03M sodium hydroxide and subsequent precipitating.

The crude starch was hydrolyzed to glucose by the method as shown in Fig. 3. Five g of crude starch were dissolved in 300 ml of 0.5M hydrochloric acid and refluxed for 6 hours. After neutralization with sodium hydroxide, the resulting glycolysis solution was washed with ethyl acetate. The aqueous phase was evaporated to a small volume for desalting by ethanol, and then the supernatant containing glucose was evaporated to dryness. The crude glucose was acetylated by acetic anhydride in the presence of anhydrous sodium acetate. The acetylglucose (glucose pentaacetate) was extracted with dichloromethane from the reaction mixture. The dichloromethane phase was washed repeatedly and then evaporated to dryness. The acetylglucose was first recrystallized with n-hexane/diethyl ether mixture. The acetylglucose was purified by two more recrystallizations from ethanol. An aliquot of crystals was combusted at each recrystallization step to measure its specific radioactivity.

12. Column chromatographic separation of metabolites

A column chromatographic technique was employed for the separation of metabolites in dichloromethane phase and aqueous phase (XAD-4 methanol eluate) from shoot and straw. A 5g of silica gel containing 10% water was used as the adsorbent which was introduced in an open glass column equipped with stopcock. The compositions of eluting solvent were as follows:

[Dichloromethane phase]
ORGF1: n-hexane/ethyl acetate (50/50) 50 ml
ORGF2: ethyl acetate 50 ml
ORGF3: methanol/ethyl acetate (10/90) 50 ml
ORGF4: acetic acid/methanol (5/95) 50 ml

[Aqueous phase]
AQUF1: ethyl acetate/methanol (50/50) 50 ml
AQUF2: methanol 50 ml
AQUF3: acetic acid/methanol (5/95) 50 ml

Each eluate was compared chromatographically with the original fraction by TLC or HPLC to define the eluate in which the target metabolite was present. In addition, the eluate was co-chromatographed with reference compounds by TLC for identification.

13. Isolation of metabolites

The rice straw from experimental group 3 (20g) and group 4 (30g) were extracted separately by the previously outlined procedure. The resulting dichloromethane phase and aqueous phase (XAD-4 methanol eluate) were worked up as follows.

(1) Dichloromethane phase (Fig. 4)

This fraction was chromatographed on a column of deactivated silica gel to obtain ORGF1, ORGF2, ORGF3 and ORGF4. Each fraction from experimental group 3 was mixed with that from experimental group 4, and the target metabolite was isolated by combination of TLC and HPLC separations. The detailed procedure is shown in Fig. 4. The final purification of NTN33893 eluted in ORGF2 was achieved by a Sep-Pak® Florisil cartridge (Waters). NTN33893 was recovered from it by ethyl acetate and acetonitrile (each 10 ml). NTN38014 isolated from ORGF4 was derivatized with acylating reagents (HFBA, PFPA) and co-chromatographed by TLC with those from reference NTN 38014. The acylation method was described in part 16.

(2) Aqueous phase (Fig. 5)

The aqueous phases from experimental group 3 and 4 were mixed, chromatographed on a column of deactivated silica gel and subjected to TLC separation. The overall procedure is shown in Fig. 5. A Sep-Pak® Accell CM cartridge (Waters) was applicable to purify NTN38014 isolated by TLC. The acylation (HFBA) mixture of NTN38014 was cleaned up by column chromatography on 10g of silica gel (activated 5 hours at 130°C). The reagents and undesired by-products were each eluted with 50 ml of n-hexane/ethyl acetate mixture (80/20) and the same composite solvent (50/50). NTN38014 - HFBA derivative was recovered by eluting the column with 50 ml of n-hexane/ethyl acetate mixture (20/80).

14. Chromatography

(1) Thin layer chromatography (TLC)

TLC was employed for purification, isolation, characterization and quantification of metabolites by use of the following 5 solvent systems. For characterization and quantification purposes, TLC plates (precoated 0.25 mm-thickness silica gel $60F_{254}$, E. Merck) were used. The purification and isolation was achieved on silica gel $60F_{254}$ plates with 0.5 mm thickness (E. Merck). Table IV shows the Rf values of reference compounds.

T.S.A: ethyl acetate/isopropanol/water (65/23/12,v/v)
T.S.B: dichloromethane/acetonitrile (50/50,v/v)
T.S.C: dichloromethane/ethanol/water/acetic acid (65/25/3/3,v/v)
T.S.D: ethyl acetate/methanol/water/acetic acid (65/25/10/1,v/v)
T.S.E: ethyl acetate/acetonitrile/ammonia water (80/17/3,v/v)

The radioactive spots on TLC plate were detected by TLC linear analyzer (RITA-3200, RAYTEST) or autoradiography (ARG) with X ray film (IX-150, IX-400, Fuji). The

reference compounds were visualized by irradiation of UV-lamp.

(2) High performance liquid chromatography (HPLC)

Characterization, purification and quantification of metabolites were performed by HPLC and TLC. The HPLC system and column packings are described below. Table V shows the further details of HPLC operating conditions. The gradient elution systems H.S.A and H.S.B were used for characterization and quantification of metabolites, while the isocratic elution system was used mainly for preparative separation. Table VI shows the retention times (Rt) of reference compounds in H.S.A and H.S.B.

Elution systems:	M600E type multi-solvent system (Waters)
Injector:	U6K manual injector (Waters)
	231-401 type autosampling injector (Gilson)
Detector:	490 multi wave length UV detector (Waters)
	Ramona 5-LS radioactivity monitor (RAYTEST)
Column:	LiChrospher RP-Select B (4 mm x 25 cm, 5 μm)
	LiChrospher RP-8 (4 mm x 25 cm, 5 μm) (both E. Merck)

15. Radioassay

The radioactivity was measured by liquid scintillation counter (LSC), Model LS-3801 or LS-5801 (both Beckman). The scintillation cocktails used were Ready Gel (Beckman) for aqueous samples and Ready Organic (Beckman) for organic samples and silica gel scrapings from TLC plate. Solid samples (rice the plants, extracted residue, lignin, protein, starch) as well as n-hexane phase from shoots were combusted by a sample oxidizer (Tri-carb 306, Packard). The resulting radioactive carbon dioxide was trapped in Carbo-Sorb (Packard) and radioassayed with Permafluor V (Packard). For efficient combustion, wet shoot samples were combusted after Combustaid® reagent (Packard) was added.

16. Acylation of NTN38014

Acylating reagents (100 μl) and triethylamine (60 μl) were added dropwise to NTN38014 dissolved in 2 ml of acetonitrile. The reaction mixture was stirred for an hour at 60°C while stirring. The reaction vessel was tightly stoppered. Heptafluorobutyric anhydride (HFBA) was more reactive with NTN38014 than pentafluoropropionic anhydride (PFPA). The reaction was stopped by adding 30 ml of water, and NTN38014 acyl derivative was extracted with three 30 ml portions of n-hexane.

17. Spectrometry

(1) Gas chromatograph/mass spectrometry (GC/MS)

Instrument: DX-303 Mass spectrometer (JEOL)

Column: 3% Silicone OV-210/Chromosorb W (AW-DMCS) 80-100 mesh 3 mm i.d. x 1.5 m
Carrier gas: He 40 ml/min
Injection temp.: 260°C
Oven temp.: program 200°C → 260°C (20°C/min)
Ionization voltage: 70 eV
Ionization mode: Electron Impact (EI)

(2) Nuclear Magnetic Resonance (NMR)

Samples were dissolved in CD_3OD (E. Merck) or $CDCl_3$ (Aldrich) and measured by an AC-250 FT-NMR Spectrometer (Bruker).

[RESULTS AND DISCUSSION]

1. Absorption and residue levels

Table VII shows the raw weights of rice plants treated with ^{14}C-NTN33893. The amounts of radioactivity in individual parts and residue levels are summarized in Table VIII.

About 4% of applied radioactivity was found in immature shoot collected 65 days posttreatment (exp. group 1). The radioactivity level in rice plants was not so increased thereafter. The mature rice plants harvested 124 days posttreatment contained only 4.4% of the initial radioactivity. Almost all of the radioactivity in mature rice was found in straw (98%). Relatively larger amounts of radioactivity was translocated into rice straw treated with exaggerated ^{14}C-NTN33893 (6.9% of the total radioactivity). Regarding percentage of the other parts, there was no difference between the normal and exaggerated groups, however ca. 4-fold concentration was observed in the exaggerated group.

The shoot and straw showed a similar residue level, 1.3 - 1.4 ppm NTN33893 equivalent, comparing on the basis of dry weight (exp. group 1 and 2). The total terminal residues in hulled grain from exp. group 2 and 3 were 0.014 ppm and 0.064 ppm NTN33893 equivalent, respectively(0.03% of the total radioactivity).

2. Extractability

Extraction efficiency tests of radioactive residues in shoot and straw were conducted (Appendix 2 and 3). In the results, 80% methanol (shoot) and 80% acetonitrile (straw) were confirmed to be optimum solvents by using Polytron® extractor. Green material was extracted finally with water-free solvents. The grain were not examined concerning the extraction efficiency, since the radioactivity level was extremely low. Therefore, the extraction of grain was followed the method for straw.

Table IX exhibits the distribution of radioactivity in extractable and unextractable fractions from shoot, straw and grain. The extractable radioactivities from shoot and straw exceeded more than 60% of the total residues. The major components of those radioactivities were water soluble, accounting for about 40%. n-Hexane soluble components were negligible.

In contrast, about 70% of the radioactive residues in grain were unextractable bound residues. Most of the extractable component was soluble in dichloromethane.

3. Isolation and identification of metabolites

(1) Dichloromethane soluble metabolites

Fig. 6 shows TLC and HPLC chromatograms of dichloromethane phase from straw (exp. group 2) before separation by column chromatography into four subfractions (ORGF1, ORGF2, ORGF3, ORGF4). Fig. 7 shows comparative

HPLC chromatograms of the subfractions (ORGF2-4). The ORGF1 was compared with others only by TLC due a large amount of matrix (interference, Fig. 8, 9). Eleven metabolites detected by HPLC (A - K) were characterized, and they were assigned as follows.

A: This metabolite appeared in ORGF3 and its HPLC-Rt (retention time) coincided with none of the reference compounds. TLC analysis revealed that peak A was single component (Fig. 9). Attempt to clarify the structure by NMR was unsuccessful because only trace amounts were available (Fig. 10). In the later description, peak A is referred to M1. The relative Rf values were 1.02 (T.S.A) and 0.52 (T.S.B), and therefore M1 overlapped with NTN33893 in TLC separation with T.S.A.

B: This metabolite was estimated to be contained in ORGF2. The Rf value in TLC analysis could not be defined. Peak B was referred to M2.

C: ORGF3 contained relatively in larger amounts of this metabolite. TLC and HPLC analyses revealed that this metabolite was single component having the same chromatographic property of WAK3839. The NMR spectrum of isolated peak C, as shown in Fig. 11, was identical with that of WAK3839. From these results, this metabolite was identified as the reduced compound of NTN33893.

D: This metabolite, appearing in ORGF3, was characterized as NTN35884, an olefin analogue of NTN33893, by co-chromatography with reference compounds in TLC and HPLC analyses. Structure elucidation by NMR analysis was unsuccessful.

E: Chiefly this metabolite was eluted in ORGF2. Co-chromatography with reference compounds by TLC and HPLC analyses as well as NMR confirmation (Fig. 12) were attempted. In the results, peak E was identified in all aspects as WAK4103 which was produced by mono-hydroxylation at 5-position in the five-membered ring.

F: ORGF3 contained this metabolite. TLC, HPLC and NMR analyses led to a conclusion that peak F was identical with a cyclic urea NTN33519 (Fig. 13).

G: ORGF2 included this metabolite. No reference compounds displayed identical chromatographic behavior with peak G. The NMR spectrum of isolated peak G, as shown in Fig. 14, was insufficient to establish the structure. In the later description, peak G is referred to as unknown M3. The relative Rf values were 1.09 (T.S.A) and 0.87 (T.S.B).

H: This metabolite was found from ORGF3. The Rf values were uncertain, since single spot of peak H was not observed in TLC analysis. Peak H was referred to as M4.

I: Peak I was the major component of the dichloromethane phase and eluted principally in ORGF2. Comparison of Peak I with reference compounds by TLC, HPLC and NMR (Fig. 15) analyses resulted in the identification as unchanged NTN33893.

J: This minor metabolite was eluted in ORGF1. The relative Rf values were close to that of WAK4103 in T.S.A (1.18) and that of parent in T.S.B (1.05). This metabolite is referred to as M5 in the later description.

K: This metabolite was the single polar component eluted in ORGF4. The acylation of peak K with HFBA or PFPA led to production of nonpolar derivatives. Both HFBA and PFPA derivatives of isolated peak K exhibited the same behavior as those obtained from the reference NTN38014. Accordingly, peak K was identified as NTN38014, a denitro or an imine analogue of NTN33893, although NMR analysis was unsuccessful because of impurity.

(2) Water soluble metabolites

The HPLC and TLC profiles of the intact aqueous phase from shoot (XAD-4 methanol eluate) are shown in Fig. 16. Fig. 17 shows the comparative HPLC chromatograms of AQUF1, AQUF2 and AQUF3 obtained by column chromatographic fractionation of the aqueous phase. Five HPLC peaks (L -P) were characterized as follows.

L: This metabolite was eluted in AQUF1 and not retained through reversed phase HPLC column. The behavior of peak L on TLC coincided with that of chloronicotinic acid, CNA, and therefore the isolation was accomplished by TLC scraping and ethyl acetate/water (pH 1) partitioning. The HPLC-Rt varied depending upon the degree of purification. Co-injection of the isolated metabolite and the reference compound into the HPLC column was successful to identify peak L as CNA.

M: This metabolite appeared in AQUF1. As a result of co-chromatography with reference compounds, peak M was identified as WAK4103 which was also detected as a dichloromethane soluble metabolite.

N: AQUF3 contained this metabolite. The behavior on TLC and HPLC was analogous to those of two metabolites (O and P) described next. Attempt to isolate for structure analysis was unsuccessful. Peak N was retained on Sep-Pak® Accell CM cartridge, indicating a basic substance, although irreversible adsorption to the anion exchange resin occurred. This metabolite showed a relative Rf

value of 0.24 in T.S.A and is described as M6 hereafter.

O: AQUF3 contained this metabolite. In all chromatographic analyses, peak O exhibited close behavior to peak P (= NTN38014). Structure analysis by NMR failed, since isolated peak O contained plant matrices together with peak P. Its basic character was proved, in analogy with M6, by irreversible anion exchange when applied to Sep-Pak® Accell CM cartridge. Peak O showed a relative Rf value of 0.18 in T.S.A, and in the later description it is referred to as M7.

P: Its HPLC-Rt coincided with NTN38014. The acyl derivative obtained by treatment with NFBA was subjected to structure analyses by NMR (Fig. 18) and/or GC/MS (Fig. 19). The GC/MS spectrum gave a molecular ion M/Z = 406 (M^+) and a characteristic fragment ion M/Z =237 ($M^+ - C_3F_7$), indicating that the derivative was mono-acylated NTN38014. It was estimated that the acyl group was introduced in the imino moiety of NTN38014.

TLC profiles of n-hexane, dichloromethane and aqueous phases of straw from experimental group 3 (exaggerated dose) are shown in Fig. 20 - 22. NTN38014 was revealed to emerge in all phases even though it was of great polarity. The gradient HPLC method surpassed TLC with regard to separative ability, particularly in the case of separation of dichloromethane soluble metabolites. Therefore, the individual metabolites were determined mainly by the HPLC gradient system, unless otherwise stated.

4. Metabolism of NTN33893 in rice plants

Table X shows the relative amounts of metabolites extractable from shoot and straw treated with the normal rate of ^{14}C-NTN33893. The recovery of radioactivity from HPLC column ranged from 101% to 109%.

Qualitative and quantitative differences were not appreciable between shoot and straw. NTN38014 was the major radioactive component in aqueous phase of both shoot and straw, accounting for about 43% (shoot) and 36% (straw) of the total radioactivities. A part of NTN38014 was extractable with dichloromethane.

Unchanged NTN33893 was found in amounts of about 8% of the total radioactivities from shoot and straw. The other metabolites, WAK3839, WAK4103, NTN33519, NTN35884 and CNA were found to be less than 2% each, and they decreased with time. Of the unknown metabolites in dichloromethane phase, M1 and M3 were detectable in amounts of around 0.2%, whereas M2, M4 and M5 were found less than that level. Unknown M6 and M7, defined as water soluble basic metabolites, increased slightly with time and reached 2.6% (M6) and 2.7% (M7) at the harvest stage of rice plants.

The unextractable component was characterized by allowing the extraction conditions to vary stepwise. The results are

summarized in Table XI. By refluxing with methanol, NTN33893, NTN33519 and NTN38014 were released in quantities up to 1.0 - 1.3% of the total radioactivity. NTN38014 was also solubilized along with lignin in amounts of 1.3% (shoot) and 1.4% (straw) of the total radioactivities. Essentially the extent of incorporation of radioactivity into lignin was negligible throughout the entire growth of rice plants(3% in straw).
The alkaline methanol treatment was affective on a point of extractability of bound residues, about 63% of which were released. The major component of the released radioactivity was revealed to be NTN33519 which accounted for 9 -12% of the total radioactivity. Significant amounts of NTN38014 and CNA were also solubilized.
NTN33519 was a minor metabolite found in the shoot and straw extractable fractions and its structure indicates little possibility to produce bound residue. Therefore, it might be an artifact produced during the alkaline methanol extraction from NTN33893 or NTN33893-like metabolite(s). In fact, NTN33893 was labile and converted almost quantitatively to NTN33519 under the extraction conditions described (Appendix 4). On the other hand, NTN38014 was stable, showing that the amounts of NTN38014 released by alkaline methanol reflected the intact bound NTN38014. CNA was solubilized in quantity, particularly from straw bound residues, accounting for 10-fold larger amounts than that of extractable CNA.

The straw bound residues were characterized also by changing the extraction solvents in a stepwise fashion. Table XII shows the amounts of extractable and unextractable metabolites found in straw treated with an exaggerated route of ^{14}C-NTN33893. Approximately 33% of the total radioactivity was unextractable. The extraction efficiencies of 4 kinds of alkaline methanol appeared to be closely related to the ionic strength. In other words, the extraction percentage increased with increasing alkaline strength. Treatment with 2M sodium hydroxide/methanol (2/3) resulted in releasing 85% of the bound radioactivity. NTN33893, NTN33519, NTN38014 and CNA were confirmed to be present in all extracts (Fig. 23). The amounts of NTN33519 and CNA exceeded those extractable with aqueous acetonitrile. With regard to CNA, 1M sodium carbonate/methanol mixture was the most suitable extractant, releasing 7.6% of the total radioactivity as CNA. NTN33519, as expected, was not as prevalent under conditions causing no or limited decomposition of NTN33893. However, intensive extraction conditions with significant decomposition of NTN33893 and/or related compounds gave rise to increased amounts of NTN33519. By treatment with 2M sodium hydroxide/ methanol mixture, NTN33519 was released in amounts of 8.7% of the total radioactivity.

5. Characterization of the total terminal residues in hulled grain

Table XIII demonstrates the results concerning characterization of the terminal residues in grain from exp. group 2 (normal) and group 3 (exaggerated). The corresponding TLC and HPLC profiles are shown in Fig. 24 - 26.

There was no significant differences with respect to quality or quantity of metabolites between the two dose experiments examined. In the dichloromethane phase, NTN33893 was the major component, accounting for about 12% of the total radioactivity in grain. Of the metabolites identified, NTN35884 and WAK4103 were observed in relatively larger amounts, both being found at about 1.5%. Trace amounts of WAK3839 and NTN38014 were also confirmed. WAK4103 was the major component in the aqueous phase in which NTN33893, NTN35884 and NTN38014 were also found. Additionally CNA and unknown metabolites assumed to be M6 and M7 were present.

Approximately 70% of the total radioactivity in grain was composed of bound residues. Fractionation of the bound residues resulted in the following distribution profile (% of the bound residue) : 67% in crude starch, 28% in crude protein, 5% in methanol extract by refluxing. No radioactivity was present in crude amino acid fraction. The radioactivities in crude starch and crude protein corresponded to 48% and 20%, respectively, of the total terminal residues in grain.

Glutelin, a rice protein, was precipitated by weak acidification of the alkaline extract for the purpose of evaluating the incorporation of radioactivity in glutelin. In the results, as shown in Table XIV, 16% of the total radioactivity in grain was coprecipitated along with glutelin. Moreover the specific radioactivity was almost constant before and after purification (ca. 18000 dpm/g). These results led to an estimation of either incorporation of radioactivity originated from NTN 33893 into protein or chemical binding of NTN33893 and/or its metabolites with protein.

The crude starch was acid-hydrolyzed for glycolysis, and the resulting glucose was acetylated to determine the incorporation of radioactivity in it. Table XV shows the overall procedure and the results. No radioactivity was lost during the hydrolysis. The first acetylglucose crystals retained radioactivity, although the initial radioactivity found in starch decreased to 57%. This might reflect the yield of acetylation. The recrystallization of acetylglucose was repeated twice more to analyze the structure. It was revealed that the third crystals were β-form of acetylglucose together with trace α-form by NMR analysis (Fig. 27). The variation of specific radioactivities of acetylglucose was small throughout the recrystallization procedure (7443 dpm/g, mean value). Using the specific radioactivities of hulled grain and acetylglucose, the distribution of radioactivity in the starch was calculated to be 59% of the grain. Accordingly, about 60% of the total terminal residues in hulled grain was considered to be attributable to the natural constituent starch in which radioactivity was derived

from so-called carbonic acid assimilation [J. P. Wargo et al., 1975].

6. Identification percentages and residue levels of individual metabolites

Table XVI shows the identification or characterization percentages as well as residue levels of individual metabolites in shoot and straw (both normal dose). Table 17 shows the corresponding data obtained from hulled grain (exaggerated dose).

Comparing the metabolites in shoot with those in straw, there was no significant variation, although the sum amounts (free + bound) decreased on the whole. Both shoot and straw contained NTN33893 in amounts of about 9% of the total radioactivities. NTN38014 comprised 53% (shoot) and 46% (straw) of the total radioactivity, and most of which was extractable. NTN33519 and CNA increased slightly with the growth of rice plants and particularly in straw both metabolites were predominantly present in unextractable fraction. Thus NTN33519 and CNA including NTN38014 appeared likely to be closely associated with formation of bound residues. However, it was necessary to take into account that possibly NTN33519 was an artifact producted from NTN33893 or NTN33893-like metabolites during the intensive extraction. The identification percentages were 79.2% (shoot) and 73.8% (straw).

Most of the total terminal residues in grain was attributable to incorporation of radioactivity into starch and protein (rice glutelin) which comprised 64% of the total radioactivity. Of the identified components, only NTN33893, WAK4103 and NTN35884 exceeded 0.001 ppm. It seems possible therefore that the mode of producing terminal residues in hulled grain was not comparable to that in straw. The identification percentage in grain was 83.8%.

7. Storage stability

Seven different ^{14}C-compounds (NTN33893, NTN38014, WAK4103, WAK3839, NTN33519 and chloronicotinic acid) were added to control samples of grain and straw. Samples were extracted with 80% acetonitrile by Polytron® at 0-day (grain and straw), 21-day (straw) and 52-day (grain) after storage at -25°C. Acetonitrile was evaporated and the aqueous phase was partitioned with n-hexane (discarded). After the remaining aqueous was lyophilized, the residue was dissolved with methanol. ^{14}C-compound in methanol solution was determined by HPLC. (HPLC condition: LiChrospher RP-select B, 4 mm i.d. x 250 mm, 5 μm, H.S.B. gradient system).

Concentration of each ^{14}C-compound at 0-day (grain and straw), 21-day (straw) and 52-day (grain) after storage (-25°C) was shown in Appendices 15 and 16. Recoveries of the parent compound and other 6 metabolites was 90 - 122% in grain and 81 - 114% in straw, which fact suggests that the parent compound and these

metabolites don't degrade in grain and straw during storage at -25°C.

8. Metabolic pathway

The proposed metabolic pathway of NTN33893 is postulated in Fig. 28.

The major metabolic route was denitration producing NTN38014. The other metabolic routes, involving conversions into WAK3839, WAK4103, NTN35884 and NTN33519, were all minor in comparison with denitration. Besides these paths without any structural cleavage, an elimination of the chloropyridinyl moiety and subsequent oxidation to yield CNA was also ascertained. In shoot and straw, bound residues as a consequence of second phase metabolism were formed through first phase metabolites, NTN38014 and CNA. The distribution pattern of metabolites in hulled grain differed from that in straw, e.g. NTN38014 was a minor metabolite in grain. This difference might be possible due to restricted translocation of metabolites from straw into grain rather than distinct metabolic profile. The incorporation of radioactivity into the glucose unit of starch was considered to be a result of utilization of NTN33893 as a carbon source following complete mineralization to CO_2 in soil or rice.

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J. P. Wargo, R. C. Honeycutt and I. L. Adler: Characterization of Bound Residues of Nitrofen in Cereal Grains, J. Agric. Food Chem., 26, 1156 (1975)

Table I. Code names and structures of NTN 33893 and its related reference compounds used in this study

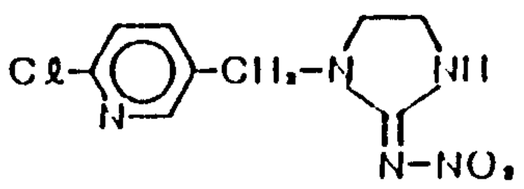
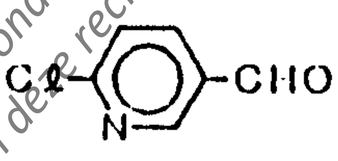
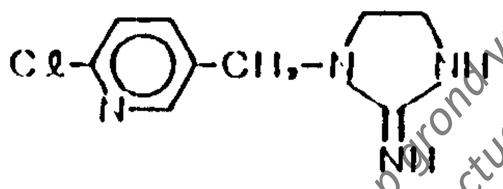
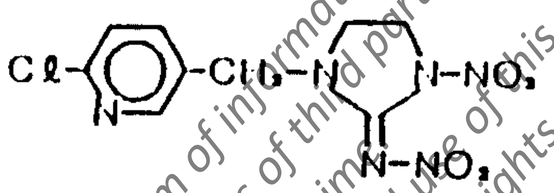
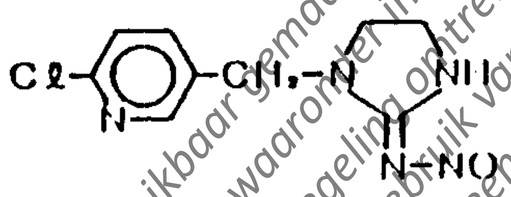
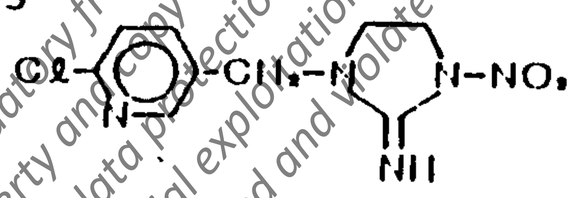
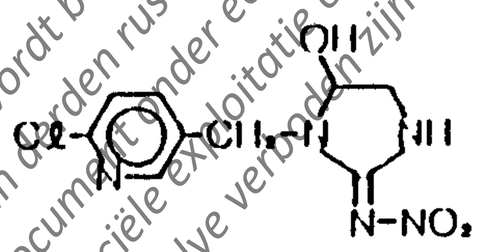
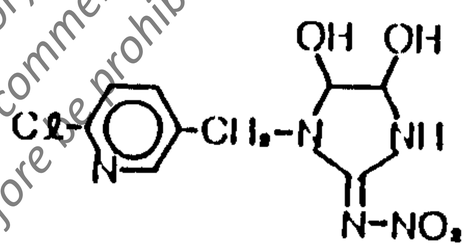
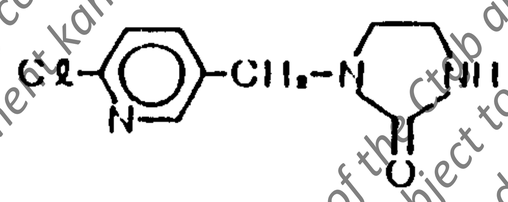
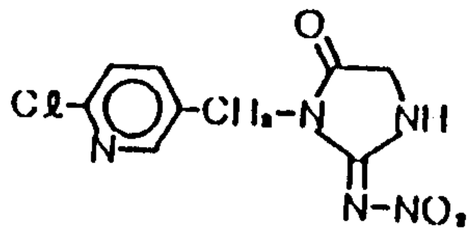
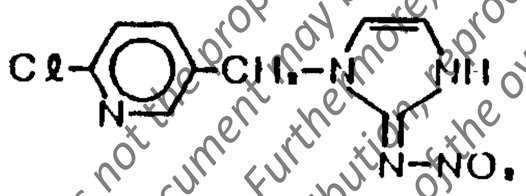
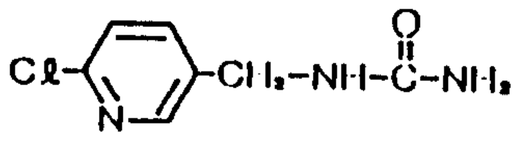
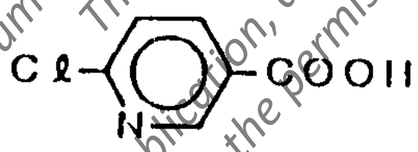
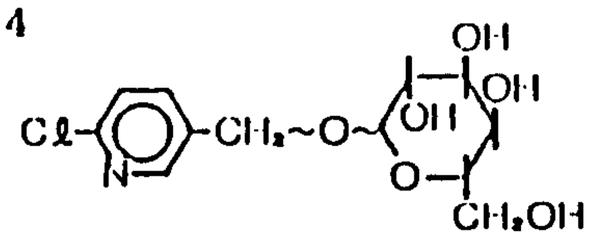
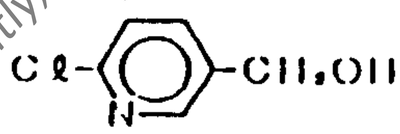
Code Name	Structure	Code Name	Structure
NTN 33893		PAD	
NTN 38014		DIJ 9979	
WAK 3839		DIJ 10533	
WAK 4103		WAK 3772	
NTN 33519		WAK 3738	
NTN 35884		NTN 36749	
CHA		RBH 1114	
PAC			

Table II. Physico-chemical properties of paddy soil used in this study

Texture	
Coarse sand (%)	3.1
Sand (%)	25.7
Silt (%)	40.8
Clay (%)	30.4
Classification	Light Clay (International) Silty Clay Loam (USDA)
Organic Carbon (%)[1]	3.2
pH[2]	5.6
Cation Exchange Capacity[1] (meq/100g)	20.5

1) dried soil basis
2) pH in H_2O (25°C)

Table III. Experimental groups for ^{14}C-NTN33893 rice metabolism study

Exp. group	1	2	3	4
$^{14}C/^{12}C$	^{14}C			^{12}C
Application method carrier	Planting hole treat. acetone sol.			Soil treat. 2% Gr
Planting vessel	Wagner pot			Plant box
No. of vessel	2 pots/group			1 box
Dose level	Normal		Exaggerated	
Dose rate kg a.i./ha mg a.i./vessel μCi/vessel	0.32 1.6 238		1.26 6.3 952	50 mg a.i./kg 2500 -
Planting rate (bundle/vessel)	1 (3 seedlings)			8 (24 seedlings)
Date applied	14/6/1989			
Date transplanted	14/6/1989			
Dates fertilized (2.0kg N/10a)	16/8/89	16/8/89, 23/8/89		22/6/89* 16/8/89 23/8/89
Dates collected	18/8/89	16/10/89		18/8/89 16/10/89
Plant parts analyzed	Immature shoot	Grain, mature straw, chaff, panicle		Immature shoot, mature straw

* 8.0kg N/10a

Table IV. TLC-Rf values of NTN33893 and its related reference compounds

Compound	Rf values				
	T.S.A	T.S.B	T.S.C	T.S.D	T.S.E
NTN33893	0.55(1.00)	0.64(1.00)	0.74(1.00)	0.68(1.00)	0.42(1.00)
NTN38014	0.02(0.04)	0 (0)	0.09(0.12)	0.12(0.18)	0 (0)
WAK3839	0.37(0.67)	0.21(0.33)	0.56(0.76)	0.56(0.82)	0.15(0.36)
WAK4103	0.69(1.25)	0.50(0.78)	0.69(0.93)	0.74(1.09)	0.23(0.55)
NTN33519	0.46(0.84)	0.14(0.22)	0.69(0.93)	0.61(0.90)	0.19(0.45)
NTN35884	0.51(0.93)	0.38(0.59)	0.59(0.80)	0.65(0.96)	0.06(0.14)
CNA	0.24(0.44)	0 (0)	0.58(0.78)	0.54(0.79)	0 (0)
PAC	0.70(1.27)	0.56(0.88)	0.74(1.00)	0.75(1.10)	0.46(1.10)
PAD	0.78(1.42)	0.82(1.28)	0.84(1.14)	0.81(1.19)	0.58(1.38)
DIJ9979	0.31(0.56)	0.01(0.02)	0.27(0.36)	0.64(0.94)	0 (0)
	0.69(1.25)	0.73(1.14)	0.81(1.09)	0.76(1.12)	0.54(1.29)
DIJ10533	0.63(1.15)	0.35(0.55)	0.52(0.70)	0.65(0.96)	0.47(1.12)
			0.76(1.03)	0.72(1.06)	
WAK3772	0.74(1.35)	0.35(0.55)	0.66(0.89)	0.77(1.13)	0.06(0.14)
WAK3738	0.76(1.38)	0.72(1.13)	0.79(1.07)	0.81(1.19)	0.26(0.62)
NTN36749	0.06(0.11)	0.01(0.02)	0.11(0.15)	0.19(0.28)	0.11(0.26)
RBN1114	0.39(0.71)	0.01(0.02)	0.32(0.43)	0.57(0.84)	0.01(0.02)

System composition (volume ratio)
T.S.A: Ethyl acetate/Isopropanol/H_2O (65/23/12)
T.S.B: Dichloromethane/Acetonitrile (50/50)
T.S.C: Dichloromethane/Ethanol/Acetic acid/H_2O (65/25/3/3)
T.S.D: Ethyl acetate/Methanol/H_2O/Acetic acid (65/25/10/1)
T.S.E: Ethyl acetate/Acetonitrile/35% NH_4OH (80/17/3)

Values in parentheses mean relative Rf values for NTN33893

Table V. HPLC operating conditions

HPLC System	Operating conditions							
Gradient	**Elution conditions**							
H.S.A	RP-8	A: Acetonitrile B: H_2O						
	Time (m)	0-6	25	35	40	45	50	55	60
	A %	5	15	20	40	50	60	10	5
	B %	95	85	80	60	50	40	90	95
	Curve	-	4	6	6	6	1	1	1
	Flow rate	1 ml/min							
H.S.B	RP-Select B	A: Acetonitrile/H_2O (50/50), 0.005 M PIC B-8 B: H_2O, 0.005 M PIC B-8						
	Time (m)	0-5	20	30	40	45	50	
	A %	20	50	80	90	25	20	
	B %	80	50	20	10	75	80	
	Curve	-	6	6	3	1	1	
	Flow rate	1 ml/min						

Isocratic	Column	Mobile phase	Flow rate
H.S.C	RP-Select B	Acetonitrile/H_2O (25/75)	1 ml/min
H.S.D	RP-8	Acetonitrile/H_2O (20/80)	1 ml/min
H.S.E	RP-Select B	Acetonitrile/H_2O (26/74), 0.005M PIC B-8	1 ml/min

PIC B-8: Octanesulfonic acid (Low-UV, Waters)
Reagent for paired ion chromatography

Table VI. HPLC-Retention times of NTN33893 and its related reference compounds

Compound	Rt (min) in HPLC Gradient System	
	H.S.A	H.S.B
NTN33893	26.35(1.00)	26.05(1.00)
NTN38014		30.46(1.17)
WAK3839	20.32(0.77)	19.08(0.73)
WAK4103	21.26(0.81)	20.49(0.79)
NTN33519	22.46(0.85)	22.13(0.85)
NTN35884	21.45(0.81)	20.03(0.77)
CNA		15.09(0.58)
PAC		12.52(0.48)
PAD		19.37(0.74)
DIJ9979	46.30(1.76)	3.01(0.12)
		28.27(1.09)
DIJ10533	29.52(1.12)	31.07(1.19)
WAK3772	19.48(0.74)	18.51(0.71)
WAK3738	28.33(1.08)	27.49(1.06)
NTN36749		26.01(1.00)
RBN1114	9.42(0.36)	6.48(0.25)

Column packing :
A: Lichrospher RP-8, 5 µm, 4 mm i.d. x 250 mm length
B: Lichrospher RP-Select B, 5 µm,
4 mm i.d. x 250 mm length

Solvent programing : see Table V.

Table VII. Raw weights (g) of rice plants treated with ^{14}C-NTN33893

Experimental Group	DAT[1]	Parts	Pot No. (1)	Pot No. (2)
Group 1 (0.32 kg a.i./ha)	65	Shoot	165.84 (45.14)[2]	169.91 (44.85)[2]
Group 2 (0.32 kg a.i./ha)	124	Straw[3]	57.28	44.96
		Grain	35.24	32.64
		Chaff	10.38	8.12
		Rhachis	1.91	1.69
Group 3 (1.26 kg a.i./ha)	124	Straw[3]	50.73	50.88
		Grain	30.58	30.95
		Chaff	9.21	9.27
		Rhachis	1.72	1.98

1) Days after treatment
2) Dry weights calculated on the basis of water contents
3) Straw was dried a week after sampling

Table VIII. Absorption percentages and residues of radioactivity in rice plants treated with ^{14}C-NTN33893

(All values were calculated by combustion analysis, see Appendix 6-14)

Experimental Group	DAT[1]	Parts	Absorption %[2]	Residue (mg/kg)[3]
Group 1 (0.32 kg a.i./ha)	65	Shoot	4.02	0.378 (1.411)[4]
Group 2 (0.32 kg a.i./ha)	124	Straw	4.29	1.314
		Grain	0.03	0.014
		Chaff	0.05	0.094
		Rhachis	<0.01	0.038
		(Total)	(4.37)	
Group 3 (1.26 kg a.i./ha)	124	Straw	6.86	8.530
		Grain	0.03	0.064
		Chaff	0.06	0.402
		Rhachis	<0.01	0.145
		(Total)	(6.95)	

1) Days after treatment
2) % of dose applied
3) NTN33893 equivalent
4) Residue level calculated on the basis of dry weight

Table IX. Extractability of radioactivity from rice plants treated with ^{14}C-NTN33893

Fractions	% of radioactivity recovered*				
	Group-1 (65-Day)	Group-2 (124-Day)		Group-3 (124-Day)	
	Shoot	Straw	Grain	Straw	Grain
Extractable					
n-Hexane	1.3	1.3	0.6	2.2	1.9
Dichloromethane	20.7	15.4	16.3	27.1	15.1
Aqueous	42.2	43.6	14.3	38.1	10.7
(Subtotal)	(64.2)	(60.3)	(31.2)	(67.4)	(27.7)
Unextractable	35.9	39.8	68.9	32.6	72.4
Actual recovery (%) after fractionation	102	94	98	92	96

* Mean value in duplicate analysis, except straw from group 3 (single analysis)

Table X. Metabolites found in extractable fractions of shoot and straw

Fractions and Metabolites	% of radioactivity recovered	
	Shoot (Group 1, 65-Day)	Straw (Group 2, 124-Day)
n-Hexane	1.3[1]	1.3[1]
Dichloromethane		
NTN33893	7.8(37.7)[2]	8.1(51.7)
NTN38014	9.5(45.9)	3.8(24.8)
WAK3839	1.1 (5.4)	0.9 (5.8)
WAK4103	0.6 (2.7)	0.4 (2.6)
NTN33519	0.4 (1.8)	0.4 (2.6)
NTN35884	0.2 (1.1)	0.1 (0.8)
M1	0.1 (0.6)	0.2 (1.6)
M3	0.2 (1.2)	0.2 (1.5)
others	0.8 (3.6)	1.3 (8.6)[3]
Aqueous		
XAD-4/MeOH		
NTN38014	33.0(81.0)	32.4(76.7)
CNA	1.9 (4.6)	0.5 (1.1)
WAK4103	0.5 (1.3)	0.5 (1.1)
M6	1.2 (2.9)	2.6 (6.2)
M7	2.4 (5.8)	2.7 (6.4)
others	1.8 (4.4)	3.6 (8.5)
XAD-4/H_2O[4]	1.4	1.3
Total	64.2	60.3

1) Not examined
2) Values in parentheses mean percent of metabolites in each fraction
3) Including M2, M4 and M5
4) Sum of unretained component and water washing from XAD-4

Table XI. Metabolites released after three successive extractions of unextractalbe fraction from shoot and straw

Extraction Methods	Metabolites or component	% of total ^{14}C recovered	
		Shoot (65-Day)	Straw (124-Day)
MeOH Reflux	NTN33893	1.3	0.6
	NTN33519	1.2	0.2
	NTN38014	1.0	1.0
	others	2.2	1.3
	subtotal	5.7 (15.9)[1]	3.1 (7.8)
Dioxane/H_2O, Dioxane/2MHCl (9/1)	crude lignin[2]	1.7	3.0
	NTN38014	1.3	1.4
	others	0.4	0.7
	subtotal	3.4 (9.5)	5.1 (12.8)
2M NaOH/MeOH (2/3)	NTN38014	8.1	6.9
	NTN33519	9.5	11.5
	CNA	1.8	5.1
	others	3.3	1.9
	subtotal	22.7 (63.2)	25.4 (63.8)
Unextractable solid (cellulose)		4.1 (11.4)	6.2 (15.6)
Total		35.9(100.0)	39.8(100.0)

1) Percent of ^{14}C in total unextractable fraction
2) Lignin was precipitated by neutralization of dioxane extract

Table XII. Relative amounts of extractable and unextractable metabolites from straw (Group 3) released with alkaline MeOH (A, B, C, D)[1]

Fraction	Metabolites	% of total ^{14}C recovered			
Extractable[2]					
	NTN33893	17.6			
	NTN38014	33.5			
	NTN33519	1.0			
	CNA	0.8			
	WAK3839, WAK4103, NTN35884	3.7 (sum)			
	others[3]	10.8			
	(subtotal)	(67.4)			
Unextractable					
Soluble Part		A	B	C	D
	NTN33893	0.8	1.5	1.8	0.9
	NTN38014	3.6	3.9	5.2	6.7
	NTN33519	0.8	1.0	2.5	8.7
	CNA	1.2	4.4	7.6	6.4
	others[3]	2.8	2.1	2.7	4.9
	(Subtotal)	(9.2)	(12.9)	(19.8)	(27.6)
Insoluble Part		23.5	19.7	12.8	4.9

1) Alkali/MeOH (2/3) mixture solvents
A: 1M CH_3COONa, B: 1M K_2HPO_4, C: 1M Na_2CO_3, D: 2M NaOH
2) Total amounts of n-hexane, dichloromethane and aqueous phases
3) At least 9 components were included.

Table XIII. Characterization of total terminal residues found in rice grain collected 124 days posttreatment

Fractions and Metabolites	% of total ^{14}C recovered	
	Group 2 (0.32 kg a.i./ha)	Group 3 (1.26 kg a.i./ha)
Extractable	[31.1]	[27.7]
n-Hexane	0.6	1.9
Dichloromethane		
NTN33893	12.5 (77)[1]	11.5 (77)
WAK4103	1.5 (9)	1.4 (9)
NTN35884	1.6 (10)	1.5 (10)
WAK3839	0.2 (1)	0.2 (1)
NTN38014	0.2 (1)	0.2 (1)
others	0.3 (2)	0.3 (2)
Aqueous		
XAD-4/MeOH		
NTN33893	1.1 (10)	0.4 (5)
WAK4103	2.2 (20)	2.0 (24)
NTN35884	0.7 (6)	0.5 (6)
CNA	2.6 (24)[2]	0.8 (9)
M6+M7		1.8 (20)
NTN38014	2.0 (18)	1.0 (12)
others	2.4 (22)	2.0 (24)
XAD-4/H_2O[3]	3.3	2.2
Unextractable	[68.9]	[72.4]
MeOH reflux		3.8 (5)
Crude amino acid	N.E.[4]	0 (0)
Crude protein		20.4 (28)
Starch		48.2 (67)

1) Values in parentheses mean percent of metabolites in each fraction
2) Including M6 and M7
3) Sum of unretained component and water washing from XAD-4
4) Not examined

Table XIV. Characterization of crude protein fraction of rice grain from experimental group 3

Subfraction	Recovery[1] of ^{14}C	Specific Activity (dpm/g ± S.D.)
0.03M NaOH extractable component (crude protein)	20.4	
Supernatant after adjusting pH 6	4.3[2]	
Precipitate (crude glutelin) after adjusting pH 6	16.1[2]	18126 ± 1262
Re-precipitated glutelin	8.4[3]	18118 ± 599

1) Percent of ^{14}C in rice grain
2) 0.03M NaOH extract after adjusting pH 6 was centrifuged 10 min at 3000 rpm to obtain crude glutelin.
3) Crude glutelin was dissolved again in 0.03M NaOH and re-precipitated by adjusting pH 6.

Table XV. Characterization of crude starch fraction of rice grain from experimental group 3 by acid glycolysis and subsequent acetylation of glucose

Subfraction	^{14}C (dpm)	Recovery of ^{14}C (%)	% of total ^{14}C in grain	Dry wt. (g)	Spec. Activity (dpm/g ± S.D.)
Crude starch before glycolysis	84605	(100)	48.2	5.015	16871 ± 180
Hydrolysate of starch by 0.5M HCl	87840	104			
Hydrolysate after washing with EtOAc[1)]	81400	96		5.253	
Acetylglucose[2)]					
- 1 st crystals	48566	57	27.5	6.571	7391 ± 176
- 2 nd crystals	42580	50	24.1	5.790	7354 ± 163
- 3 rd crystals	38077	45	21.7	5.020	7585 ± 239
					(mean:7443)
Theoretical ^{14}C percentage in starch[3)]		58.7			

1) Hydrolysate of starch was washed by water/EtOAc partitioning before acetylation
2) Acetylglucose was obtained by reaction with sodium acetate and acetic anhydride
3) Calculated on the basis of specific radioactivities of acetylglucose and grain, see Appendix 15.

Table XVI. Total amounts of NTN33893 and its metabolites found in shoot and straw

Compound	% of total ^{14}C in straw								
	Group 1 (shoot, 65-Day)			Group 2 (straw, 124-Day)			Group 3 (straw, 124-Day)		
	Conv.[1)]	Conv. + Exha.[2)]	mg/kg[3)]	Conv.[1)]	Conv. + Exha.[2)]	mg/kg[3)]	Conv.[1)]	Conv. + Exha.[2)]	mg/kg[3)]
NTN33893	7.8	9.1	0.034	8.1	8.7	0.114	17.6	18.5	1.577
NTN38014	42.5	52.9	0.200	36.2	45.5	0.598	33.5	40.2	3.426
NTN33519	0.4	11.1	0.042	0.4	12.1	0.159	1.0	9.7	0.827
CNA	1.9	3.7	0.014	0.5	5.6	0.074	0.8	7.2	0.614
WAK3839	1.1	1.1	0.004	0.9	0.9	0.012	1.8	1.8	0.153
WAK4103	1.1	1.1	0.004	0.9	0.9	0.012	1.4	1.4	0.119
NTN35884	0.2	0.2	<0.001	0.1	0.1	0.001	0.5	0.5	0.043
M-1	0.1	--[4)]	<0.001	0.2	--[4)]	0.003	0.2	--[4)]	0.019
M-2	<0.1	--	<0.001	0.2	--	0.003	0.1	--	0.006
M-3	0.2	--	<0.001	0.2	--	0.003	0.3	--	0.026
M-4	--	--	--	0.1	--	0.001	--	--	--
M-5	--	--	--	0.1	--	0.001	0.1	--	0.008
M-6	1.2	--	0.005	2.6	--	0.034	1.9	--	0.164
M-7	2.4	--	0.009	2.7	--	0.036	2.2	--	0.190
Total	58.9	79.2	0.312	53.2	73.8	1.051	61.4	79.3	7.172

1) Amounts found in extractable fraction by conventional extraction
2) Sum amounts of metabolite found in extracts by conventional and exhaustive extraction methods.
3) Residue levels (conv. + exha.) equivalent to NTN33893
4) Not analyzed

Table XVII. Total amounts of NTN33893, its metabolites and radioactive natural components characterized as terminal residues in grain (Group 2, 0.32 kg a.i./ha, 124-Day, Group 3, i.26 kg a.i./ha, 124-Day)

Compound or components	Group 2 (0.32 kg a.i./ha)		Group 3 (1.26 kg a.i./ha)	
	% of total ^{14}C in grain	Residue (mg/kg) [1]	% of total ^{14}C in grain	Residue (mg/kg) [1]
NTN33893	13.6	0.002	11.9	0.008
WAK4103	3.7	<0.001	3.4	0.002
NTN35884	2.3	0.001	2.0	0.001
NTN38014	2.2	<0.001	1.2	<0.001
CNA	2.6	<0.001	0.8	<0.001
WAK3839	0.2	<0.001	0.2	<0.001
^{14}C-Glutelin	--[2]	--[2]	16.1	0.010
^{14}C-Starch	--[2]	--[2]	48.2	0.031
Total	24.6	0.003	83.8	0.054

1) NTN33893 equivalent
2) Not analysed

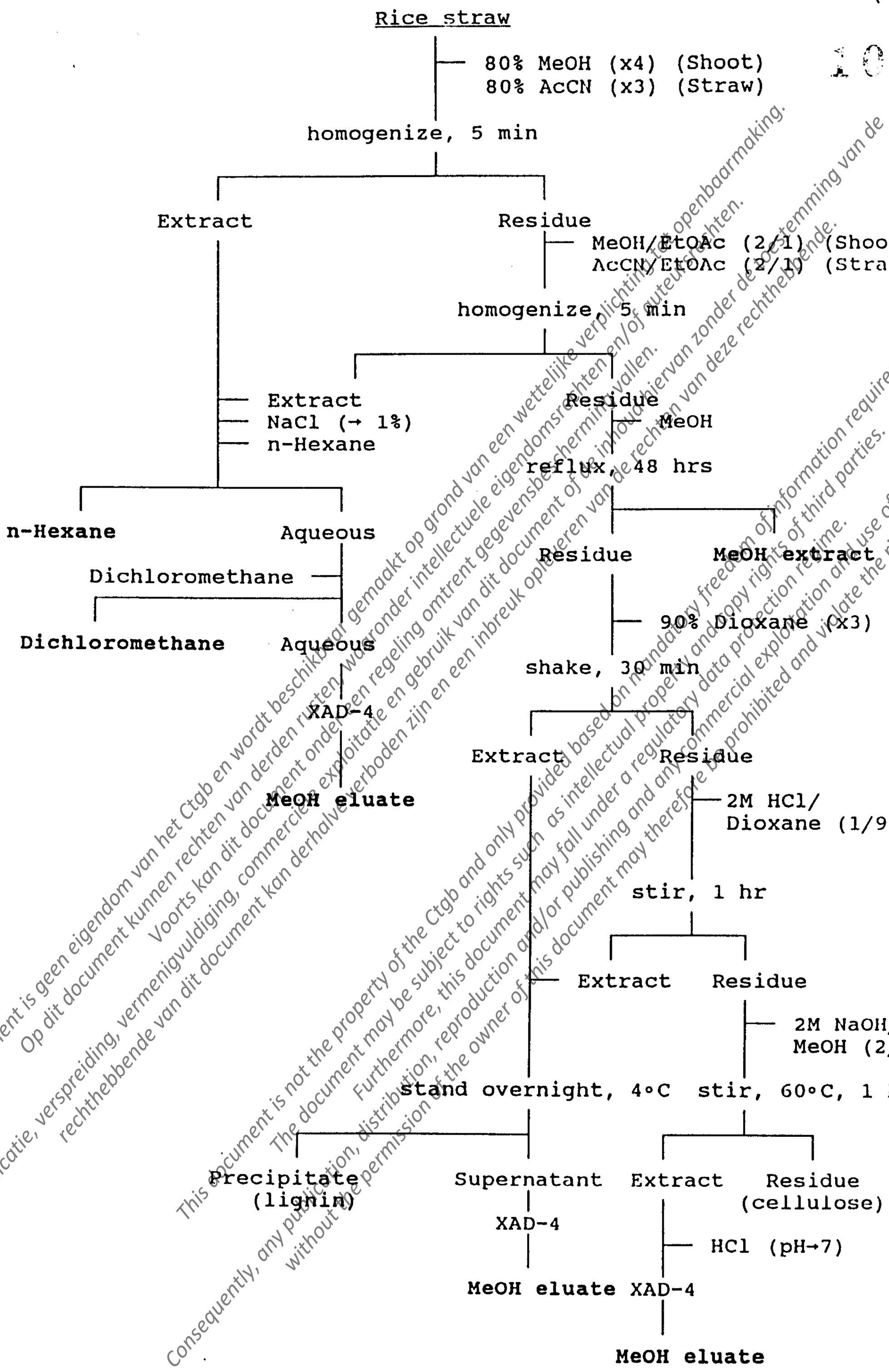


Fig. 1. Extraction scheme for ^{14}C-NTN33893 residues in shoot and straw

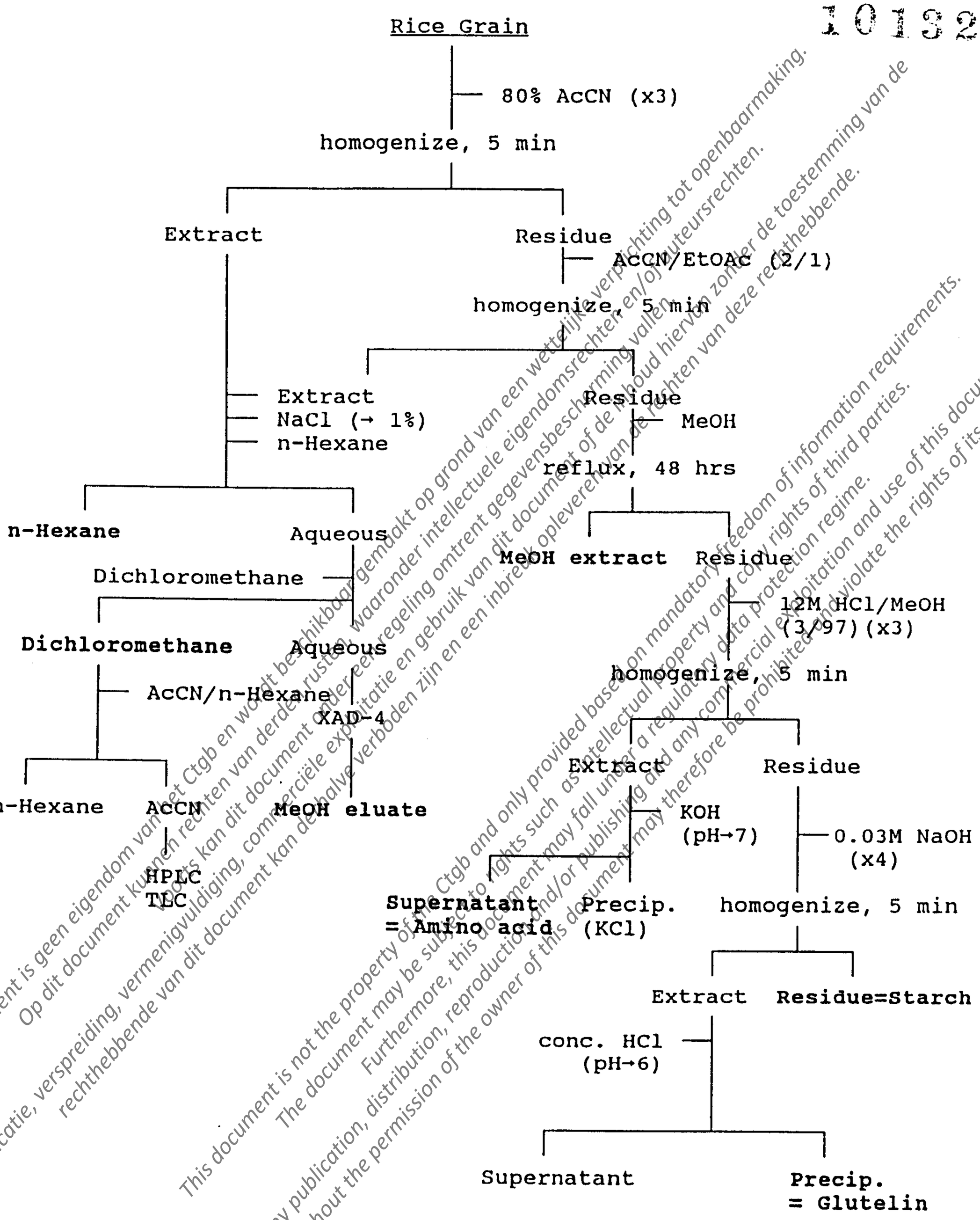


Fig. 2. Extraction scheme for ^{14}C-NTN33893 residues in rice grain

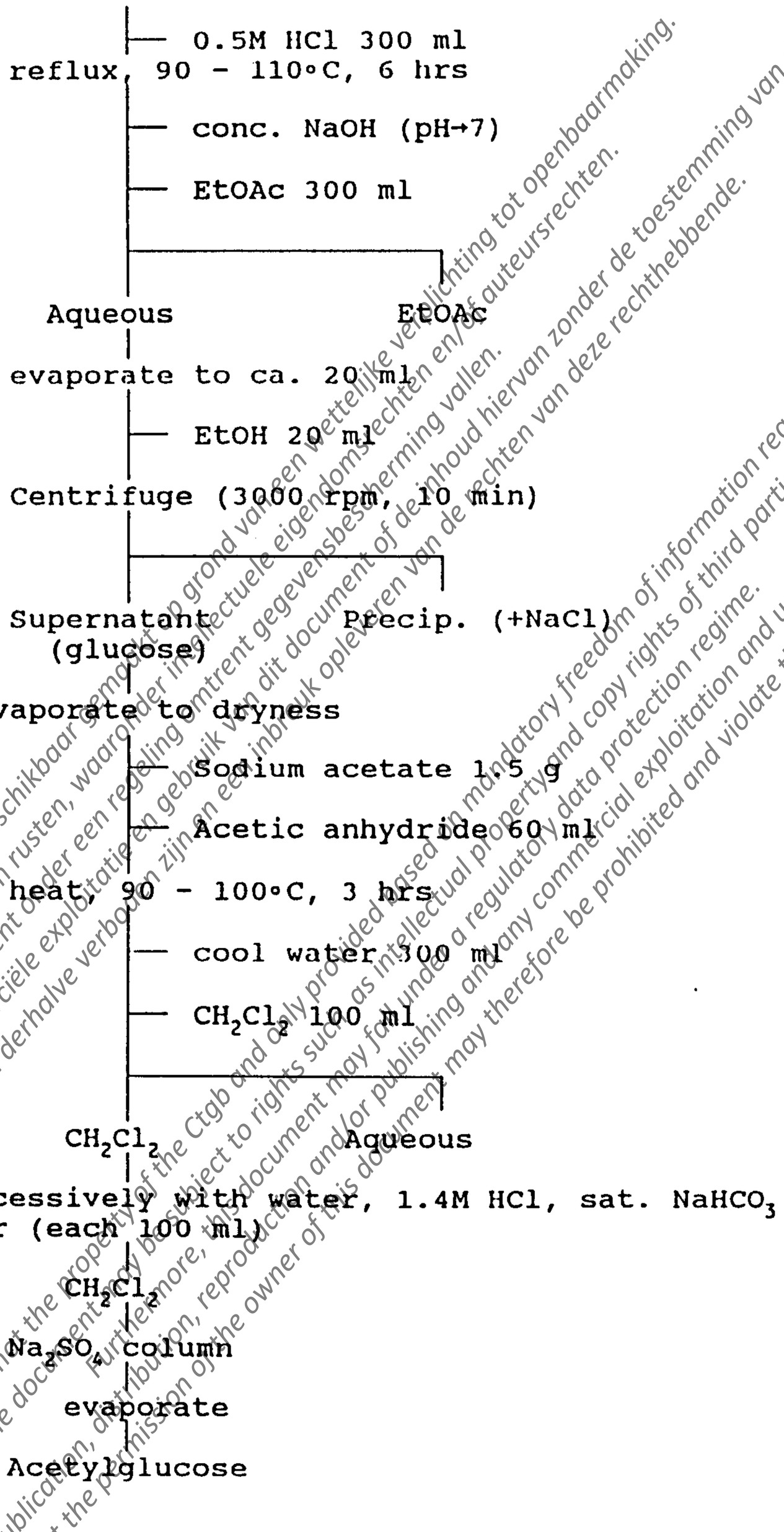
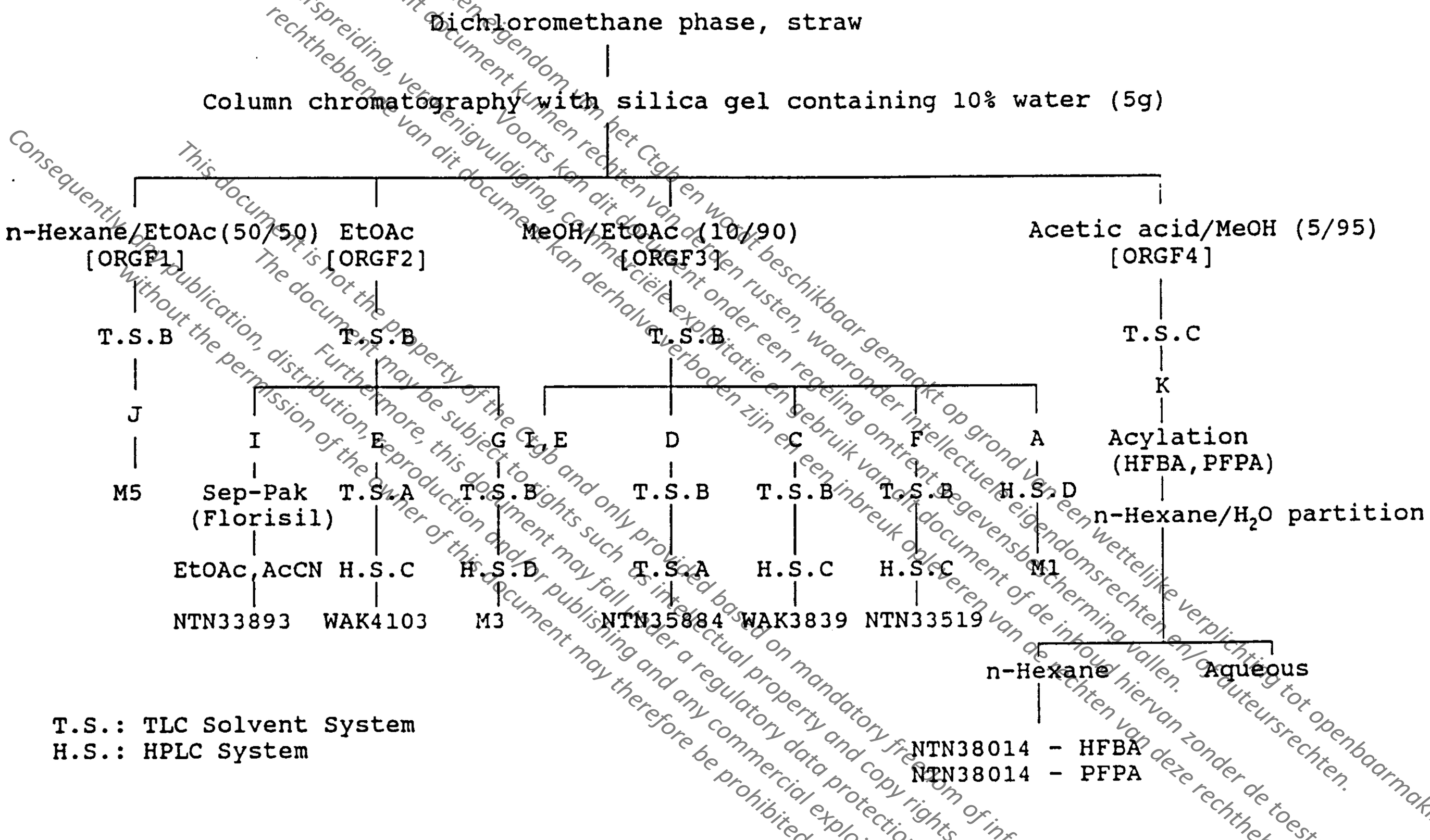


Fig. 3. Schematic diagram for glycolysis of starch and acetylation of glucose



T.S.: TLC Solvent System
H.S.: HPLC System

Figure 4. Schematic diagram for working up of dichloromethane phase from straw for metabolite isolation

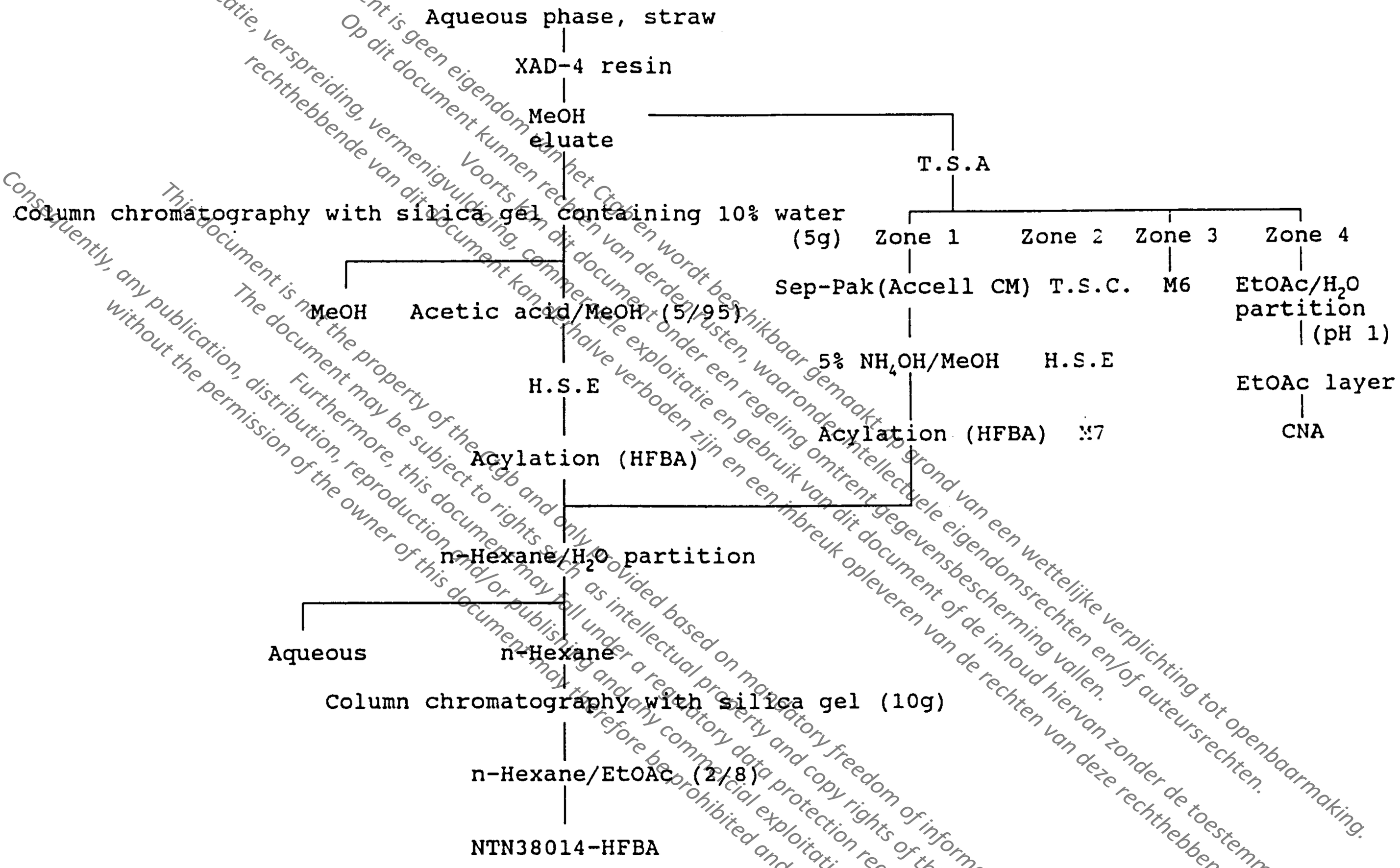
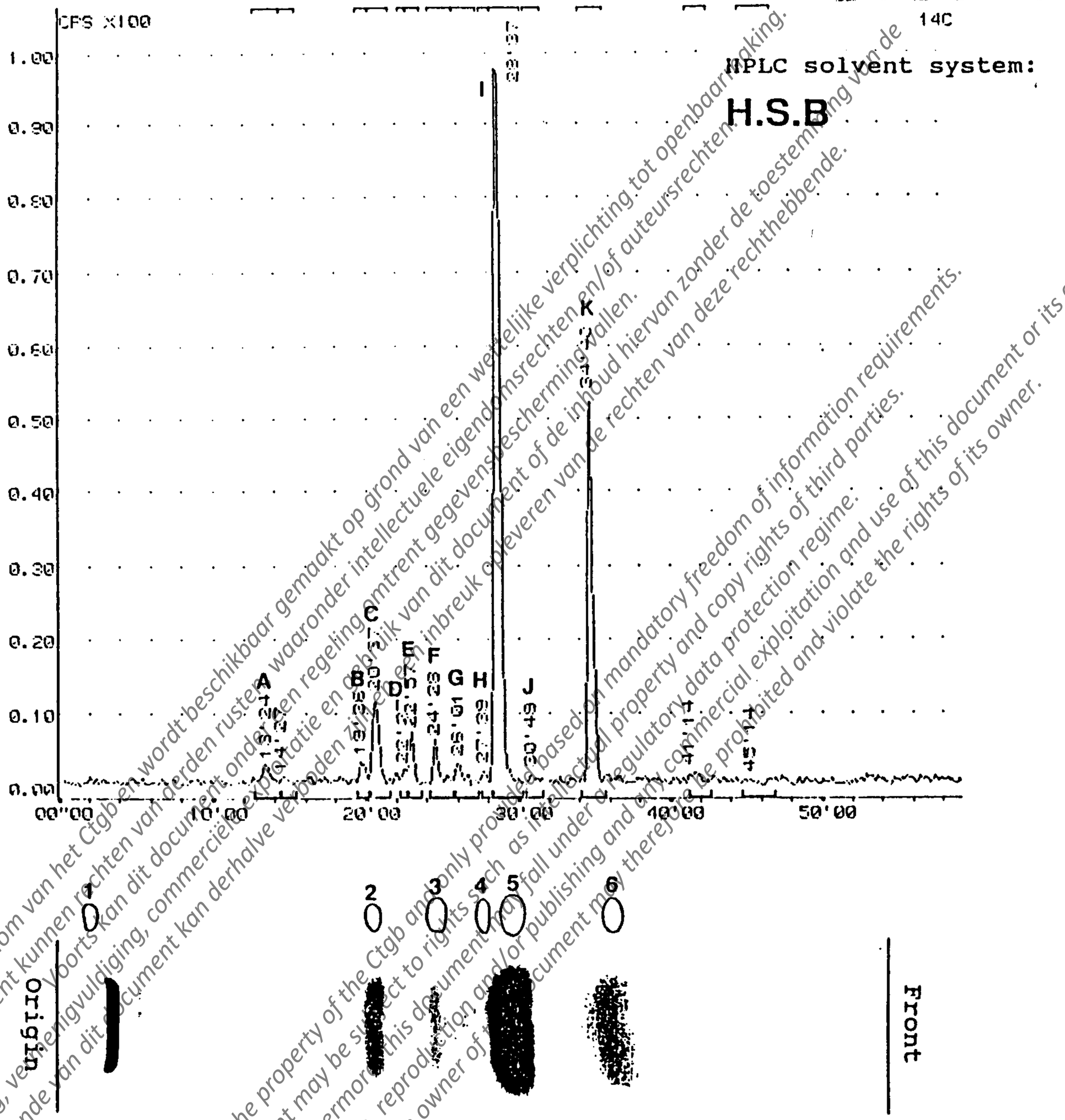


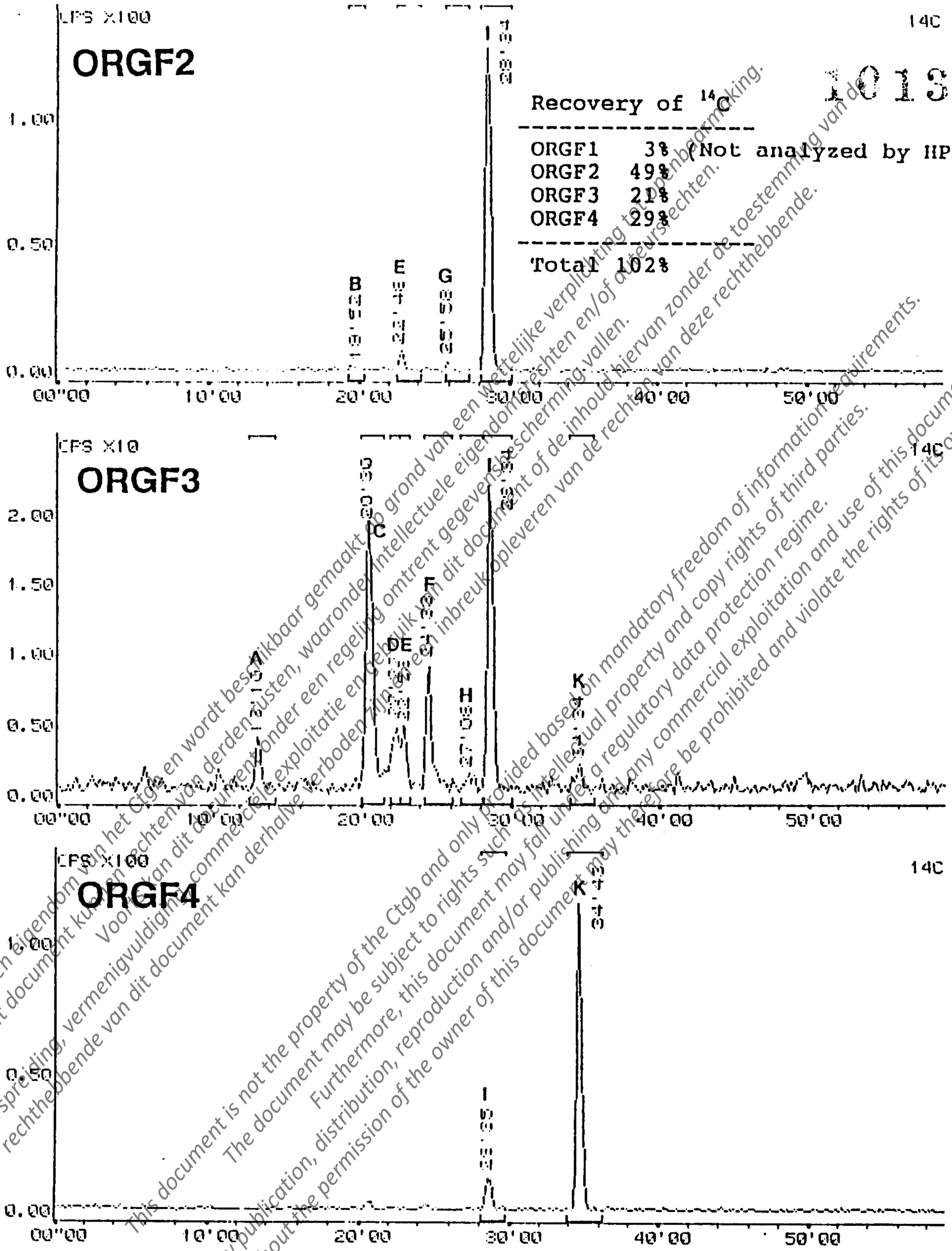
Figure 5. Schematic diagram for working up of aqueous phase from straw for metabolite isolation



TLC solvent system: Ethyl acetate/Isopropanol/H_2O(65/23/12)
(T.S.A)

HPLC solvent system:
H.S.B

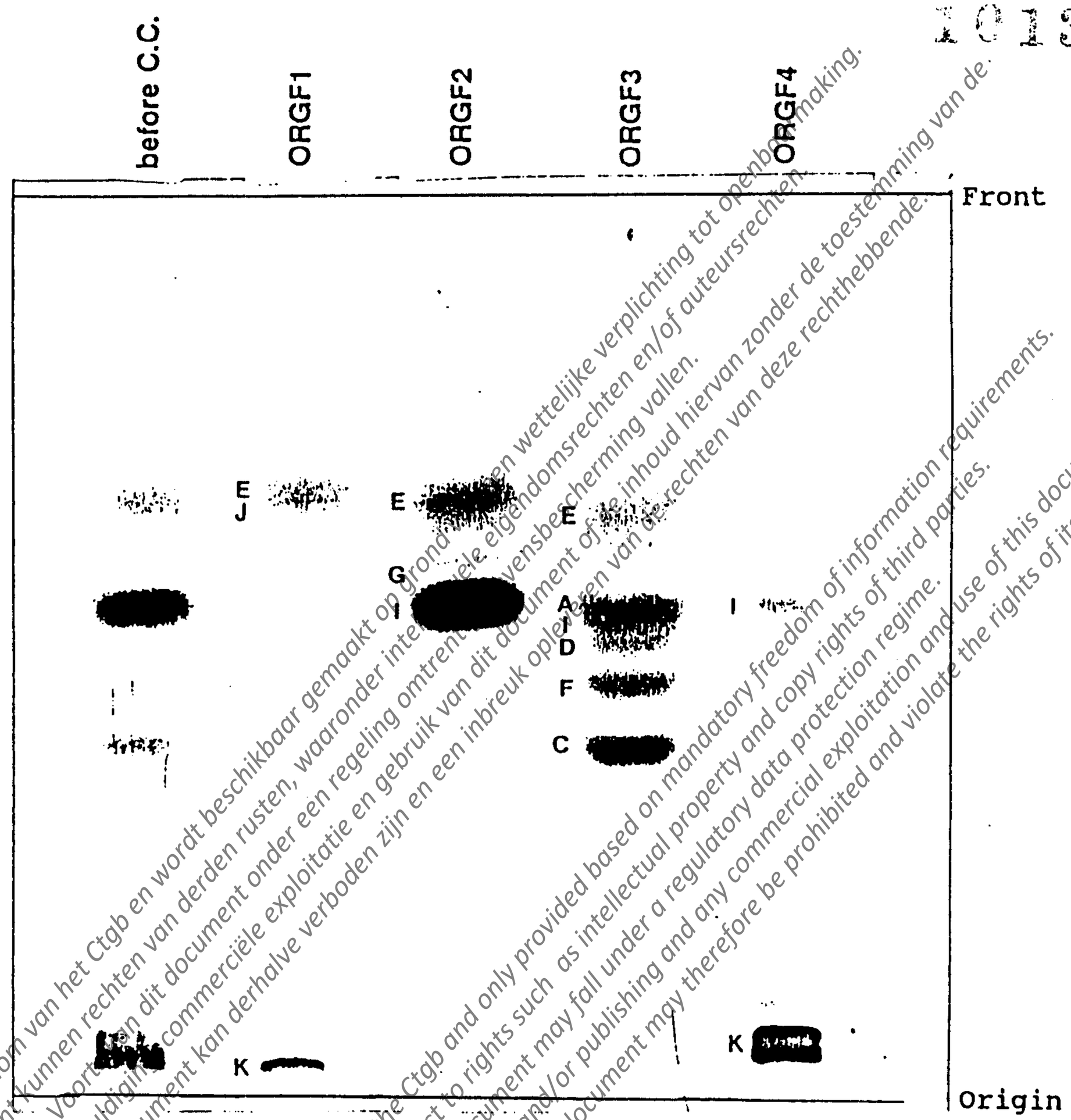
Fig.6. HPLC and TLC chromatograms showing separation of metabolites in dichloromethane phase from straw (Group 2)
Reference compounds: 1.NTN38014 (k) 4.NTN35884 (D)
2.WAK3839 (C) 5.NTN33893 (I)
3.NTN33519 (F) 6.WAK4103 (E)



Recovery of ^{14}C

ORGF1	3% (Not analyzed by HPLC)
ORGF2	49%
ORGF3	21%
ORGF4	29%
Total	102%

Fig.7. Comparative HPLC chromatograms (H.S.B) of ORGF2, ORGF3 and ORGF4 fractionated by column chromatography of dichloromethane phase from straw (Group 2)

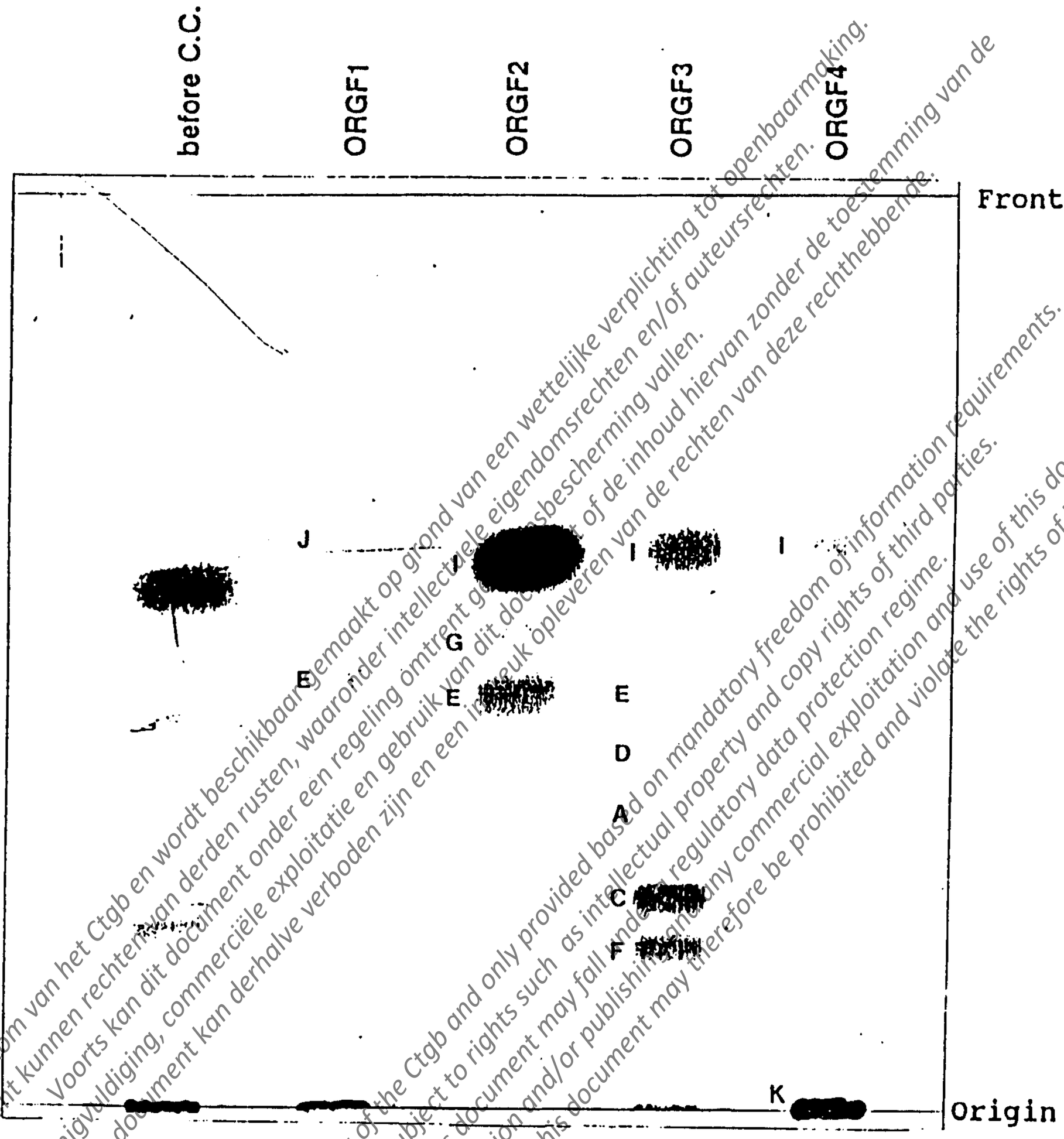


A = M1
C = WAK3839
D = NTN35884
E = WAK4103
F = NTN33519
G = M3
I = NTN33893
K = NTN38014

Fig.8. TLC chromatograms of dichloromethane phase from straw (group 2) before and after fractionation by column chromatography(C.C.)

A,D,E,F,G,I,K : HPLC peak No.(see Fig.6 and 7)

Solvent:Ethyl acetate/Isopropanol/H_2O(65/23/12)(T.S.A)



A = M1
C = WAK3839
D = NTN35884
E = WAK4103
F = NTN33519
G = M3
I = NTN33893
K = NTN38014

Fig.9. TLC chromatograms of dichloromethane phase from straw (Group 2) before and after fractionation by column chromatography(C.C.)

A,D,E,F,G,I,K : HPLC peak No.(see Fig.6 and 7)

Solvent:Dichloromethane/Acetonitrile(50/50)(T.S.B)

NR 1284 (ESR/ENG)

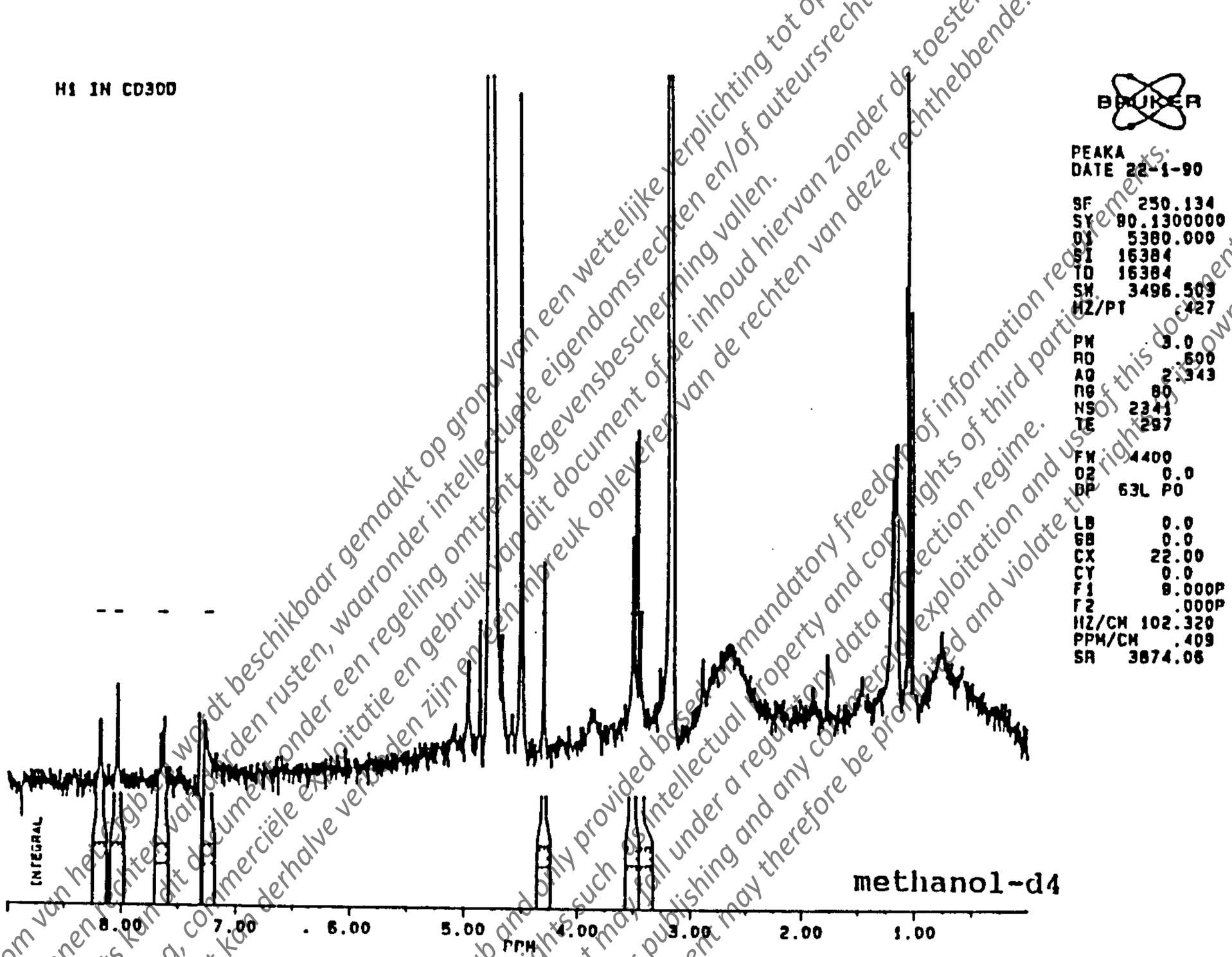
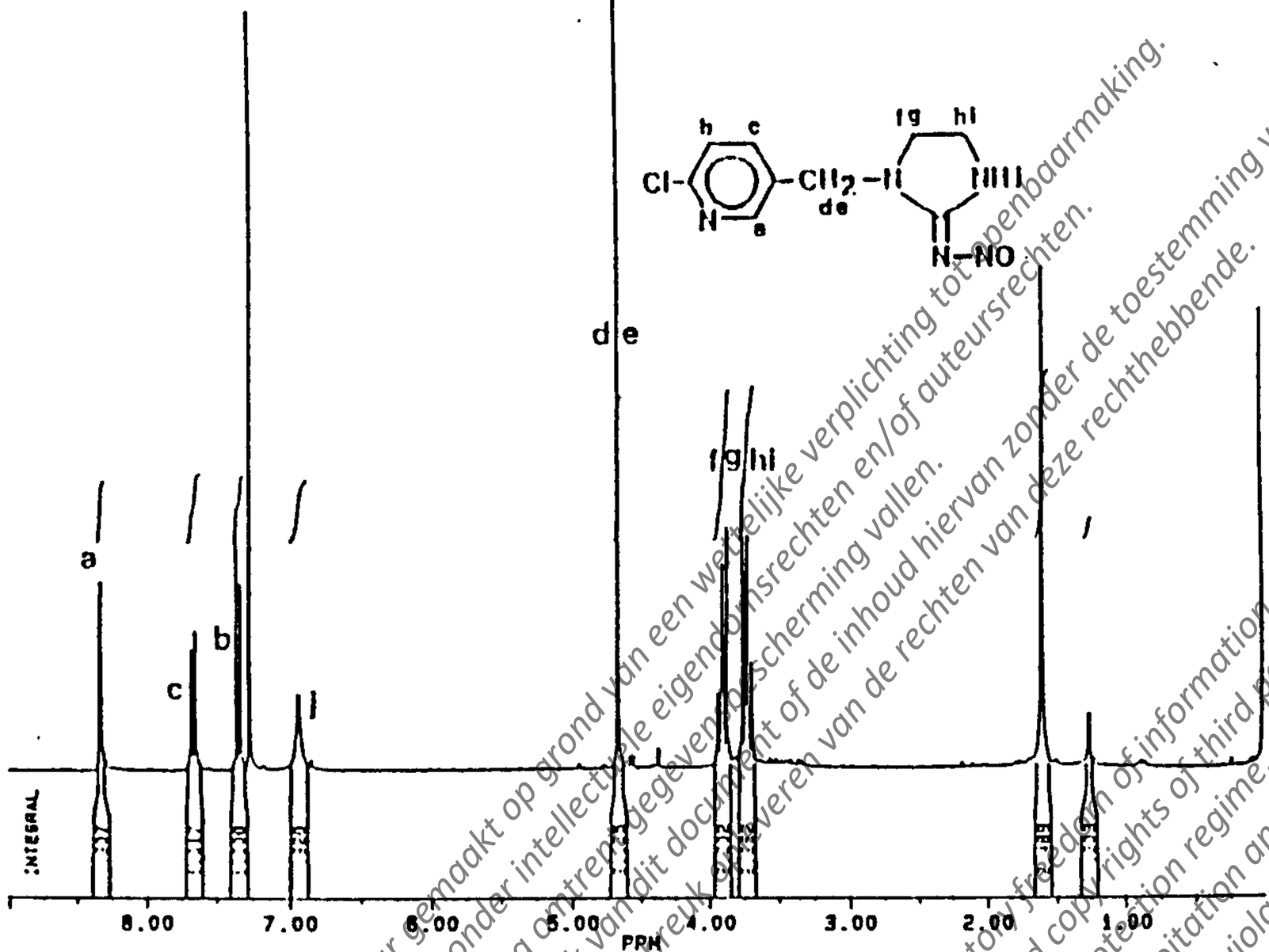


Fig.10. ^{1}H-NMR(250MHz) spectrum of M1 (HPLC Peak No. A)

WAK3839 IN CDCL3

(A)



BRUKER

IIGANTNF.HFI
DATE 2-3-88

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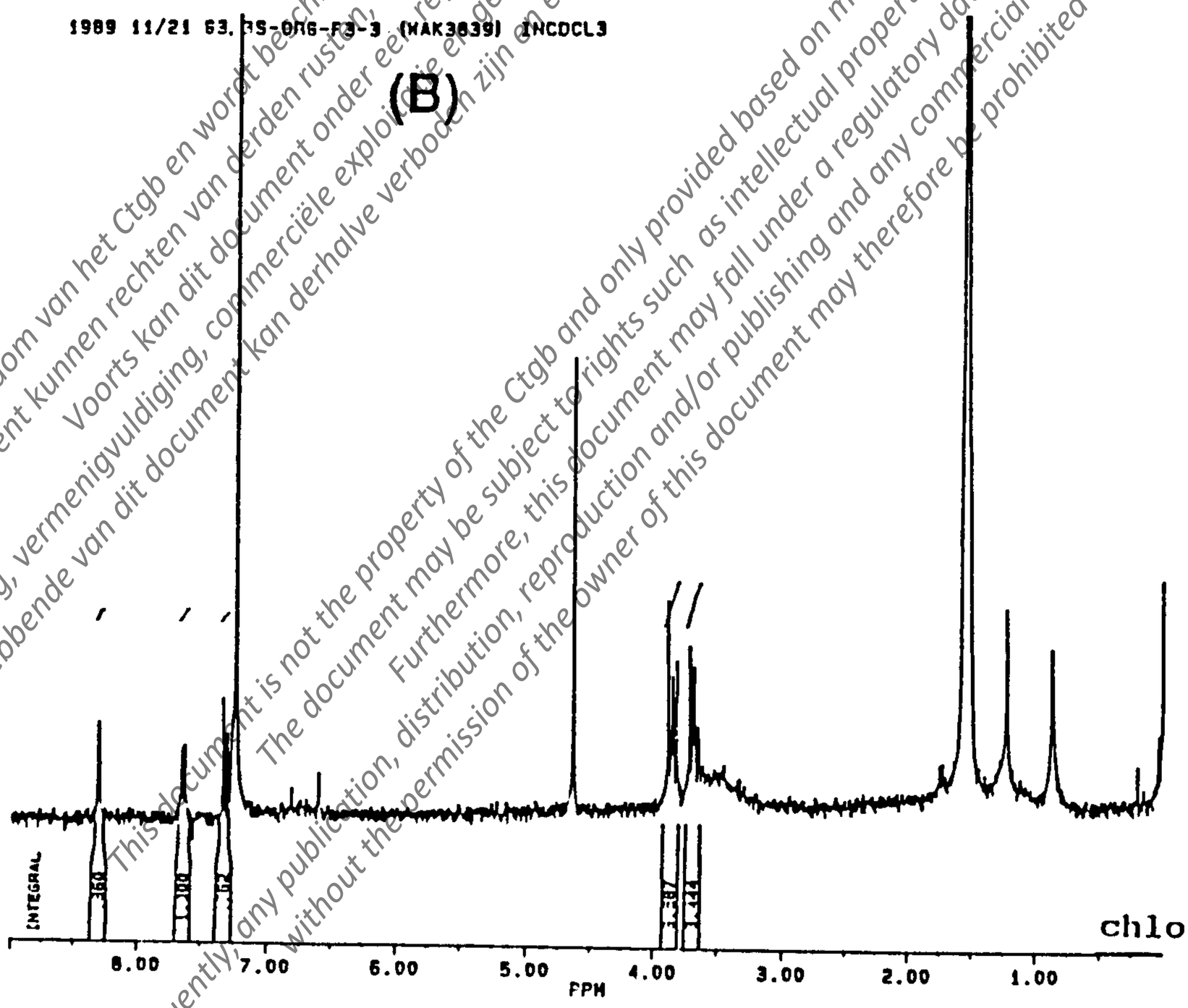
LB	.250
GB	0.0
CX	22.00
CY	0.0
F1	9.001P
F2	.002P
HZ/CM	102.320
PPM/CM	.409
SN	2853.56

1989 11/21 63.[illegible]S-ORG-F3-3 (WAK3839) INCDCL3

(B)



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KKWAK383.002
DATE 21-11-89

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CX	22.00
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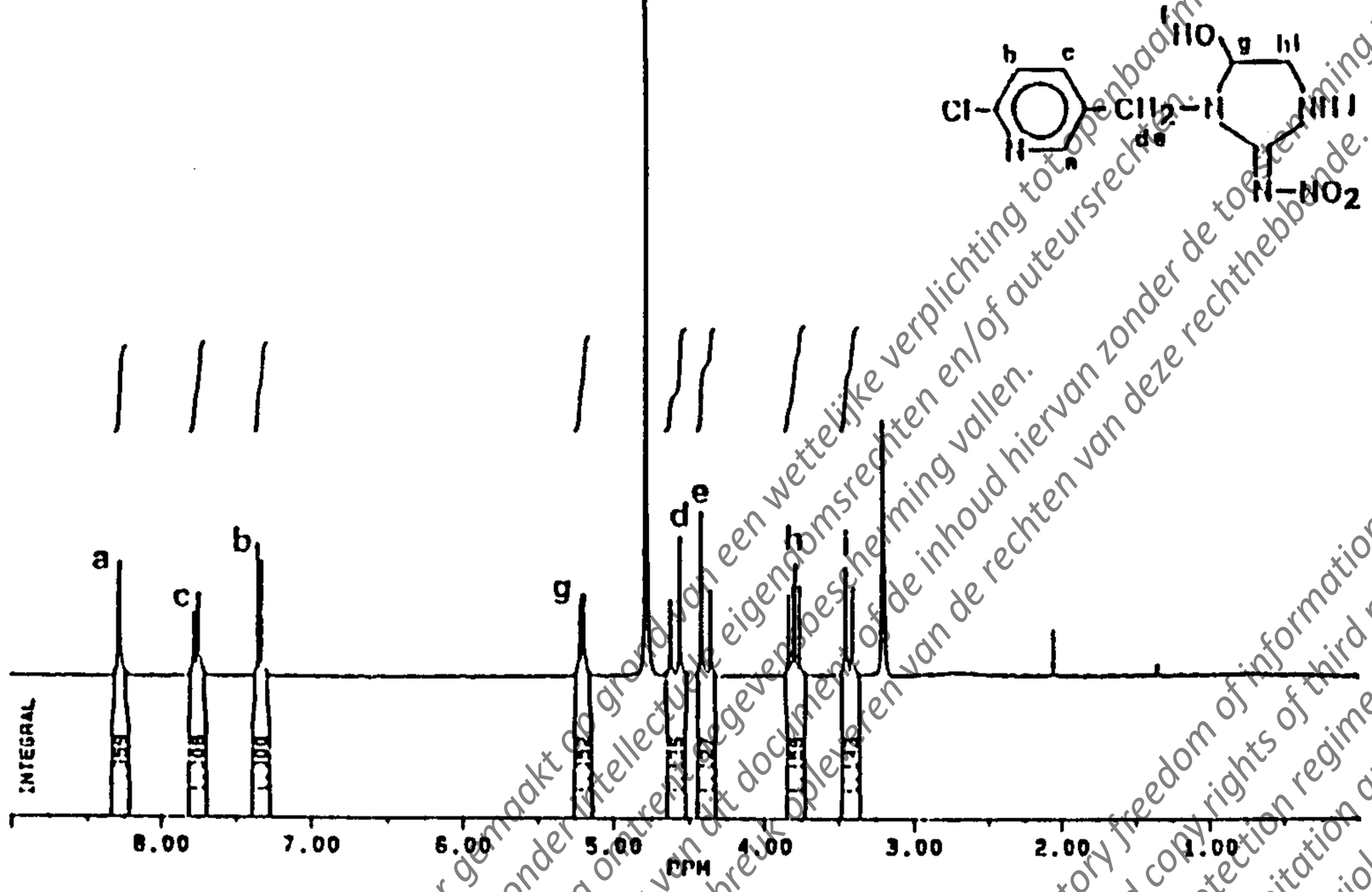
F2	.002P
HZ/CM	102.320
PPM/CM	.409
SR	2861.67

Fig.11. ^{1}H-NMR(250MHz) spectra of WAK3839
(A)Reference compound
(B)HPLC Peak No. C

WAK4103 IN CD3OD

(A)



WAK4103 (CD3OD)

(B)

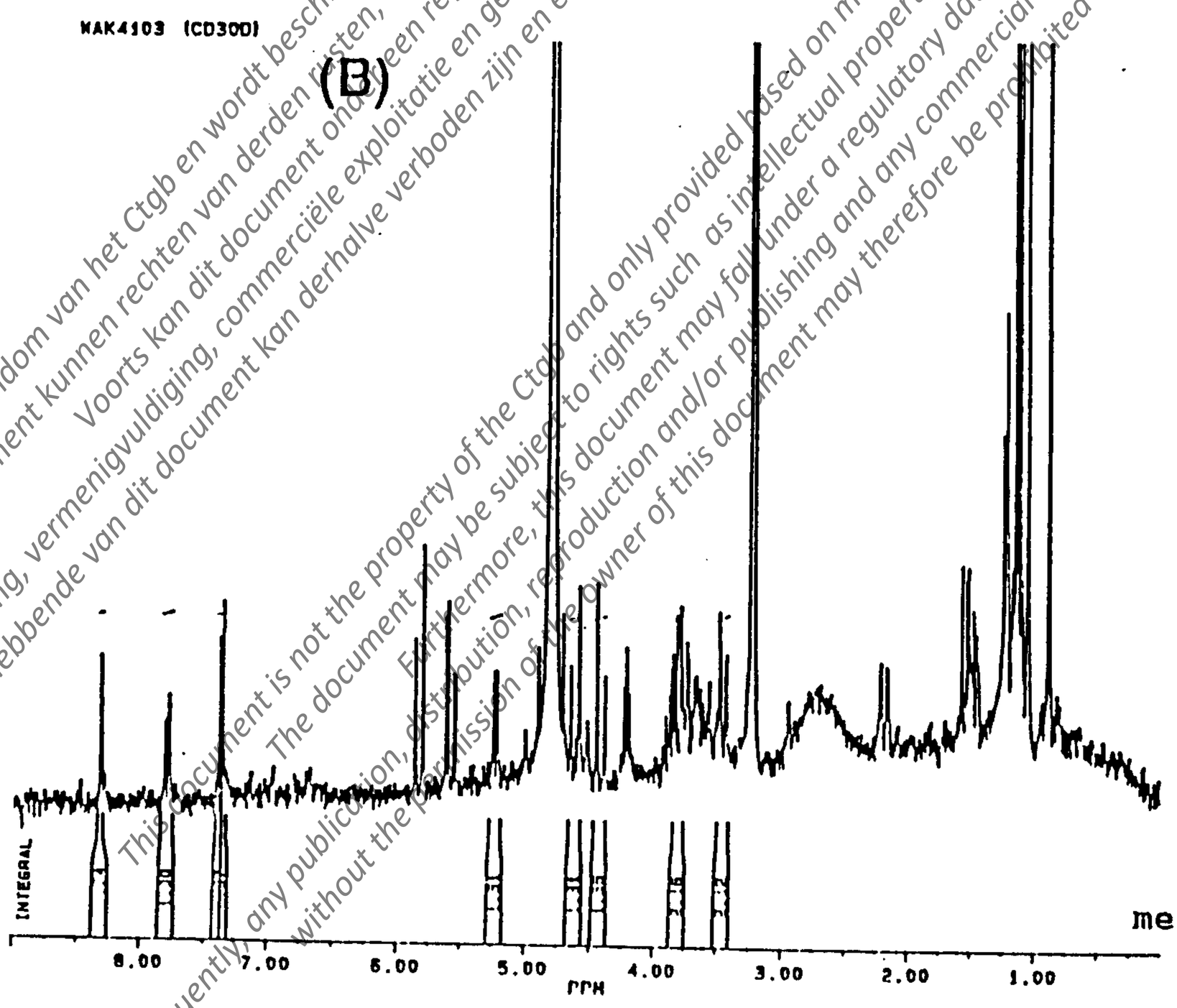
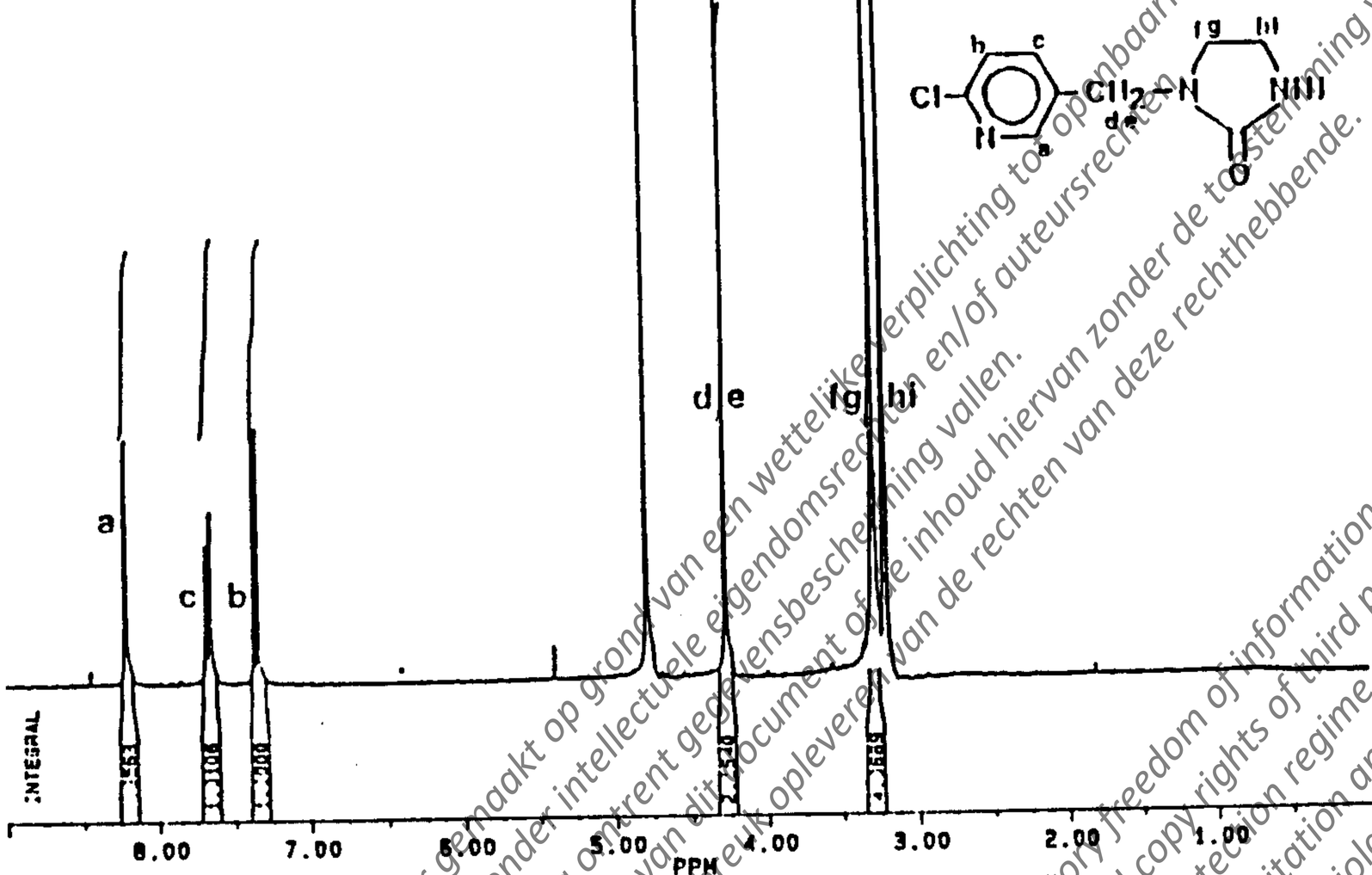


Fig.12. ^{1}H-NMR(250MHz) spectra of WAK4103
(A)Reference compound
(B)HPLC Peak No. E

NTN33519 IN CD300

(A)



KKB9053.005
DATE 2-3-88

SF 250.134
SY 90.1300000
O1 5380.000
SI 16384
TD 16384
SW 3496.503
HZ/PT .427

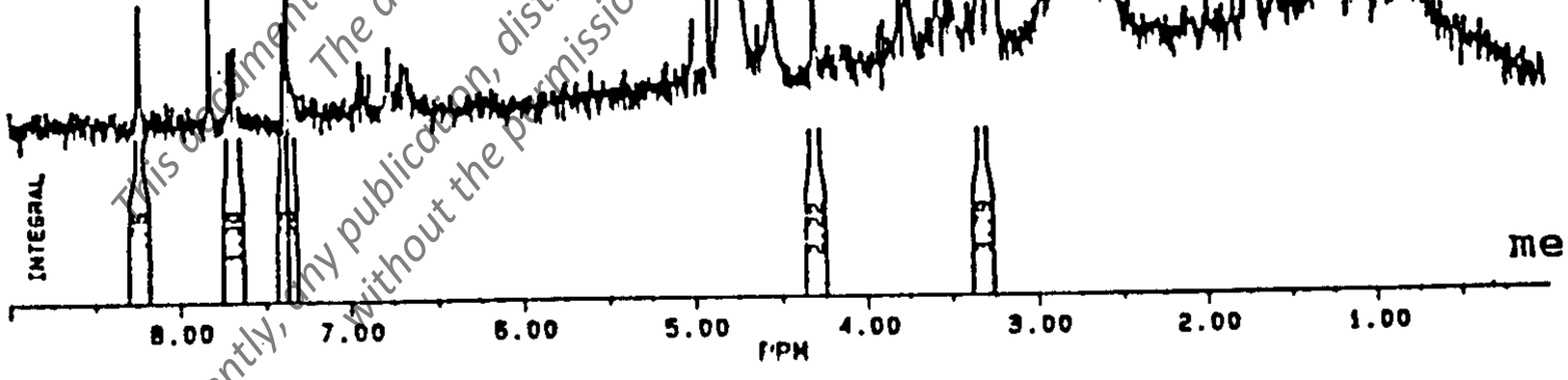
PW 3.0
RD .600
AQ 2.343
RG 80
NS 32
TE 297

FW 4400
O2 0.0
DP 63L PO

LB .250
GB 0.0
CX 22.00
CY 0.0
F1 9.000P
F2 .000P
HZ/CM 102.320
PPM/CM .409
SR 3865.10

NTN33519 (CD300)

(B)



PEAKF
DATE 22-11-89

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SY 90.1300000
O1 5380.000
SI 16384
TD 16384
SW 3496.503
HZ/PT .427

PW 3.0
RD .600
AQ 2.343
RG 80
NS 400
TE 297

FW 4400
O2 0.0
DP 63L PO

LB .250
GB 0.0
CX 22.00
CY 0.0
F1 9.000P
F2 .000P
HZ/CM 102.320
PPM/CM .409
SR 3858.27

methanol-d4

Fig.13. ^{1}H-NMR(250MHz) spectra of NTN33519
(A)Reference compound
(B)HPLC Peak No. F

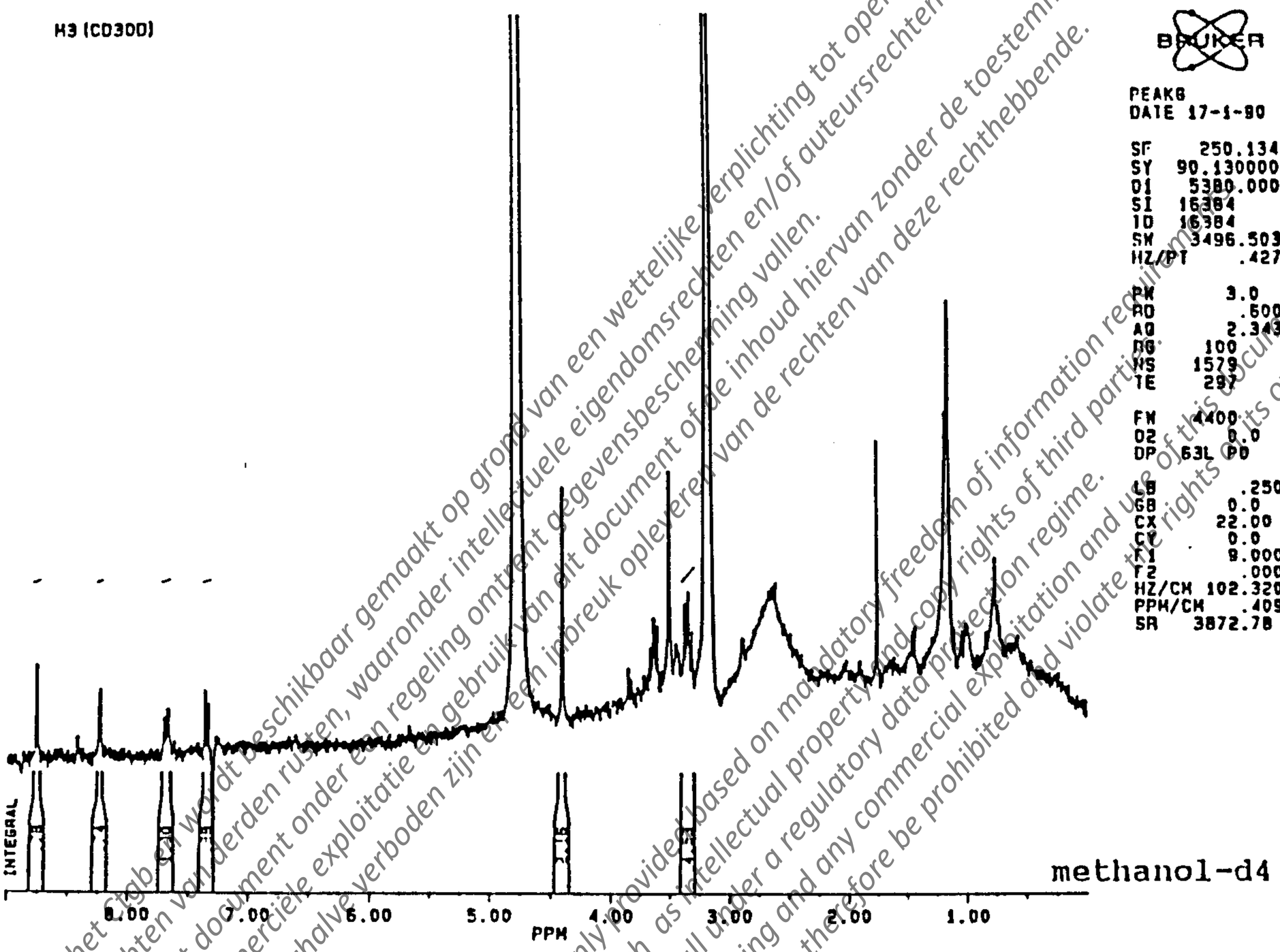
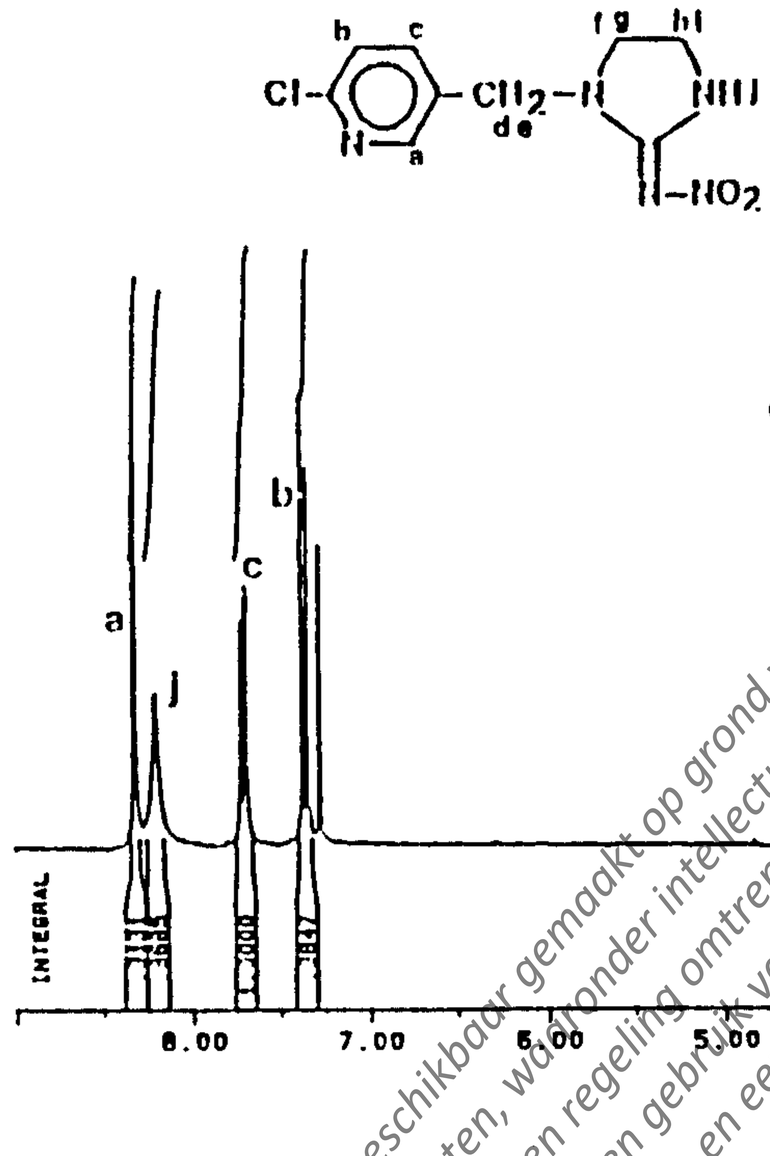


Fig.14. ^{1}H-NMR(250MHz) spectrum of M3 (HPLC Peak No. G)

NTN33893 IN CDCL3

(A)



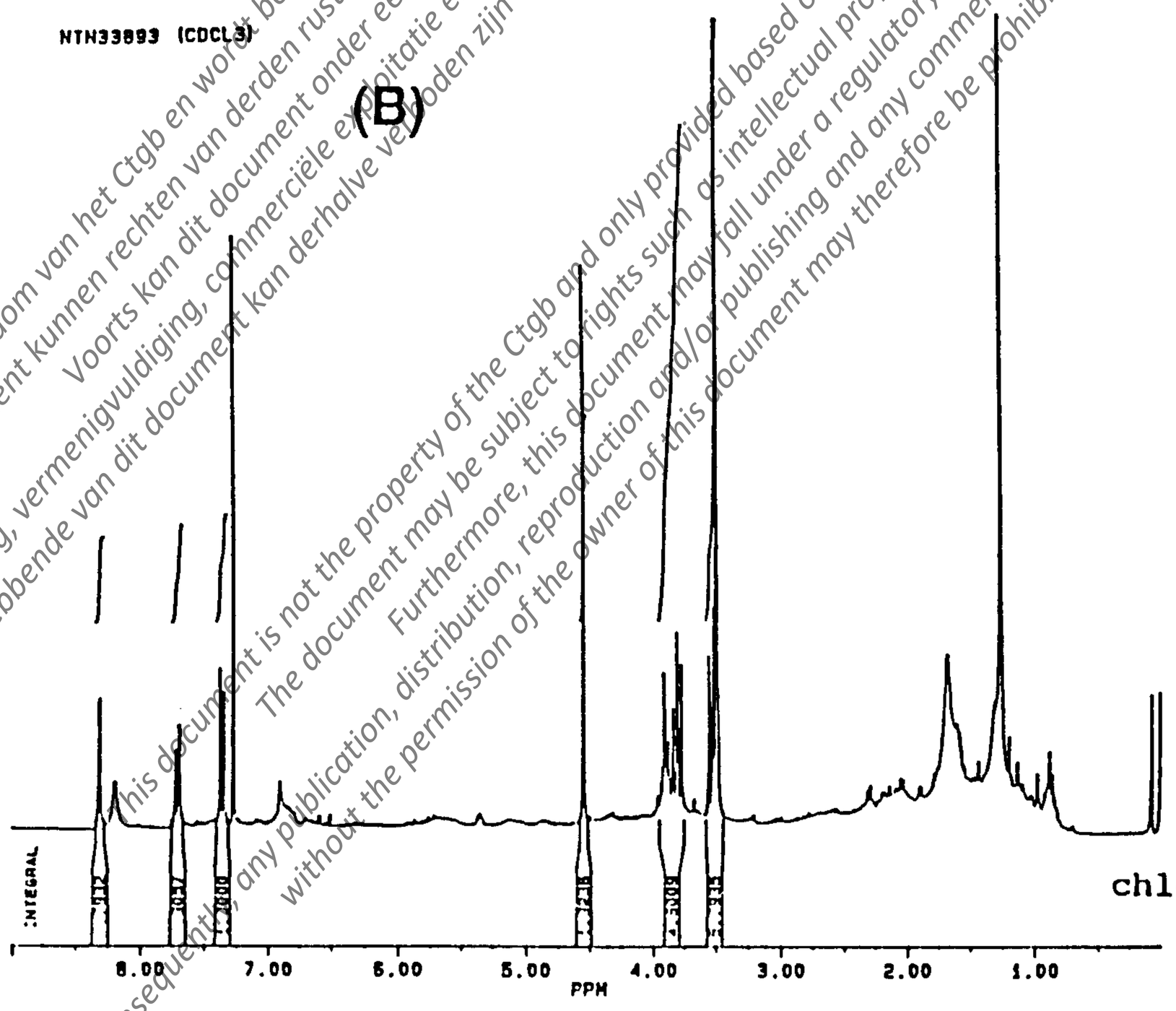
BRUKER

KK89053.001

SF	250.133
SY	90.1300000
OI	4000.000
SI	16384
TD	16384
SW	3496.503
HZ/PT	.427
PW	3.0
RD	.600
AQ	2.343
NG	40
NS	8
TE	297
FW	4400
O2	0.0
DP	63L PO
LB	.250
GB	0.0
CX	22.00
CY	0.0
F1	9.0010
F2	.002P
HZ/CM	102.320
PPM/CM	.408
SN	2851.86

NTN33893 (CDCL3)

(B)



BRUKER

PEAKI	
DATE	20-11-89
SF	250.133
SY	90.1300000
OI	4000.000
SI	16384
TD	16384
SW	3496.503
HZ/PT	.427
PW	3.0
RD	.600
AQ	2.343
NG	64
NS	1241
TE	297
FW	4400
O2	0.0
DP	63L PO
LB	.250
GB	0.0
CX	22.00
CY	0.0
F1	9.001P
F2	.002P
HZ/CM	102.320
PPM/CM	.409
SA	2853.14

chloroform-d1

Fig.15. ^{1}H-NMR(250MHz) spectra of NTN33893
(A)Reference compound
(B)HPLC Peak No. I

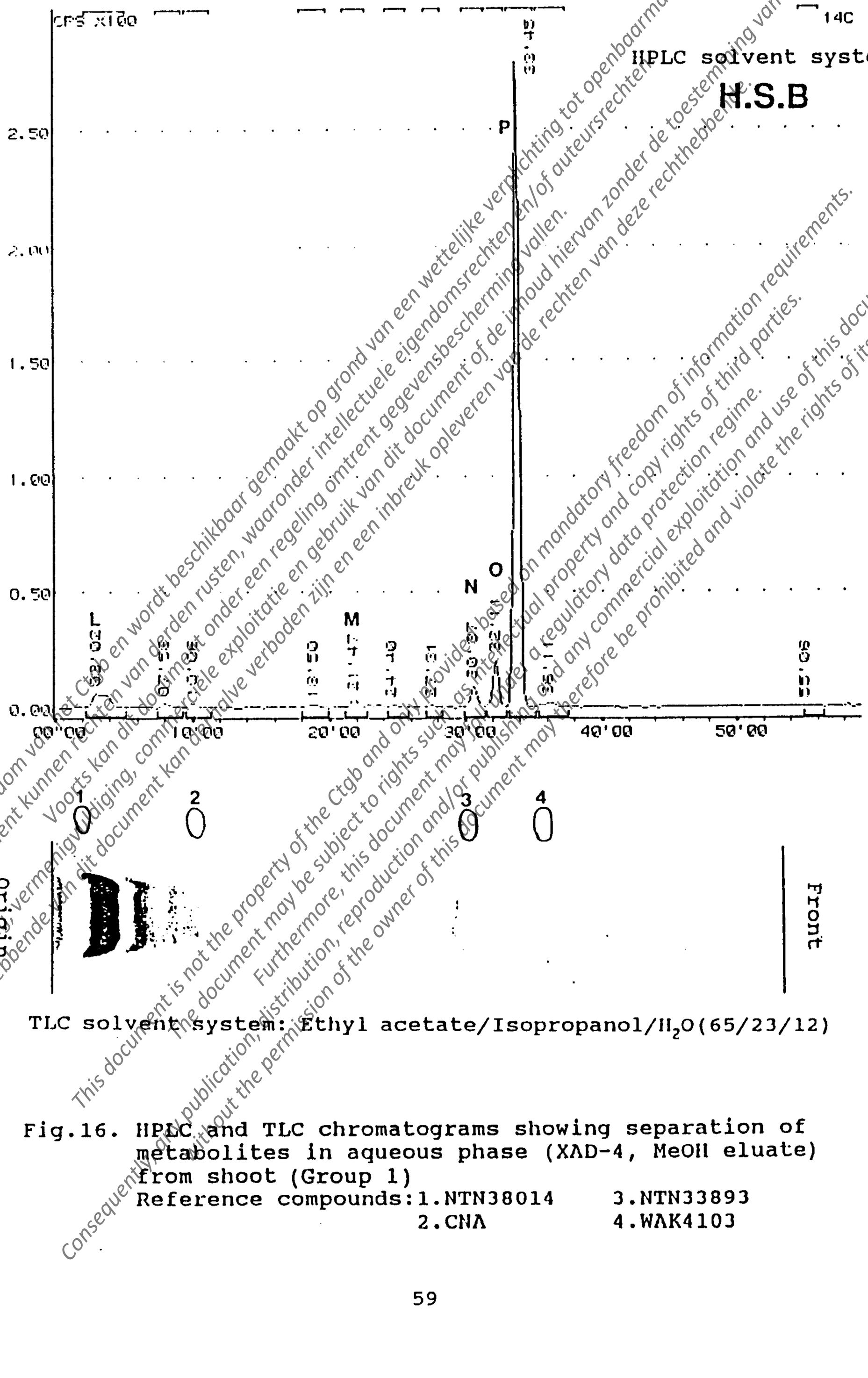
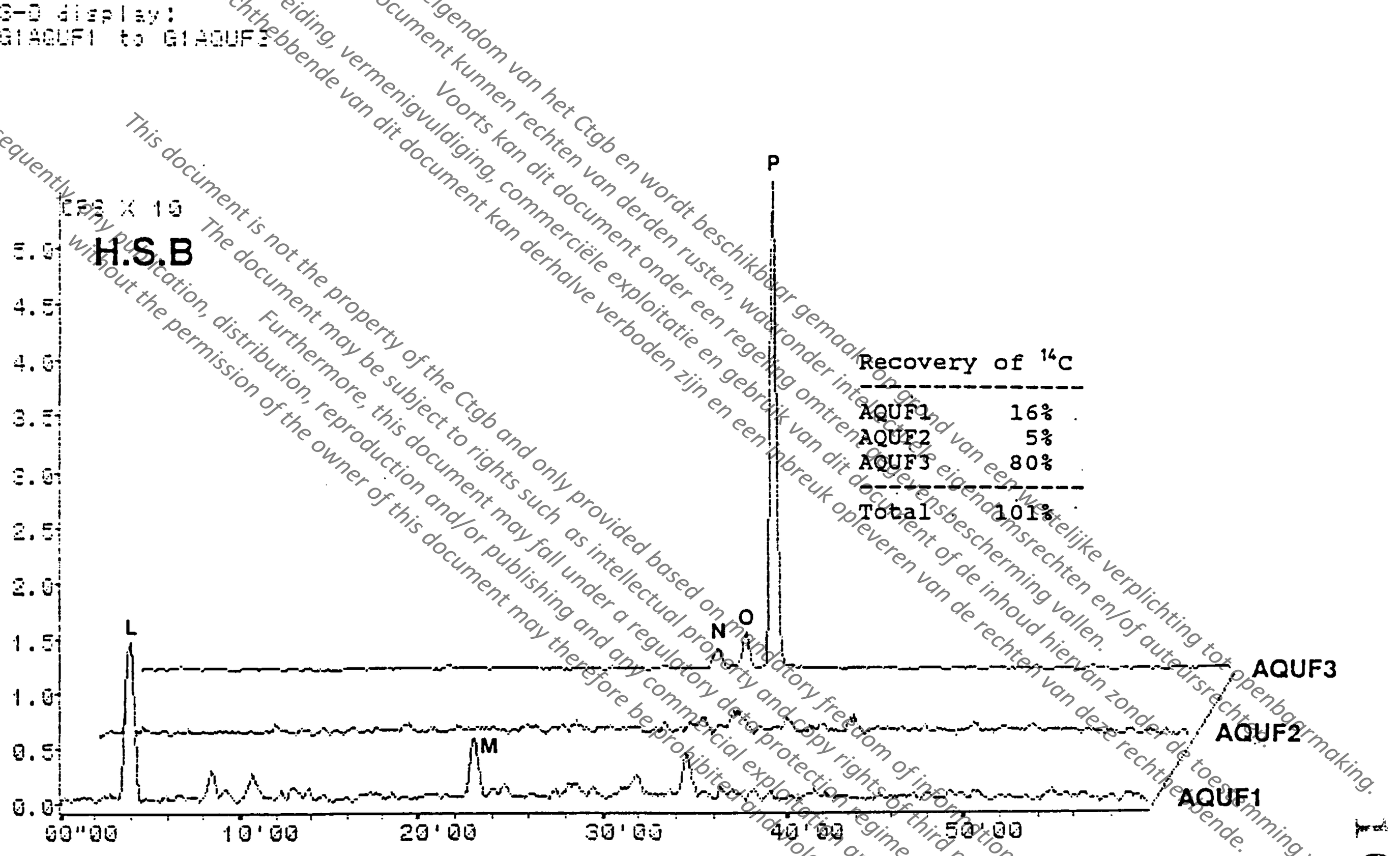
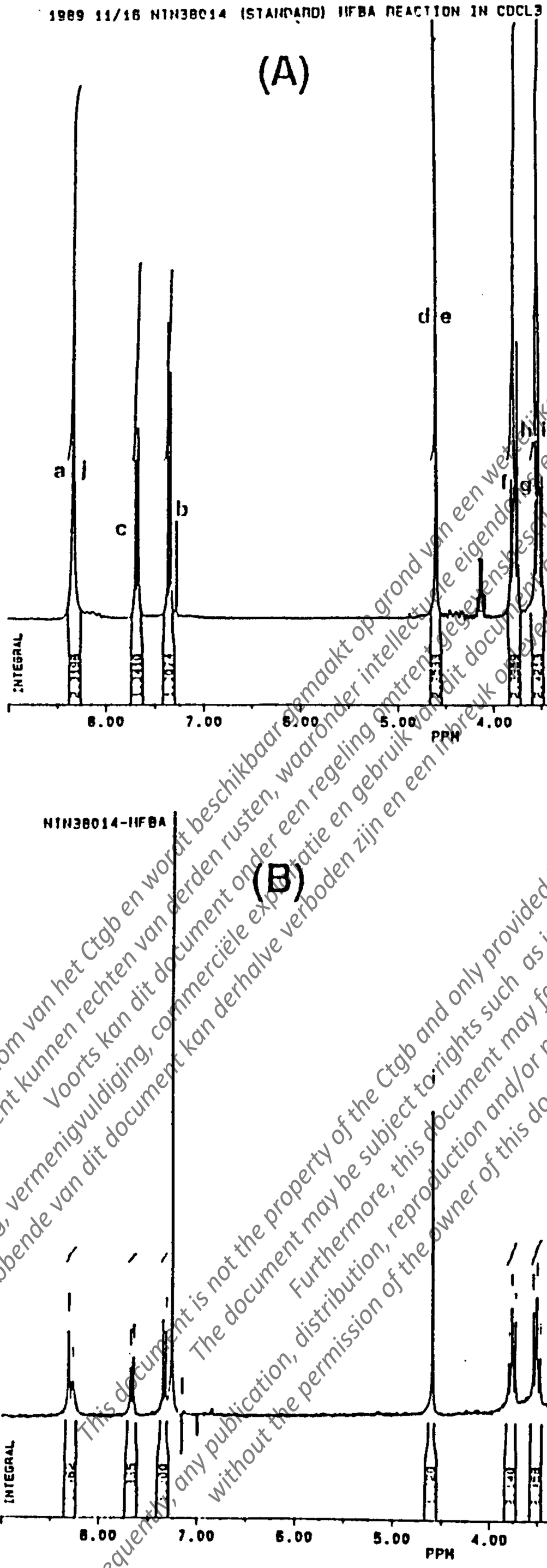


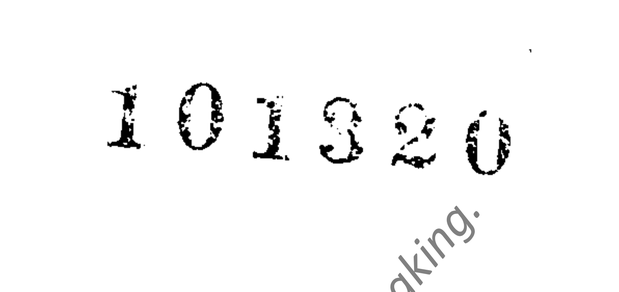
Fig.16. HPLC and TLC chromatograms showing separation of metabolites in aqueous phase (XAD-4, MeOH eluate) from shoot (Group 1)
Reference compounds: 1.NTN38014 3.NTN33893
2.CNA 4.WAK4103



Recovery of ^{14}C	
AQUF1	16%
AQUF2	5%
AQUF3	80%
Total	101%

Fig.17. Comparative HPLC chromatograms (H.S.B) of AQUF1, AQUF2 and AQUF3 fractionated by column chromatography of aqueous phase from shoot (Group 1)





KKHFBA.001
DATE 16-11-89

SF	250.133
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OI	4000.000
SI	16384
TD	16384
SW	3496.503
HZ/PT	.427
PW	3.0
RD	.600
AQ	2.343
NB	64
NS	42
TE	297
FW	4400
D2	0.0
DP	63L PO
LB	.250
GB	0.0
CX	22.00
CY	0.0
F1	8.001P
F2	.002P
HZ/CM	102.320
PPM/CM	.409
SN	2852.28

PEAKP
DATE 12-3-90

SF	250.133
SY	90.1300000
OI	4000.000
SI	16384
TD	16384
SW	3496.503
HZ/PT	.427
PW	3.0
RD	.600
AQ	2.343
NB	80
NS	121
TE	297
FW	4400
D2	0.0
DP	63L PO
LB	.250
GB	0.0
CX	22.00
CY	0.0
F1	9.001P
F2	.002P
HZ/CM	102.320
PPM/CM	.409
SN	2855.27

Fig.18. ^{1}H-NMR(250MHz) spectra of HFBA derivatives of NTN38014
(A)Reference compound
(B)HPLC Peak No. P

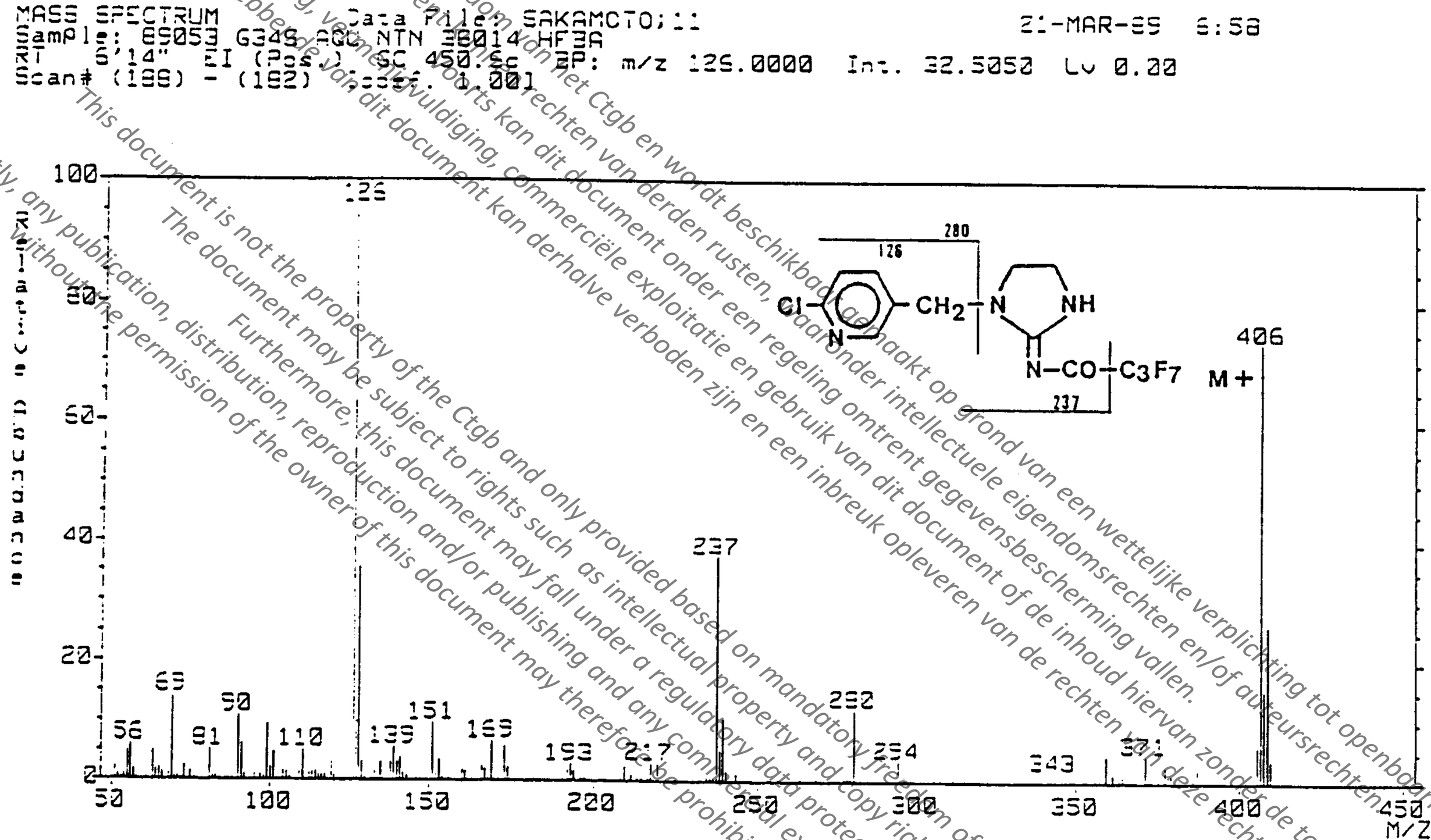
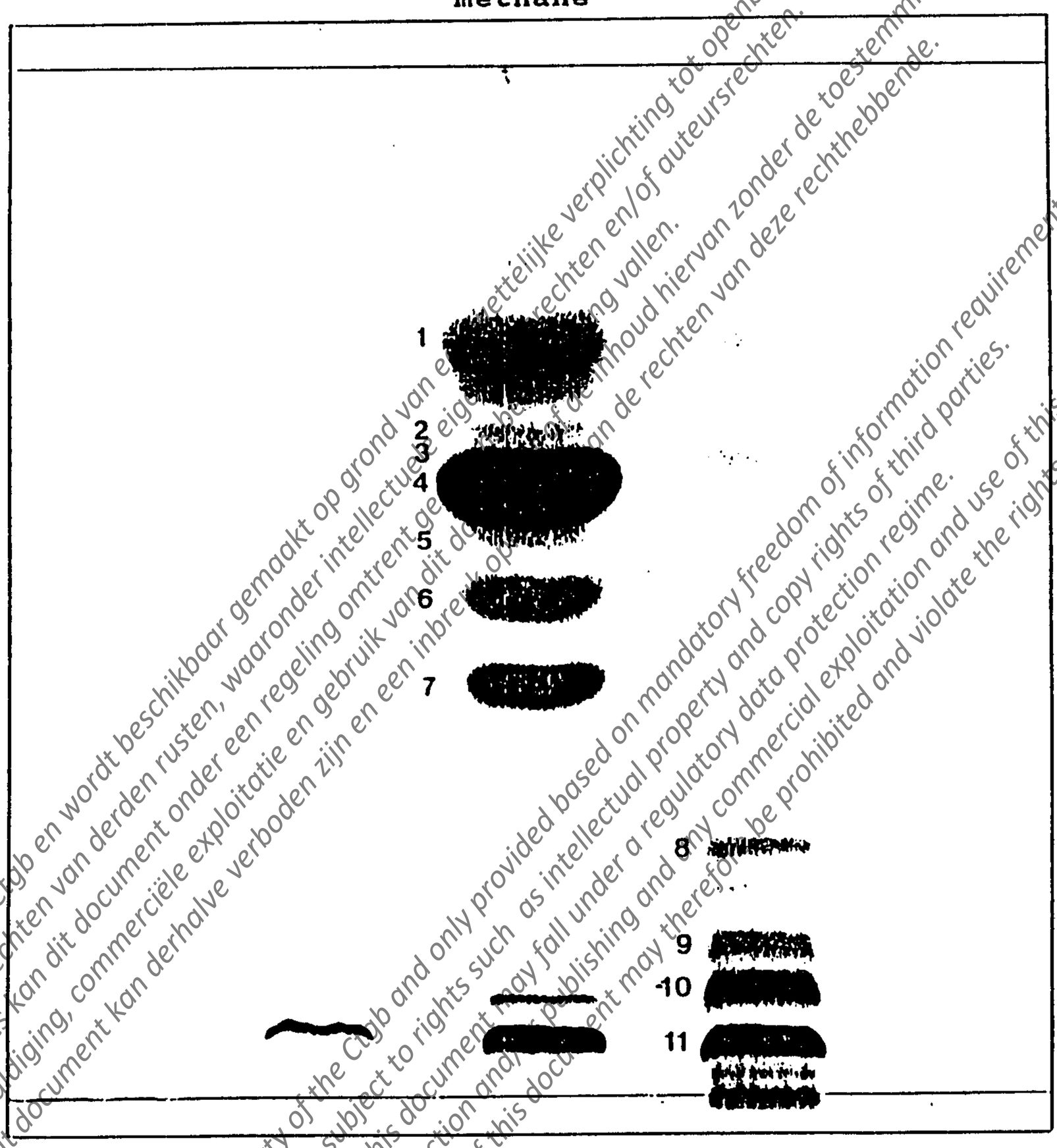


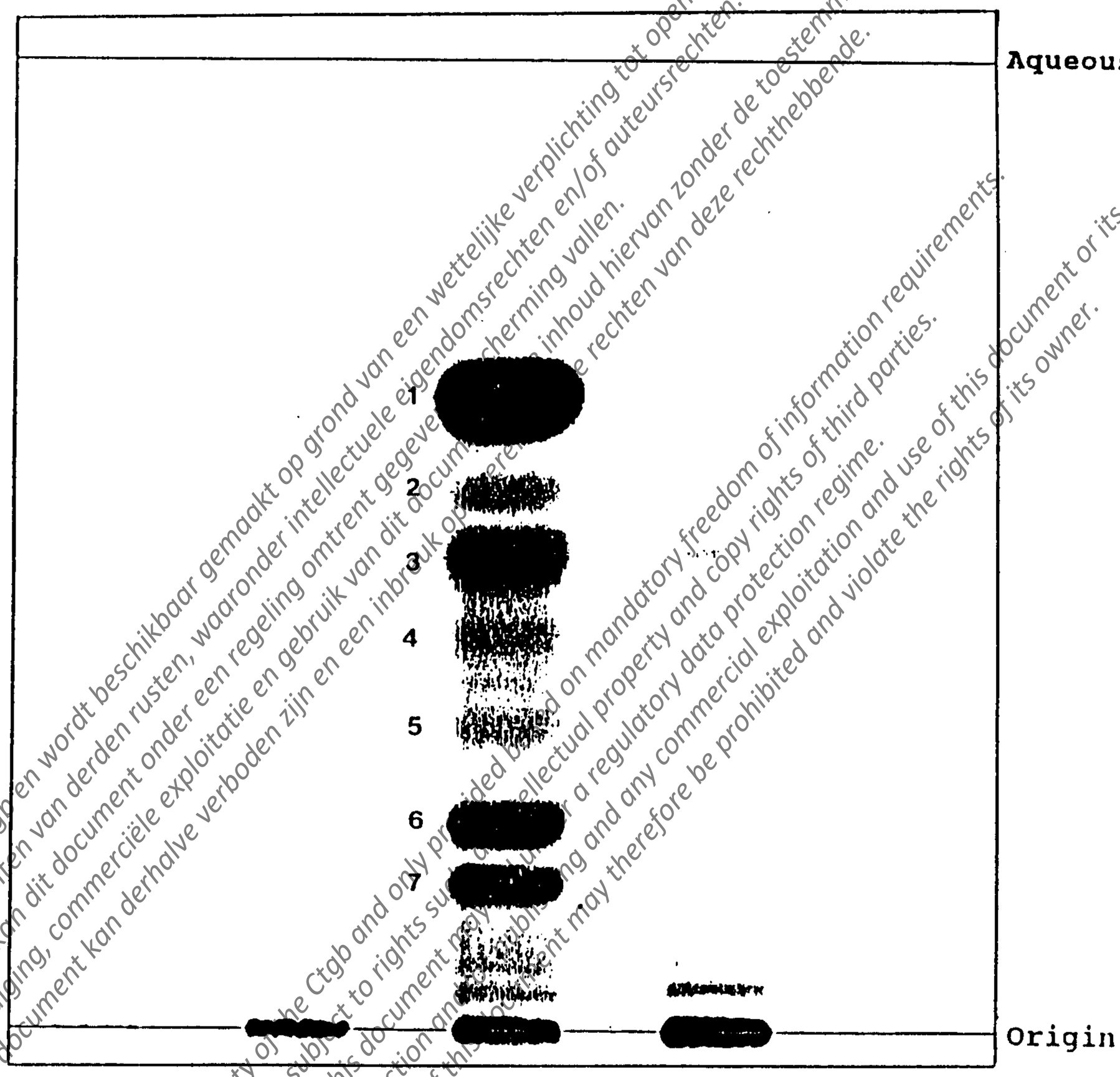
Fig.19. GC/MS spectrum of HFBA derivative of NTN38014 isolated from rice plant



1. WAK4103	7. WAK3839
2. M3	8. CNA
3. M1	9. M6
4. NTN33893	10. M7
5. NTN35884	11. NTN38014
6. NTN33519	

Fig.20. Comparative TLC chromatogram showing separation of metabolites in n-hexane, dichloromethane and aqueous phases from straw (Group 3)

Solvent:Ethyl acetate/Isopropanol/H_2O(65/23/12)(T.S.A)



1. NTN33893	5. M1
2. M3	6. WAK3839
3. WAK4103	7. NTN33519
4. NTN35884	

Fig.21. Comparative TLC chromatogram showing separation of metabolites in n-hexane, dichloromethane and aqueous phases from straw (Group 3)

Solvent:Dichloromethane/Acetonitrile(50/50)(T.S.B)

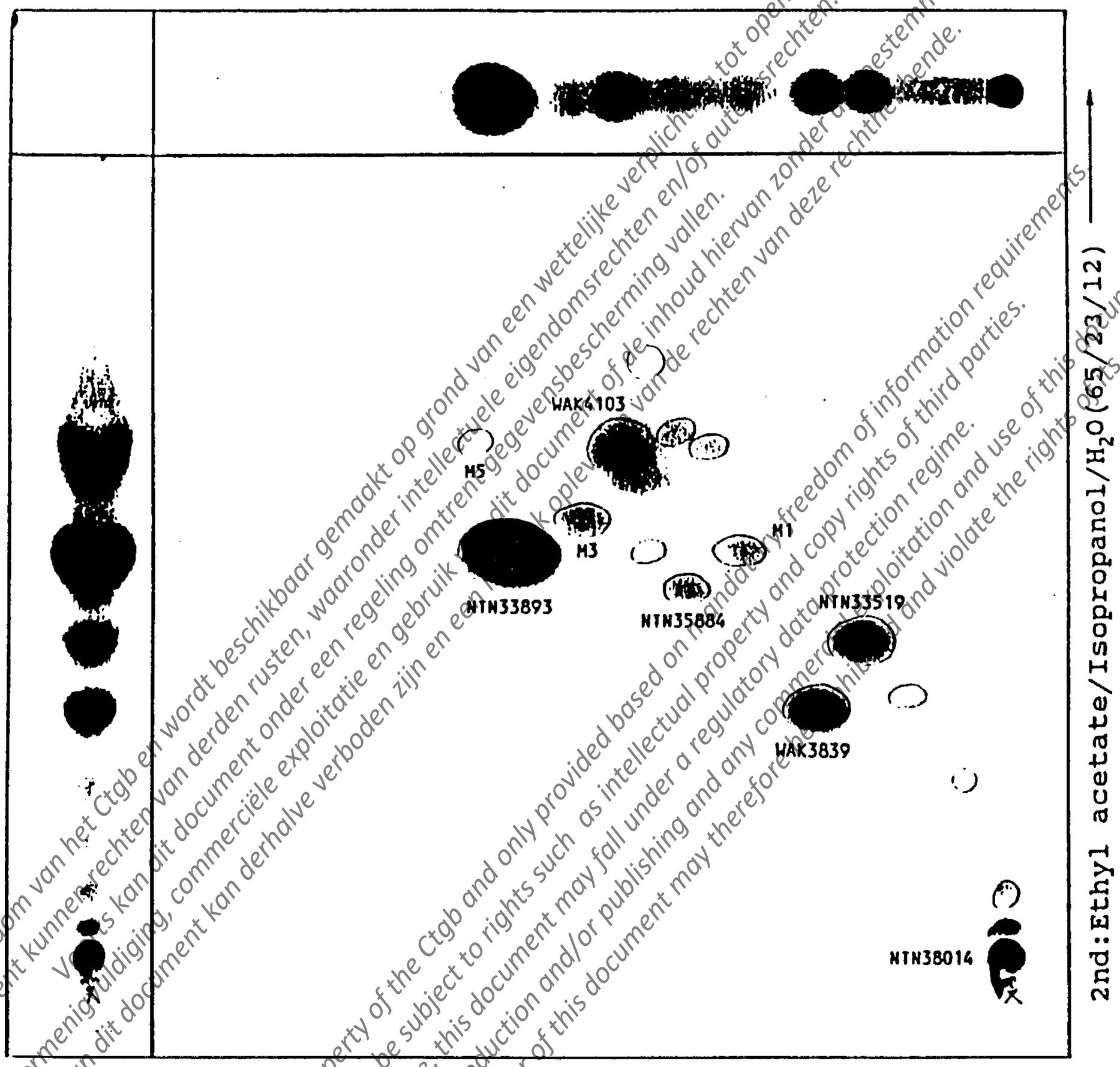
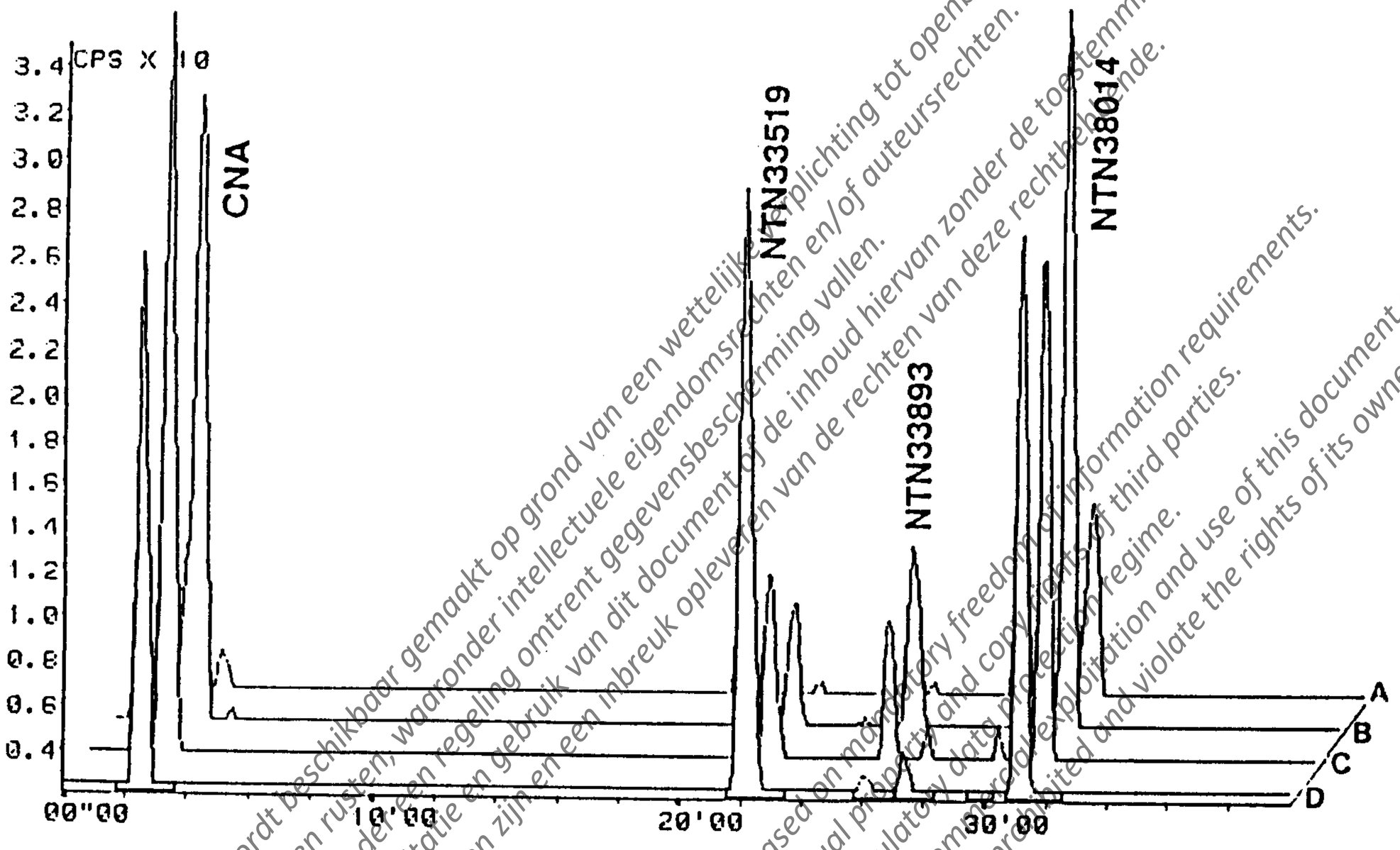


Fig.22. Two-dimensional TLC chromatogram showing separation of metabolites in dichloromethane phase from straw (Group 3)

3-D display:
G3RES.005 to G3RES.002



No.	Name of measurement	Trace	Z-value	Norm
1	G3RES.005	14C	1.000	1.00
2	G3RES.004	14C	2.000	1.00
3	G3RES.003	14C	3.000	1.00
4	G3RES.002	14C	4.000	1.00

Normalisation: Manual
Linearisation: Manual

Fig.23. Comparative HPLC chromatograms (H.S.B) of alkaline methanol extracts from straw (Group 3)

A: 1M CH_3COONa/MeOH(2/3)
B: 1M K_2HPO_4/MeOH(2/3)
C: 1M Na_2CO_3/MeOH(2/3)
D: 2M NaOH/MeOH(2/3)

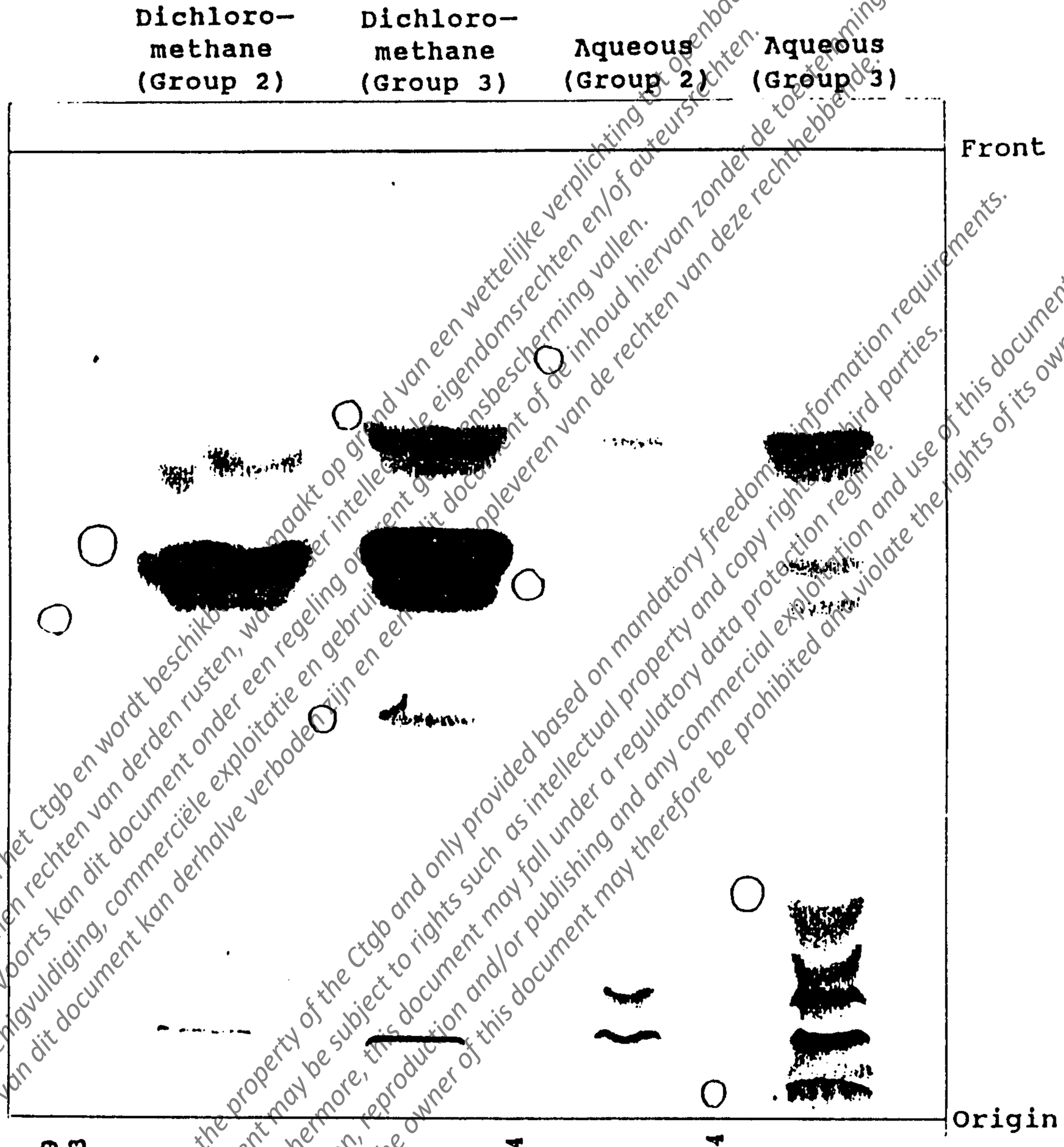


Fig.24. Comparative TLC chromatograms showing separation of metabolites in dichloromethane and aqueous phases from grain (Group 2,3)

Solvent:Ethyl acetate/Isopropanol/H_2O(65/23/12)(T.S.A)

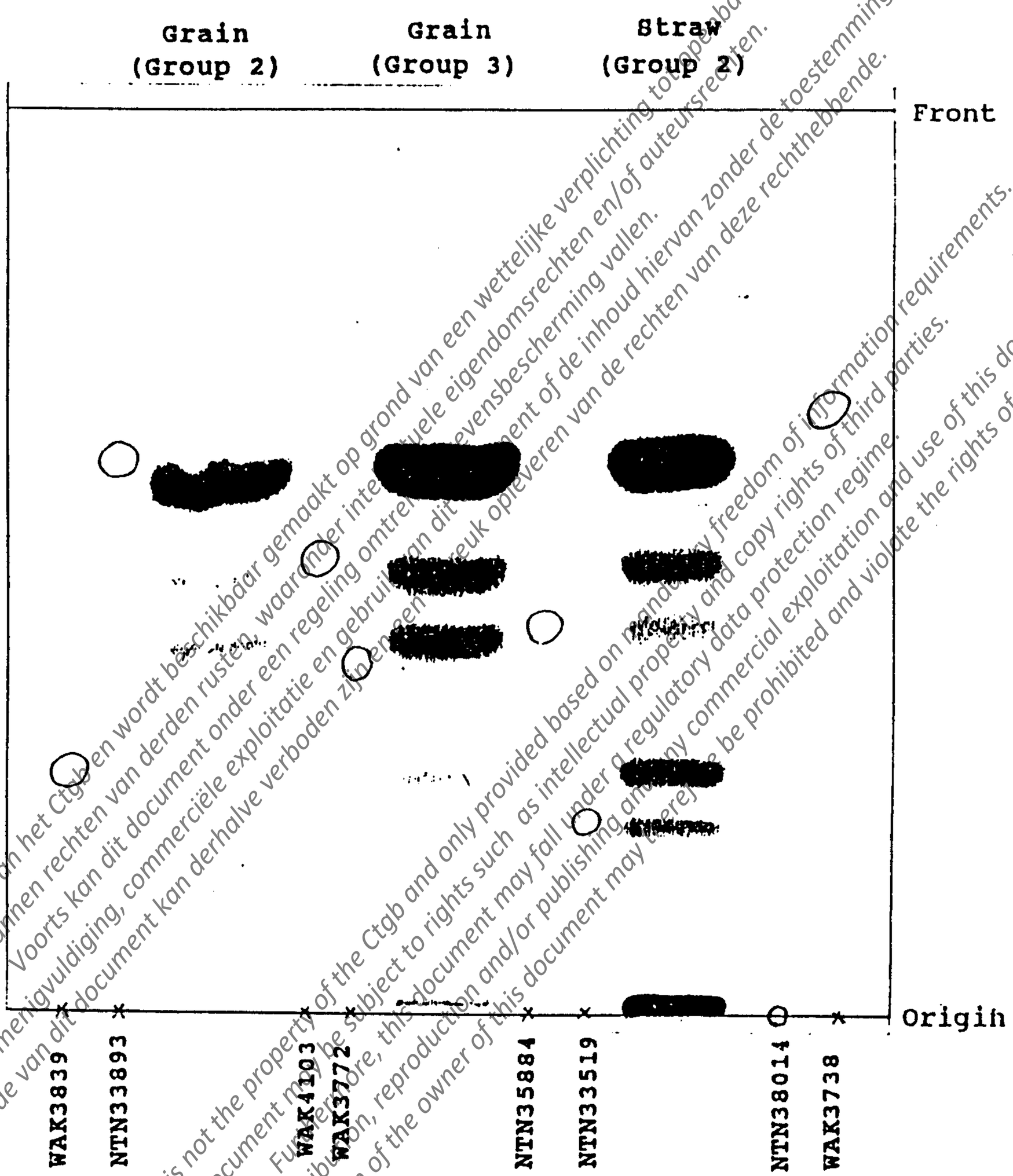


Fig.25. Comparative TLC chromatograms showing separation of metabolites in dichloromethane phases from grain and straw (Group 2,3)

Solvent:Dichloromethane/Acetonitrile(50/50)(T.S.B)

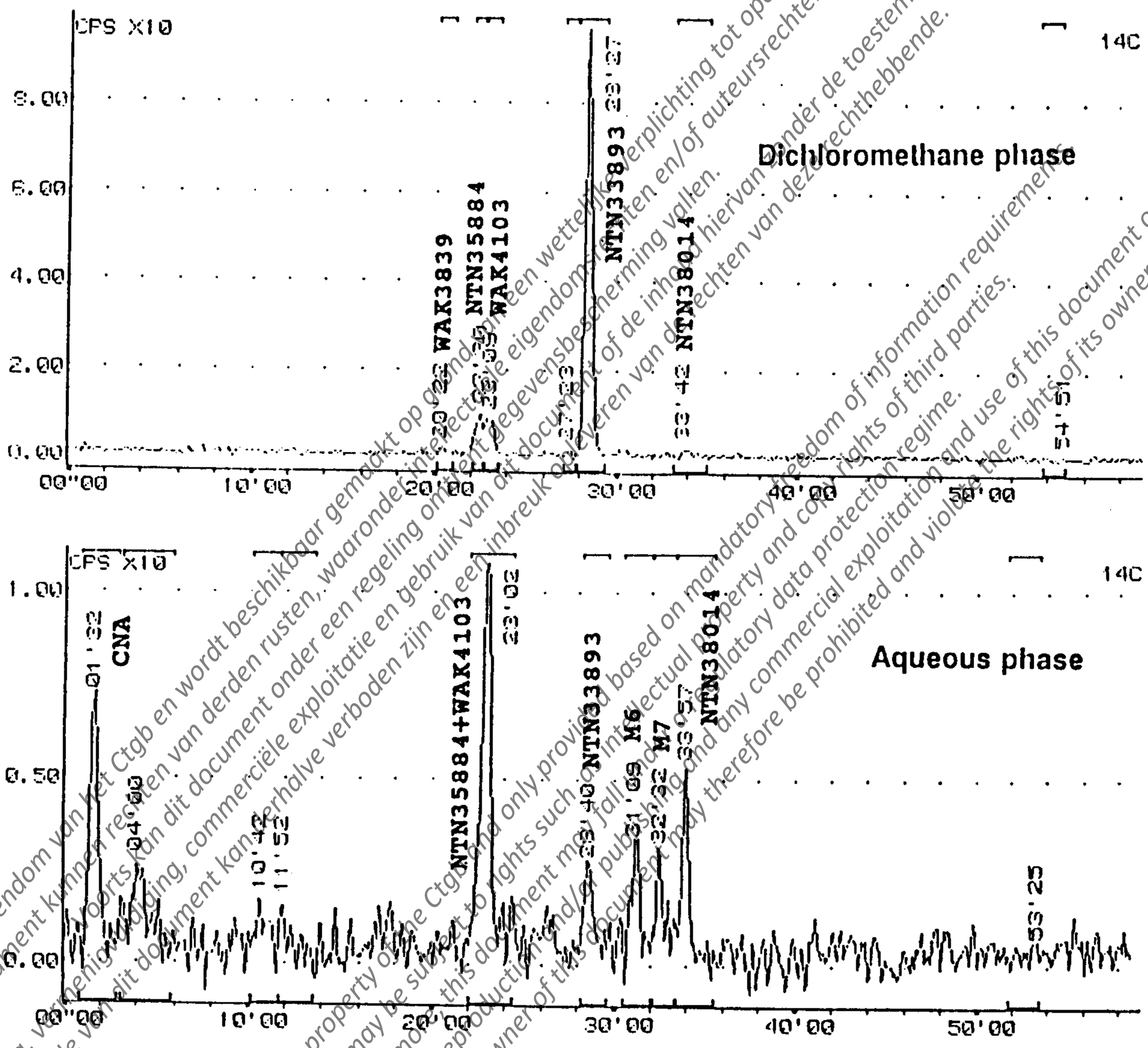


Fig.26. Comparative HPLC chromatograms (H.S.B) of metabolites in dichloromethane and aqueous phases from grain (Group 3)

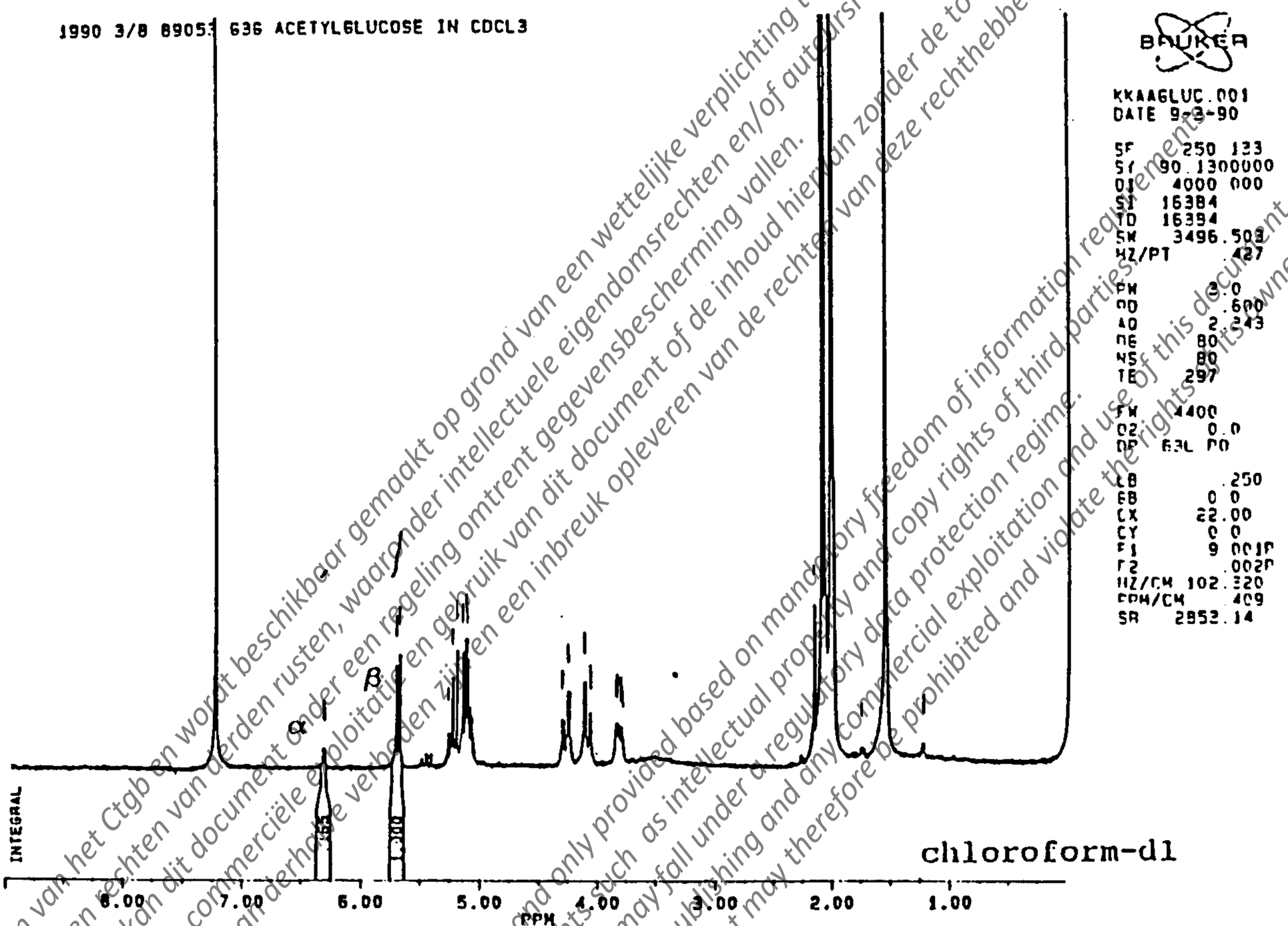


Fig.27. ^{1}H-NMR(250MHz) spectrum of acetylglucose induced from rice grain (3rd crystals)

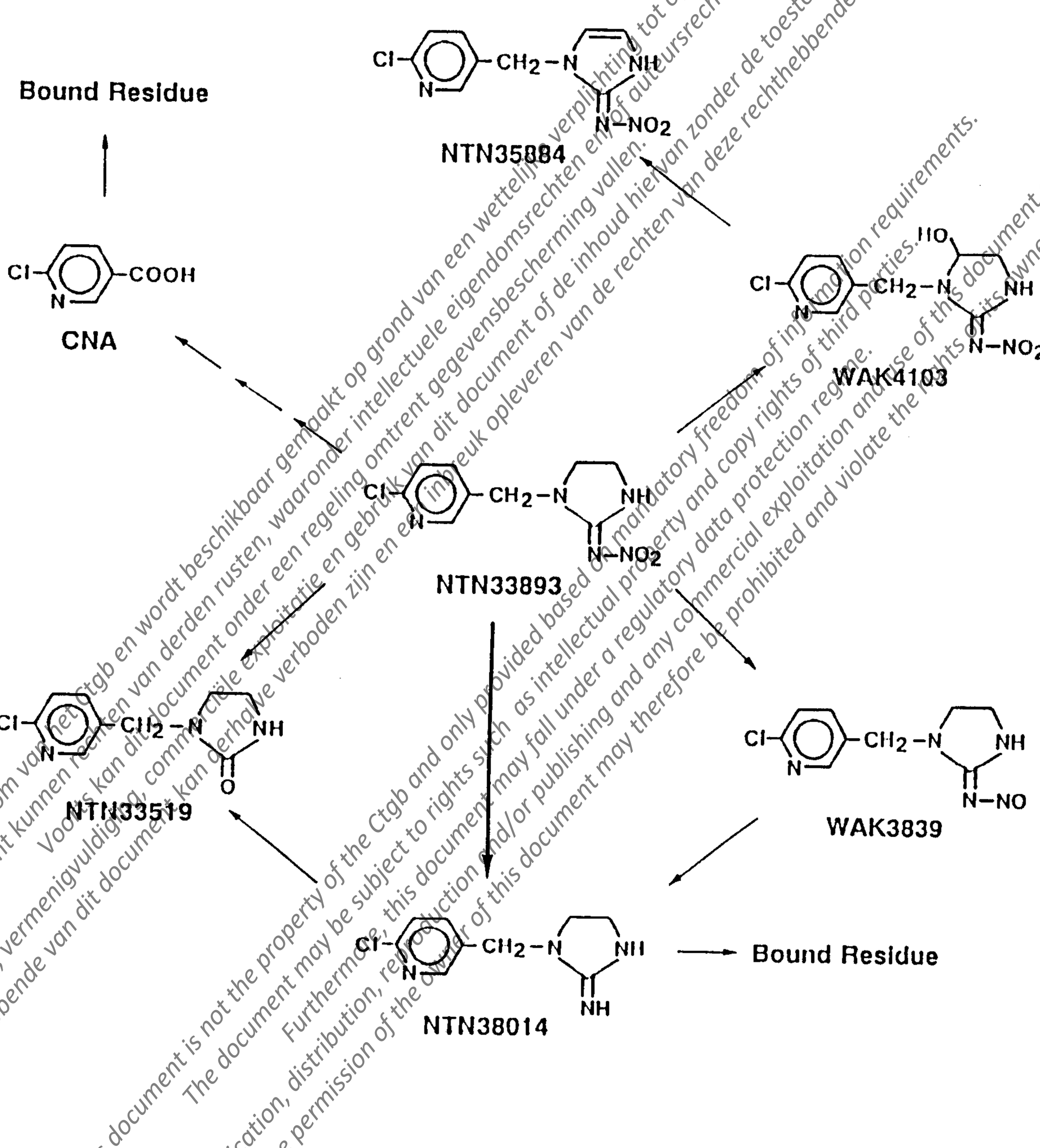


Fig.28. Proposed metabolic pathway of NTN33893 in rice plants

Appendix 1. Recipe of NTN33893 2% granule

Formulation date : Feb/3/1989

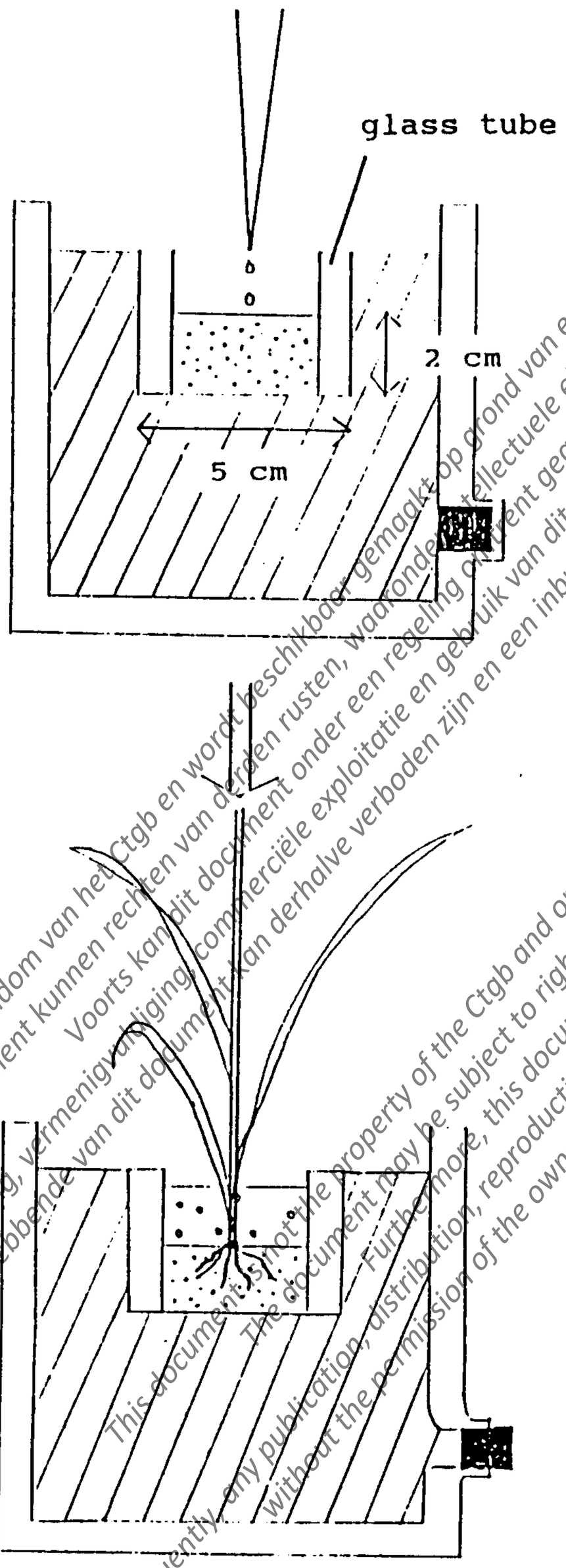
Formulation No. : FLN05

Batch size : 150 kg

Composition :

	Charge value (W/W, %)	as 100 %
NTN33893 premix 50	4.32	2.16
Na-Ligninsulfonate (CSL-LS)	0.50	
Benthonite (Kunigeru V-2)	35.00	
Talc (GTA)	60.18	

Appendix 2. A Diagram of treatment of test substances and transplanting of rice plant



Appendix 3. Extraction efficiency test
(Shoot, Group 1, 65-Day)

Solvent	No. of extraction	^{14}C extracted	
		Polytron Ext.	Shaking Ext.
80% AcCN	1 st	52.6	50.6
	2 nd	10.2	10.8
	3 rd	2.7	2.6
	Total	65.5	64.0
80% MeOH	1 st	55.2	51.1
	2 nd	11.1	10.7
	3 rd	3.2	2.6
	Total	69.5	64.4
EtOAc/MeOH (1/2)	1 st	52.1	49.4
	2 nd	8.8	8.9
	3 rd	2.1	4.6
	Total	63.0	62.9

Polytron extraction: 2g sample / 15 ml solvent
5 min / extraction
ambient temp.

Shaking extraction: 2g sample / 15 ml solvent
30 min / extraction
ambient temp.

Appendix 4. Extraction efficiency test (Straw, Group 2, 124-Day)

Solvent	No. of extraction	^{14}C extracted (%) Polytron Ext.	^{14}C extracted (%) Shaking Ext.
80% AcCN	1 st	55.5	54.5
	2 nd	15.2	11.5
	3 rd	5.1	3.4
	Total	75.8	69.4
80% MeOH	1 st	57.5	55.6
	2 nd	12.1	10.5
	3 rd	3.5	2.9
	Total	73.1	69.0

Polytron extraction: 1 g sample / 20 ml solvent
5 min / extraction
ambient temp.

Shaking extraction: 1 g sample / 20 ml solvent
30 min / extraction
ambient temp.

Appendix 5. Stability of NTN33893 and NTN38014 under various extraction methods

Extraction methods	Temp. and Time	Compound Tested	Recovery or yield (%) of NTN 33893	NTN 33519	NTN 38014
MeOH Reflux	100-110°C 48 hrs	NTN33893	67-69	21-22	
2M HCl/Dioxane (1/9)	Room temp. 1 hr	NTN33893	87-89	4-7	
1M CH_3COONa/MeOH (2/3)	60°C 1 hr	NTN33893*	95		
1M K_2HPO_4/MeOH (2/3)	60°C 1 hr	NTN33893*	98		
1M Na_2CO_3/MeOH (2/3)	60°C 1 hr	NTN33893*	18	48	
		NTN38014*			113
2M NaOH/MeOH (2/3)	60°C 1 hr	NTN33893	2-3	85-92	4-7
		NTN38014*			85

* Stability was tested by using non-radioactive compound.

Appendix 6. Combustion analysis of shoot
(Group 1, 0.32kg a.i./ha, 238μCi/pot, 65-Day)

Sample ID	(A) fresh wt(g)	(B) wt (g) combusted	(C) dpm	(D) mg/kg (fresh)	(E) total ^{14}C (μCi)	(F) absorption (%)
G-1-(1)	165.84	0.2011	25061	0.373	9.31	3.91
		0.2032	24353	0.358	8.95	3.76
		0.2029	22530	0.332	8.29	3.49
		0.2002	22157	0.331	8.27	3.47
		0.2001	21918	0.327	8.18	3.44
		0.2070	22071	0.319	7.97	3.35
		0.2103	22471	0.319	7.98	3.35
		0.2045	23189	0.339	8.47	3.56
		0.2028	22125	0.326	8.15	3.42
G-1-(1) average				0.336	8.40	3.53
S.D.				0.018	0.45	0.19
[Dry wt basis]				[1.234]		
G-1-(2)	169.91	0.2011	26516	0.394	10.09	4.24
		0.2013	30922	0.459	11.76	4.94
		0.2084	27934	0.401	10.26	4.31
		0.2117	30150	0.426	10.90	4.58
		0.2012	26675	0.396	10.15	4.26
		0.2030	27283	0.402	10.29	4.32
		0.2031	28725	0.423	10.82	4.55
		0.2035	29647	0.436	11.15	4.68
		0.2151	34916	0.485	12.42	5.22
		0.2010	24759	0.368	9.43	3.96
G-1-(2) average				0.419	10.73	4.51
S.D.				0.034	0.88	0.37
[Dry wt basis]				[1.587]		
G-1 average				0.378	9.57	4.02
S.D.				0.059	1.65	0.69
[Dry wt basis]				[1.411]		

(D) mg/kg (fresh) : [(C)/334510 (dpm/μg)]/(B)
(E) total ^{14}C (μCi) : (C)/(B) x (A)/2220000 dpm
(F) absorption (%) : (E)/238 (μCi) x 100

Appendix 7. Combustion analysis of straw
(Group 2, 0.32kg a.i./ha, 238μCi/pot, 124-Day)

Sample ID	(A) dry wt(g)	(B) wt (g) combusted	(C) dpm	(D) mg/kg (dry)	(E) total ^{14}C (μCi)	(F) absorption (%)
G-2-(1)	57.28	0.1026	47176	1.375	11.86	4.98
		0.1144	53831	1.407	12.14	5.10
		0.1040	47378	1.362	11.75	4.94
		0.0958	45766	1.428	12.33	5.18
		0.1134	52131	1.374	11.86	4.98
G-2-(1)	average			1.389	11.99	5.04
	S.D.			0.027	0.24	0.10
G-2-(2)	44.96	0.1095	42609	1.163	7.88	3.31
		0.0972	39625	1.219	8.26	3.47
		0.1022	41990	1.228	8.32	3.50
		0.1128	50138	1.329	9.00	3.78
		0.1056	44288	1.254	8.49	3.57
G-2-(2)	average			1.239	8.39	3.53
	S.D.			0.060	0.41	0.17
G-2 average				1.314	10.19	4.29
S.D.				0.106	2.55	1.07

(D) mg/kg (dry) : [(C)/334510 (dpm/μg)]/(B)
(E) total ^{14}C (μCi) : (C)/(B) x (A)/2220000 dpm
(F) absorption (%) : (E)/238 (μCi) x 100

Appendix 8. Combustion analysis of straw
(Group 3, 1.26kg a.i./ha, 952μCi/pot, 124-Day)

Sample ID	(A) dry wt(g)	(B) wt (g) combusted	(C) dpm	(D) mg/kg (dry)	(E) total ^{14}C (μCi)	(F) absorption (%)
G-3-(1)	50.73	0.0993	308581	9.29	71.01	7.46
		0.1067	302794	8.48	64.85	6.81
		0.1025	336079	9.80	74.93	7.87
		0.0991	278328	8.40	64.18	6.74
		0.0992	297800	8.97	68.60	7.21
	G-3-(1) average			8.99	68.71	7.22
	S.D.			0.58	4.46	0.47
G-3-(2)	50.88	0.1065	256014	7.19	55.09	5.79
		0.1007	296808	8.81	67.55	7.10
		0.1136	322466	8.49	65.06	6.83
		0.1058	250561	7.08	54.28	5.70
		0.0950	276997	8.72	66.83	7.02
	G-3-(2) average			8.06	61.76	6.49
	S.D.			0.85	6.53	0.69
G-3 average				8.53	65.24	6.86
S.D.				0.66	4.91	0.52

(D) mg/kg (dry) : [(C)/334510 (dpm/μg)]/(B)
(E) total ^{14}C (μCi) : (C)/(B) x (A)/2220000 dpm
(F) absorption (%): (E)/952 (μCi) x 100

Appendix 9. Combustion analysis of grain
(Group 2, 0.32kg a.i./ha, 238μCi/pot, 124-Day)

Sample ID	(A) dry wt(g)	(B) wt (g) combusted	(C) dpm	(D) mg/kg (dry)	(E) total ^{14}C (μCi)	(F) absorption (%)
G-2-(1)	35.24	0.1077	465	0.013	0.07	0.03
		0.1059	480	0.014	0.07	0.03
		0.0987	417	0.013	0.07	0.03
		0.1089	484	0.013	0.07	0.03
		0.1072	504	0.014	0.07	0.03
G-2-(1) average				0.013	0.07	0.03
S.D.				0.001	0	0
G-2-(2)	32.64	0.1069	484	0.014	0.07	0.03
		0.0975	483	0.015	0.07	0.03
		0.1015	471	0.014	0.07	0.03
		0.1003	466	0.014	0.07	0.03
		0.0987	472	0.014	0.07	0.03
G-2-(2) average				0.014	0.07	0.03
S.D.				0.0004	0	0
G-2 average				0.014	0.07	0.03
S.D.				0.001	0	0

(D) mg/kg (dry) : [(C)/334510 (dpm/μg)]/(B)
(E) total ^{14}C (μCi) : (C)/(B) x (A)/2220000 dpm
(F) absorption (%): (E)/238 (μCi) x 100

Appendix 10. Combustion analysis of grain
(Group 3, 1.26kg a.i./ha, 952µCi/pot, 124-Day)

Sample ID	(A) dry wt(g)	(B) wt (g) combusted	(C) dpm	(D) mg/kg (dry)	(E) total ^{14}C (μCi)	(F) absorption (%)
G-3-(1)	30.58	0.0961	1856	0.058	0.27	0.03
		0.1033	2016	0.058	0.27	0.03
		0.1040	2061	0.059	0.27	0.03
		0.1106	2186	0.059	0.27	0.03
		0.1059	2133	0.060	0.28	0.03
G-3-(1) average				0.059	0.27	0.03
S.D.				0.001	<0.01	0
G-3-(2)	30.95	0.0988	2272	0.069	0.32	0.03
		0.1044	2411	0.069	0.32	0.03
		0.1081	2493	0.069	0.32	0.03
		0.0962	2252	0.070	0.33	0.03
		0.1027	2316	0.067	0.31	0.03
G-3-(2) average				0.069	0.32	0.03
S.D.				0.001	0.01	0
G-3 average				0.064	0.30	0.03
S.D.				0.007	0.04	0

(D) mg/kg (dry) : [(C)/334510 (dpm/µg)]/(B)
(E) total ^{14}C (µCi) : (C)/(B) x (A)/2220000 dpm
(F) absorption (%) : (E)/952 (µCi) x 100

Appendix 11. Combustion analysis of chaff
(Group 2, 0.32kg a.i./ha, 238μCi/pot, 124-Day)

Sample ID	(A) dry wt(g)	(B) wt (g) combusted	(C) dpm	(D) mg/kg (dry)	(E) total ^{14}C (μCi)	(F) absorption (%)
G-2-(1)	10.38	0.1045	2195	0.063	0.10	0.04
		0.1040	3070	0.088	0.14	0.06
		0.1005	3015	0.090	0.14	0.06
		0.1048	3024	0.086	0.13	0.05
		0.0996	2864	0.086	0.13	0.05
G-2-(1) average				0.083	0.13	0.05
S.D.				0.011	0.02	0.01
G-2-(2)	8.12	0.1040	3532	0.102	0.12	0.05
		0.1004	3579	0.107	0.13	0.05
		0.1074	3803	0.106	0.13	0.05
		0.1011	3545	0.105	0.13	0.05
		0.1056	3653	0.103	0.13	0.05
G-2-(2) average				0.105	0.13	0.05
S.D.				0.002	0.004	0
G-2 average				0.094	0.13	0.05
S.D.				0.016	0	0

(D) mg/kg (dry) : [(C)/334510 (dpm/μg)]/(B)
(E) total ^{14}C (μCi) : (C)/(B) x (A)/2220000 dpm
(F) absorption (%) : (E)/238 (μCi) x 100

Appendix 12. Combustion analysis of chaff
(Group 3, 1.26kg a.i./ha, 952μCi/pot, 124-Day)

Sample ID	(A) dry wt(g)	(B) wt (g) combusted	(C) dpm	(D) mg/kg (dry)	(E) total ^{14}C (μCi)	(F) absorption (%)
G-3-(1)	9.21	0.0999	14560	0.436	0.60	0.06
		0.1027	15108	0.440	0.61	0.06
		0.1102	15927	0.432	0.60	0.06
		0.1075	15797	0.439	0.61	0.06
		0.0992	14536	0.438	0.61	0.06
G-3-(1) average				0.437	0.61	0.06
S.D.				0.003	0.01	0
G-3-(2)	9.27	0.1010	12317	0.365	0.51	0.05
		0.1049	12712	0.363	0.51	0.05
		0.1106	13538	0.366	0.51	0.05
		0.0968	11909	0.368	0.51	0.05
		0.1014	12710	0.375	0.52	0.05
G-3-(2) average				0.367	0.51	0.05
S.D.				0.005	0.004	0
G-3 average				0.402	0.56	0.06
S.D.				0.049	0.07	0.01

(D) mg/kg (dry) : [(C)/334510 (dpm/μg)]/(B)
(E) total ^{14}C (μCi) : (C)/(B) x (A)/2220000 dpm
(F) absorption (%) : (E)/952 (μCi) x 100

Appendix 13. Combustion analysis of rhachis
(Group 2, 0.32kg a.i./ha, 238μCi/pot, 124-Day)

Sample ID	(A) dry wt(g)	(B) wt (g) combusted	(C) dpm	(D) mg/kg (dry)	(E) total ^{14}C (μCi)	(F) absorption (%)
G-2-(1)	1.91	0.0953	1170	0.037	0.01	<0.01
		0.0998	1147	0.034	0.01	<0.01
		0.0921	1052	0.034	0.01	<0.01
		0.0945	1111	0.035	0.01	<0.01
		0.1082	1260	0.035	0.01	<0.01
		G-2-(1) average		0.035	0.01	<0.01
		S.D.		0.001	0	
G-2-(2)	1.69	0.1035	1446	0.042	0.01	<0.01
		0.1024	1427	0.042	0.01	<0.01
		0.0942	1242	0.039	0.01	<0.01
		0.1054	1458	0.041	0.01	<0.01
		0.1024	1406	0.041	0.01	<0.01
		G-2-(2) average		0.041	0.01	<0.01
		S.D.		0.001	0	
G-2 average				0.038	0.01	<0.01
S.D.				0.004	0	

(D) mg/kg (dry) : [(C)/334510 (dpm/μg)]/(B)
(E) total ^{14}C (μCi) : (C)/(B) x (A)/2220000 dpm
(F) absorption (%) : (E)/238 (μCi) x 100

Appendix 14. Combustion analysis of rhachis
(Group 3, 1.26kg a.i./ha, 952μCi/pot, 124-Day)

	(A)	(B)	(C)	(D)	(E)	(F)
Sample ID	dry wt(g)	wt (g) combusted	dpm	mg/kg total (dry)	^{14}C (μCi)	absorption (%)
G-3-(1)	1.72	0.0947	5078	0.160	0.04	<0.01
		0.1029	5527	0.161	0.04	<0.01
		0.0999	5440	0.163	0.04	<0.01
		0.1102	5850	0.159	0.04	<0.01
		0.0947	5110	0.161	0.04	<0.01
	G-3-(1) average			0.161	0.04	<0.01
	S.D.			0.001	0	
G-3-(2)	1.98	0.0874	3827	0.131	0.04	<0.01
		0.0884	3745	0.127	0.04	<0.01
		0.0873	3814	0.131	0.04	<0.01
		0.1031	4430	0.128	0.04	<0.01
		0.1106	4699	0.127	0.04	<0.01
	G-3-(2) average			0.129	0.04	<0.01
	S.D.			0.002	0	
G-3 average				0.145	0.04	<0.01
S.D.				0.023	0	

(D) mg/kg (dry) : [(C)/334510 (dpm/μg)]/(B)
(E) total ^{14}C (μCi) : (C)/(B) x (A)/2220000 dpm
(F) absorption (%) : (E)/952 (μCi) x 100

Appendix 15. Equation to calculate ^{14}C in starch on the basis of specific radioactivities of rice grain and acetyglucose

$$\frac{\text{Specific radioactivity of starch (dpm/g)}^{1)} \times 0.7^{2)}}{\text{Specific radioactivity of grain (dpm/g)}^{3)}} \times 100$$

1) $\dfrac{(C_{16}H_{22}O_{11})_n}{(C_6H_{10}O_5)_n}$ x Specific radioactivity (dpm/g) of acetylglucose (mean value, 1 st - 3 rd)

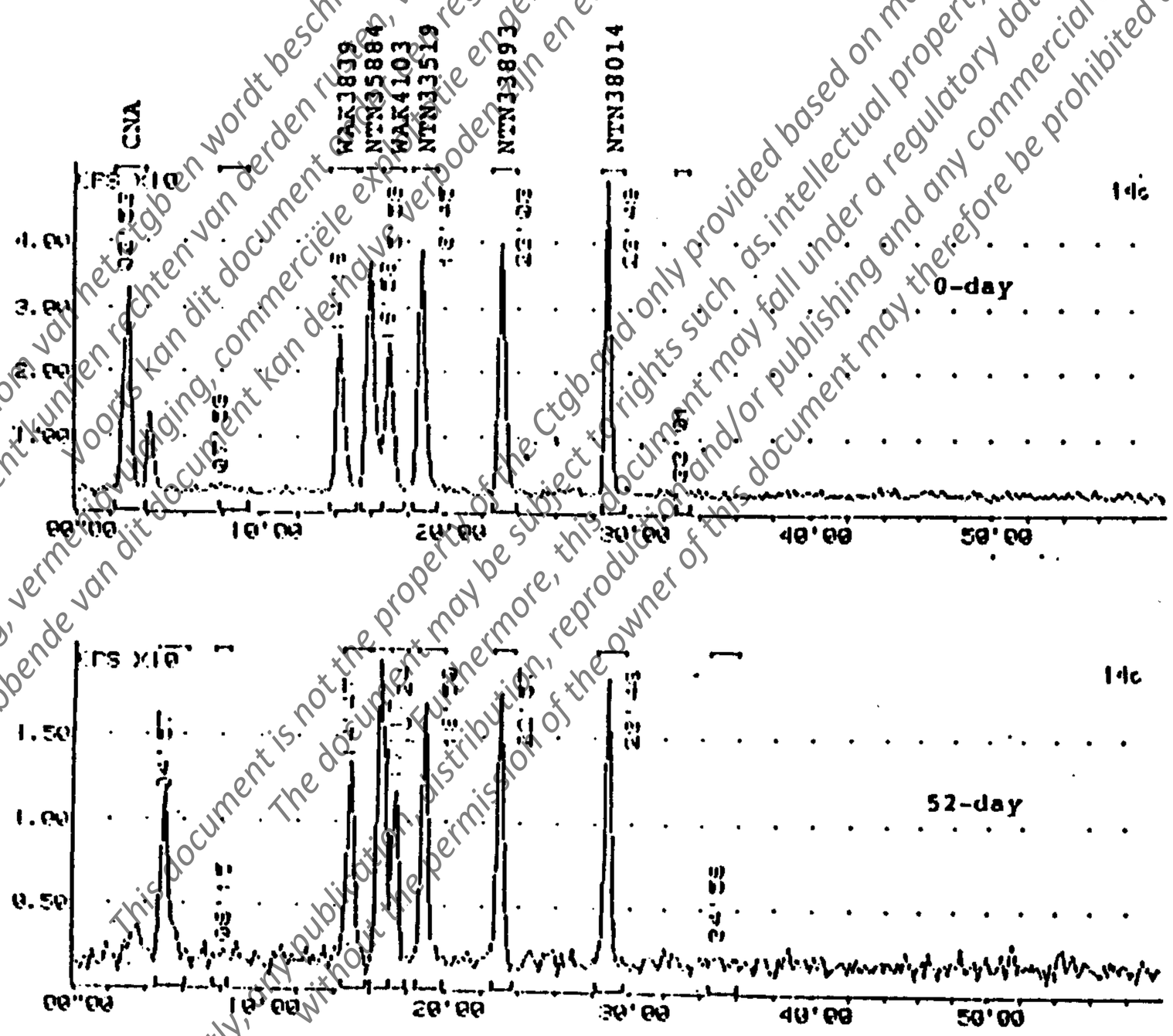
2) Starch content in rice grain

3) 21367 dpm/g

Appendix 16. Recovery of NTN33893 and metabolites after storage (-25°C) of grain

Compound	Concentration(ppm)		Recovery(%)
	0-day	52-day	
CNA	0.59	0.56	95
WAK3839	3.15	3.87	122
NTN35884	8.42	7.82	93
WAK4103	4.91	4.95	101
NTN33519	1.08	0.98	91
NTN33893	1.25	1.13	90
NTN38014	1.05	1.01	96

Fortified radioactivity: 1μCi of each metabolite was added to 1g of grain



Appendix 17. Recovery of NTN33893 and metabolites after storage (-25°C) of straw

Compound	Concentration(ppm)		Recovery(%)
	0-day	21-day	
CNA	0.74	0.84	114
WAK3839	3.66	3.69	101
NTN35884	8.23	7.90	96
WAK4103	4.77	4.72	99
NTN33519	1.07	1.09	102
NTN33893	1.12	1.10	98
NTN38014	1.11	1.03	93

Fortified radioactivity: 1µCi of each metabolite was added to 1g of straw