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Study Title

Metabolism of [^{14}C]NTN 33893 in Apples

Data Requirement

171-4 Nature of Residue (Metabolism) - Plants

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

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<u>**Good Laboratory Practice Certification**</u>

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I. SUMMARY

The metabolism of the insecticide NTN 33893 was investigated in apples after application of [pyridinyl-^{14}C-methyl]NTN 33893 directly to the fruit using a syringe. The amount applied was approximately equivalent to the recommended application rate of 375 g/ha.

Samples of the apples were taken 0 and 14 days (harvest) after the last of three applications. The total residues found in the harvested samples were 1.76 mg/kg for day 0 and 1.45 mg/kg for day 14. The following metabolites were identified by co-chromatography with reference compounds (amounts given are for day 14 and are in per cent of radioactivity recovered and mg/kg a.i. equivalents).

		%	mg/kg
Unchanged parent compound	(I)	69.0	0.996
Olefine compound	(VI)	5.7	0.082
5-Hydroxy compound	(IV)	2.7	0.039
Guanidine compound	(II)	2.4	0.038
Glucoside of 6-chloropicolyl alcohol	(X)	2.2	0.031
Urea compound	(III)	1.7	0.024
Dihydroxy compound	(VII)	1.1	0.016
Nitrosimine compound	(VIII)	0.7	0.010

In the day 14 samples at least 26 unidentified metabolites were also detected, the largest being component 28 (0.018 mg/kg), and in total amounted to 11.1 %, 0.160 mg/kg. The non-extractable residue was low at only 3 %, 0.044 mg/kg.

In two separate parallel translocation experiments, in which radioactivity was applied to leaves, less than 60 % of the radioactivity was recovered from the treated leaves. Less than 0.1 per cent of the radioactivity had translocated into either the peel or the pulp.

1. ZUSAMMENFASSUNG

Der Metabolismus des Insektizids NTN 33893 wurde in Äpfeln nach Applikation von [pyridinyl-^{14}C-methyl]NTN 33893 mit einer Pipette auf die Frucht untersucht. Die applizierte Menge entsprach etwa der empfohlenen Aufwandmenge von 375 g/ha.

Die Äpfel wurden 0 und 14 Tage (Erntereife) nach der letzten von drei Applikationen geerntet. Die Gesamtrückstände betrugen in den Proben von Tag 0 1,76 mg/kg und von Tag 14 1,45 mg/kg. Folgende Metaboliten wurden durch Co-Chromatographie mit Referenzsubstanzen identifiziert (Angaben in Prozent der wiedergefundenen Radioaktivität und in mg/kg Wirkstoffäquivalenten):

		%	mg/kg
Unveränderter Wirkstoff	(I)	69.0	0.996
Olefin-Verbindung	(VI)	5.7	0.082
5-Hydroxy-Verbindung	(IV)	2.7	0.039
Guanidin-Verbindung	(II)	2.4	0.038
Glucosid des 6-Chlorpicolyl-alkohols	(X)	2.2	0.031
Harnstoff-Verbindung	(III)	1.7	0.024
Dihydroxy-Verbindung	(VII)	1.1	0.016
Nitrosimin-Verbindung	(VIII)	0.7	0.010

Mindestens 26 nicht identifizierte Metaboliten wurden in einer Gesamtmenge von 11,1 % oder 0,160 mg/kg nachgewiesen, von denen Komponente 28 mengenmäßig die größte war (0,018 mg/kg). Der nicht-extrahierbare Rückstand war mit nur 3 % oder 0,044 mg/kg niedrig.

In zwei getrennten, parallel durchgeführten Translokationsversuchen, in welchen die Radioaktivität auf Blätter appliziert wurde, wurden weniger als 60 % der Radioaktivtät in den behandelten Blättern wiedergefunden. Weniger als 0.1 % der Radioaktivität wurde in die Schale oder Frucht der Äpfel transloziert.

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.

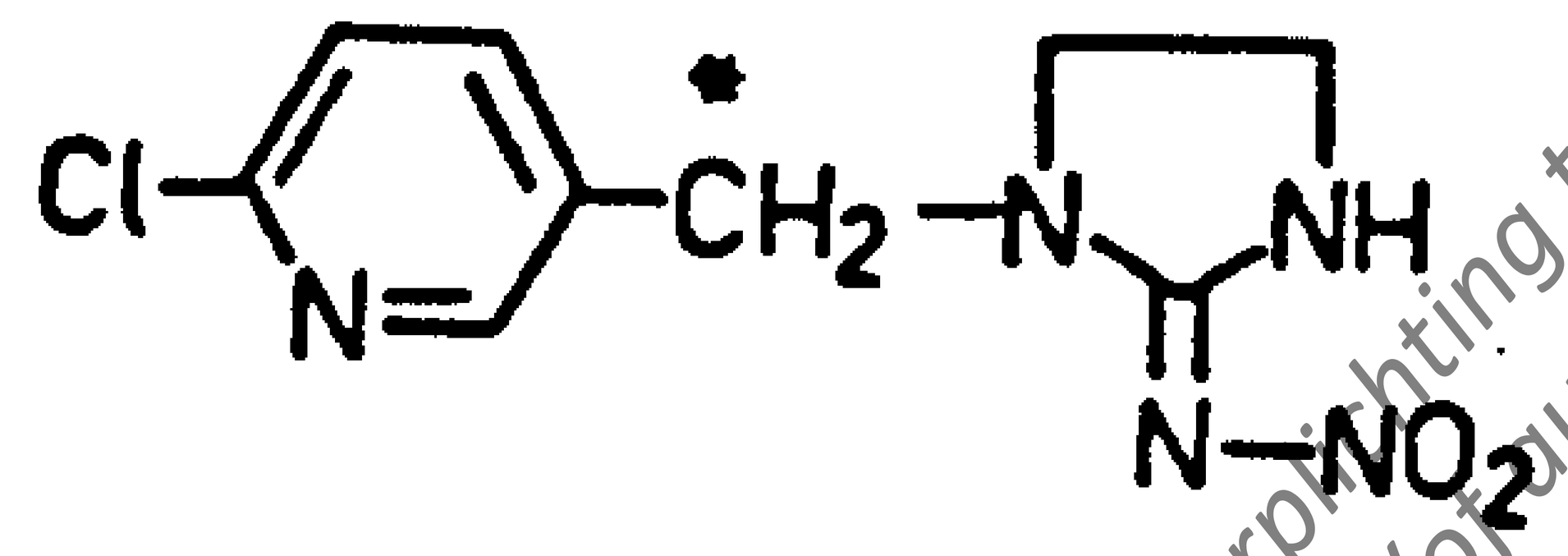
In studies reported so far (cell culture: [redacted] 1989; tomatoes: [redacted] 1989; potatoes after granular treatment: [redacted] 1992a; potatoes after spray application: [redacted], 1990; corn: [redacted] 1992b; rice: [redacted] 1990; egg plants: [redacted] 1991) the following metabolites were detected:- 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 but it was still the main component in both.

The objective of the following study was to investigate the metabolism of NTN 33893 in apples after application using an Eppendorf syringe. The study began with the first application on June 27th 1989 and finished in October 1991. The main metabolism experiment was supported by a storage stability experiment, which covered the period of the whole study, and a translocation experiment. The raw data and the original report will be archived in the central [redacted]

III. MATERIALS

A. Test Compound

Structural formula



(* denotes labelling position)

Chemical name : 1-(6-chloro-3-pyridinyl)methyl-4,5-dihydro-N-nitro-1H-imidazol-2-amine (C.A.)

CAS Number : 105 827-78-9 (1987)

Molecular Weight : 255.7

Specific Radioactivity (a.i) : 3.42 MBq/mg (92.3 µCi/mg)

Radiochemical Purity : 99.7 % (laboratory Dr. Ecker)

The pyridinyl-^{14}C-methyl radiolabelled active ingredient was formulated as a WP 25 (laboratory Dr. [redacted]. This was divided into formulation ECW 6102/6 for the main metabolism experiment and ECW 6102/7 for the translocation experiment (see appendices 1 and 2 for formulation details). The content, purity and specific radioactivity of the active ingredient in the formulation was determined, after extraction from the formulation, by HPLC and LS measurement. The identity of the active ingredient used for the formulation was confirmed by EI-MS (appendix 3) and ^{1}H-NMR (appendix 4).

B. Reference Compounds

The structural formulae of the reference compounds, used for identification of metabolites, and their R_f values in different solvent systems are given in Table 1. The reference compounds were obtained from [redacted] (6-chloronicotinic acid), [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.

C. Environmental and Growth Conditions of Apples

For the metabolism study a total of 80 apples on 4 trees of the variety Golden Delicious were used and for the translocation experiment 2 apples on 1 tree. The studies were conducted in the vegetation area (Building 6682) of the Institute of Metabolism Research, Monheim, Bayer AG. The plant containers were labelled with the study number (M 173 0295-7) and a radioactivity symbol. Water was applied to the soil in the pots so that an optimum growth of the plants was obtained. Plant protection and fertilisation measures were carried out and documented as were climatic details (appendix 5). The trees were planted in " Frimmersdorf" soil and details of the analysis of the soil are given in appendix 6.

IV. METHODS

A. Application

The stability of the parent compound in the application solutions was confirmed by TLC (SS I) before and after each application both for the metabolism (appendix 7) and translocation (appendix 8) experiments.

A.1. Metabolism Experiment

A total of 3 applications were made to the 80 apples, the first on June 27, the second on July 25 and the third on August 22 all in 1989. For the 3 applications water (16.1 ml) was added to the formulated radiolabelled NTN 33893. From this an aliquot (10 µl) was taken to check the stability of the active ingredient in the application solution. Aliquots (2 x 100 µl) were then applied uniformly to each of the 80 apples using an Eppendorf syringe with a tuft of hair attached to the tip. The following amounts were applied:-

<u>1st Application</u>

33.0 mg WP 25.1, 8.283 mg a.i., 28.286 MBq was added to water (16.1 ml). Total amount applied to each apple (2 x 100 µl) = 409.9 µg WP 25.1, 102.9 µg a.i., 0.351 MBq.

<u>2nd Application</u>

30.3 mg WP 25.1, 7.605 mg a.i., 25.971 MBq was added to water (16.1 ml). Total amount applied to each apple (2 x 100 µl) = 376.4 µg WP 25.1, 94.5 µg a.i., 0.323 MBq.

<u>3rd Application</u>

32.6 mg WP 25.1, 8.183 mg a.i., 27.945 MBq was added to water (16.1 ml). Total amount applied to each apple (2 x 100 µl) = 405.0 µg WP 25.1, 101.7 µg a.i., 0.347 MBq.

Therefore, the total amount applied to each apple during the study was the sum of the above 3 applications = 1.191 mg WP 25.1, 0.299 mg a.i., 1.021 MBq.

A.2. Translocation Experiment

The applications for the translocation experiments were made on the same days as for the metabolism experiment. At each application date 2 experiments were conducted each with one apple and the nearest 5 leaves to it. At the time of application and during the course of the experiment the apples were partly protected with plastic to minimise direct contamination i) during application and ii) later by condensation of volatilised radioactivity.

At each application date water (2.05 ml) was added to the formulated radiolabelled NTN 33893. From this an aliquot (10 µl) was taken and used for a stability check of the application solution. Aliquots (2 x 100µl) were then applied to each of 10 leaves (5 in each experiment) in the same way as the metabolism experiment. The following amounts were applied:-

<u>1st Application</u>

2.1 mg WP 25.1, 0.527 mg a.i., 1.800 MBq was added to water (2.05 ml). Total amount applied to each leaf (2 x 100 µl) = 204.9 µg WP 25.1, 51.4 µg a.i., 0.176 MBq.

<u>2nd Application</u>

2.1 mg WP 25.1, 0.527 mg a.i., 1.800 MBq was added to water (2.05 ml). Total amount applied to each leaf (2 x 100 µl) = 204.9 µg WP 25.1, 51.4 µg a.i., 0.176 MBq.

<u>3rd Application</u>

3.2 mg WP 25.1, 0.803 mg a.i., 2.742 MBq was added to water (2.05 ml). Total amount applied to each leaf (2 x 100 µl) = 312.2 µg WP 25.1, 78.4 µg a.i., 0.268 MBq.

Therefore, the total amount applied to each leaf during the study was the sum of the above 3 applications = 722.0 µg WP 25.1, 0.181 mg a.i., 0.620 MBq.

B. Sampling and Storage

All samples were deep frozen at -20°C until analysis.

B.1. Metabolism Experiment

<u>Day 0 (22/8/1989):</u>

A total of 10 apples (1362.55 g) were taken and dipped in methanol (3 x 500 ml). The apples were then separated into peel and pulp and stored. Before extraction the peel was macerated in liquid nitrogen, divided into 6 portions (5 x ca. 25 g, 1 x ca. 150 g) and then stored until analysis. The pulp was cut into small pieces, macerated with liquid nitrogen and then divided into 7 portions (5 x ca. 130-155 g, 1 x ca. 315 g, 1 x ca. 76 g) before storage.

<u>Day 14 (5/9/1989):</u>

A total of 70 apples (10654.28 g) were sampled and divided into 4 different batches:-

Batch 1: 20 apples (3279.16 g) for the first analysis were processed as for day 0 samples (methanol solution 2905 ml, peel 508.90 g, pulp 2758.58 g).

Batch 2: 19 apples (3397.97 g) were processed as for day 0 samples and kept as reserve (methanol solution 2914 ml, peel 529.86 g, pulp 2861.57 g).

Batch 3: 30 apples (3751.85 g) were cut into small pieces and stored in portions (ca. 100-125 g) for the storage stability study and validation of the residue method.

Batch 4: 1 apple (225.3g) was kept in reserve for autoradiography (not reported).

B.2. Translocation Experiment

The 2 apples (152.83 g and 141.07 g) and 10 (2 x 5) leaves (7.2 g and 5.83 g) from the 2 experiments were separately collected on day 14 (5/9/1989), the day of harvest. The apples were separated into peel and pulp before storing at -20°C.

C. Extraction

C.1. Peel and Pulp Samples for Metabolism Experiment

After being homogenised in liquid nitrogen the peel and pulp samples were extracted in the same way. The samples were macerated with methanol/water(1:1, 1x) and methanol (2x). The radioactivity in the extracts and residues was determined. The extracts were combined and partitioned against n-hexane. The n-hexane phase, which contained no radioactivity, was discarded. The methanol was evaporated from the methanol/water phase and the residual water phase added to an XAD 4 resin column. The column was eluted with water followed by methanol. The water eluate, which contained no radioactivity, was discarded. The methanol eluate was concentrated, water added, the remaining methanol removed and the residual water phase partitioned against ethyl acetate. The radioactivity in the water and ethyl acetate phases was determined. An extraction scheme is given in figure 1.

C.2. Leaves, Peel and Pulp for Translocation Experiment

For each experiment the 5 treated leaves were combined, macerated in liquid nitrogen and then air dried. Aliquots were taken from this residue and combusted.

The apples were separated into peel and pulp and the individual plant fractions extracted with methanol. The amount of radioactivity in the methanol extract was determined by LSC. The residue was air dried and aliquots combusted.

D. Purification (XAD 4) of the Methanol/Water Extracts for TLC Analysis

The aqueous remainder, obtained after evaporation of methanol from the methanol/water extracts of peel and pulp, were purified on an XAD 4 resin (Serva Heidelberg, 0.2 - 0.4 mm) column prior to TLC analysis. The resin (50 g) was pre-washed with methanol followed by water. The aqueous sample (50 ml) was added to the

column and the column eluted with water (250 ml, water eluate) followed by methanol (250 ml, methanol eluate).

E. 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)

The radioactive compounds were detected using a Linear Analyser (Raytest, TM 3000) or by X-ray film (Curix RP-1, Agfa-Gevaert). The reference compounds used in co-chromatography were visualised by UV light (254 nm).

F. High Performance Liquid Chromatography (HPLC)

HPLC Instrument	:	Varian 5000
Column	:	RP 8, 250 x 4 mm, 10µm (Merck)
Gradient	:	Methanol/Water, 0-50 % in 60 min
Flow Rate	:	1 ml/min
Fraction Collector	:	Isco Model Foxy
Detector	:	Raytest "Ramona D" Radioactive Flow-Through Detector

G. Spectroscopy

G.1. Nuclear Magnetic Resonance (NMR)

The ^{1}H-NMR spectra were recorded on a Bruker AC 300 spectrometer, the samples were dissolved in CD_3OD (EGA) for analysis.

G.2. Mass Spectrometry (MS)

The electron impact (EI) probe mass spectra were recorded on a Finnigan 8230 at 70 eV with a source temperature of 200°C.

H. Radioactivity Measurement

The radioactivity in liquid samples was determined by LSC, technical details of the measurement of radioactivity are given in appendix 9.

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

The quantitative estimation of the radioactivity on TLC plates was done by scraping off the individual radioactive zones which had been located by autoradiography. These samples were then suspended in a scintillation cocktail and measured by LSC.

I. Storage Stability Study

After being chopped into small pieces the apples (day 14) were stored at -20°C. Samples were extracted 1, 192, 274, 332, 590 and 728 days after the beginning of the storage period. The parent compound and main olefine metabolite were quantitatively determined in the extracts by TLC. The extraction procedure is outlined below and an extraction scheme is given in figure 2.

The apple samples were macerated with methanol/water (1:1) followed by methanol (2x), the extracts were combined and partitioned against n-hexane. The n-hexane phase was removed and the methanol, from the methanol/water extract, evaporated. The residual aqueous phase was partitioned against ethyl acetate and the resultant organic phase concentrated. Only the ethyl acetate phase was further investigated by 1-dimensional TLC using SS II.

V. RESULTS AND DISCUSSION

A. Metabolism Experiment

A.1. Total Residues and Distribution of Radioactivity

The total residues in the apples at days 0 and 14 were determined by summation of the radioactivity in the surface wash solution, in the peel and pulp extracts and in the peel and pulp solids and then calculated as active ingredient equivalents. This gave total residues of 1.45 mg/kg at day 14 and 1.76 mg/kg at day 0 (table 2).

Of the applied radioactivity (10.21 MBq/10 apples, 20.42 MBq/20 apples) approximately 80 % was detected in the day 0 (8.19 MBq, 1.76 mg/kg, 80.2 %) and day 14 (16.17 MBq, 1.45 mg/kg, 79.2 %) samples indicating that a low loss of radioactivity had occurred during the application period of 56 days (Table 3). After surface washing the apples and extraction of the separated peel and pulp the vast majority of the recovered radioactivity was in the surface wash solution (Table 3) for both day 0 (74.2 %, 1.31 mg/kg) and day 14 (64.9 %, 0.94 mg/kg) samples. Of the remaining radioactivity more was found in the peel (15.9 %, 0.28 mg/kg day 0; 21.1 %, 0.31 mg/kg day 14) as compared to the pulp (9.9 %, 0.17 mg/kg day 0; 14.0 %, 0.20 mg/kg day 14). The major difference observed between the day 0 and day 14 results was that a decrease had occurred in the radioactivty content of the surface wash solution with a corresponding increase in the radioactivity in the peel and pulp. Nearly all of this increase was attributed to an increase in the content of the methanol eluate from the XAD 4 column of the methanol/water extract of both peel and pulp (table 3).

For details of the raw data for the total residues see appendices 10 (day 0) and 11 (day 14).

A.2. Quantitation and Identification of Metabolites

Metabolites in surface wash solution

Quantitation of the proportions of the metabolites in the surface wash solution was achieved using 1-dimensional TLC (either SS I or SS IV, figures 3 and 4). Components 20-24 and 26-29 were determined using SS I and component 25 SS IV. The raw data for the quantitative determination of the metabolites in the surface wash solution are given in appendix 12.

The main component (table 4) of the surface wash solution was unchanged parent compound (1.163 mg/kg, day 0; 0.805 mg/kg, day 14). This was the only component present in this fraction in a sufficient quantity to be isolated by HPLC (figure 5) and identified (MS figure 6, ^{1}H-NMR figure 7).

The Electron Impact spectrum (figure 6) showed a low intensity molecular ion at m/z 255, fragmentation of the nitro group (46 mass units) led to the base ion at m/z 209, the following chlorine fragmentation gave an ion at m/z 173. The chloropicolyl fragment was also observed at m/z 126.

In the ^{1}H-NMR spectrum (figure 7) the signals were assigned in the following way: H 2 of the aromatic rings at 8.35 ppm (doublet) , H 4 at 7.82 ppm (double doublet) and H 5 at 7.46 ppm (doublet) with coupling constants $J_{(2,4}$ of 2.6 Hz and $J_{(4,5}$ of 8.5 Hz. The methylene group produced a singlet at 4.54 ppm and both methylene groups in the five membered ring produced a multiplet at 3.73 and 3.55 ppm.

Both the mass and NMR spectra of component 1 showed good agreement with the spectra of the non-radiolabelled reference parent (compound Figures 8 and 9).

All other metabolites in the surface wash solution were present in such low amounts (< 0.031 mg/kg) that isolation and subsequent identification (MS or NMR) was not attempted. Identification of the metabolites was therefore achieved by co-chromatography of

the surface wash solution with authentic reference compounds using two different 2-dimensional TLC systems (figures 3 and 4) and HPLC (figure 10). The main metabolites identified (table 4) in this way were, for both day 0 and day 14, the 5-hydroxy compound IV (0.030 and 0.025 mg/kg, resp.), the urea III (0.024 and 0.024 mg/kg, resp.) and the guanidine II (0.023 and 0.011 mg/kg, resp.). Other metabolites present in still smaller amounts were the dihydroxy compound VII and the nitrosimine compound VIII. Also present in very small quantities (< 0.019 mg/kg) were three unknown metabolites and some unidentified radioactivity at the origin.

The metabolite identified as the 5-hydroxy compound (IV) probably contained the 4-hydroxy compound (V) since in a tomato stem injection experiment the presence of the 4-hydroxy form, as well as the 5-hydroxy form, was proven spectroscopically (Dräger, Brauner and Bornatsch, 1989). Under the chromatographic conditions used these two isomers were not separated.

<u>Metabolites in peel</u>

The majority of the radioactivity detected (see table 3) in the peel (15.9 %, day 0; 21.1 %, day 14) was in the methanol eluate (13.7 %, day 0; 18.1 %, day 14) from the XAD 4 column of the methanol/water extract and therefore the water phase was not further investigated. After adding water to the methanol eluate and evaporation of the methanol, the aqueous remainder was partitioned against ethyl acetate and the results are given in Table 3. The ethyl acetate phase was quantitatively analysed by two 1-dimensional TLC systems, SS II for components 1-4 and 6 and SS III for component 5 (figures 11, 12). The water phase was quantified using only one 1-dimensional TLC system, SS IV (figure 13). The results are given in tables 5 (mg/kg) and 6 (%). The metabolites in the phases were identified by co-chromatography of the solution with reference compounds using two different 2-dimensional TLC systems for the ethyl acetate phase (figures 11 and 12), one 2-dimensional TLC system for the water phase (figure 13) and HPLC (figure 14, ethyl acetate phase only).

The main component in the ethyl acetate phase was parent compound I (6.0 %, 0.106 mg/kg, day 0; 6.9 %, 0.100 mg/kg, day 14) with the olefine VI as the major metabolite (1.6 %, 0.029 mg/kg, day 0; 2.1 %, 0.030 mg/kg, day 14). The 5-hydroxy (IV), dihydroxy (VII) and guanidine (II) compounds were also shown to be present but in very low amounts (all < 0.016 mg/kg). There were also 3 unidentified metabolites which in total came to 0.027 mg/kg or less. In the water phase only 2 of the 21 metabolites detected were identified. These were the guanidine NTN 33823 (II) (0.006 mg/kg, day 0; 0.022 mg/kg, day 14) and the glucoside RBN 1114 (X) (0.019 mg/kg, day 0; 0.027 mg/kg, day 14). The concentrations of the remaining 19 metabolites were very low, the highest being 0.010 mg/kg for day 0 and 0.013 mg/kg for day 14, hence further identification of these metabolites was not attempted.

The raw data for the quantitative determination of the metabolites in the methanol eluate of the XAD-4 column of the peel extract are given in appendix 13.

Metabolites in pulp

The pulp samples were processed in exactly the same way as the peel samples and thus only the ethyl acetate and water phases obtained from partitioning of the methanol eluate from the XAD-4 column were investigated. The quantitative distribution (see tables 5 and 6) of the radioactivity between the metabolites in the ethyl acetate phase, as determined by two 1-dimensional TLC systems as for the peel samples (SS II and SS III; figures 15 and 16), was almost identical to that obtained for the peel sample. The parent compound (I) was the main component (4.9 %, 0.087 mg/kg, day 0; 6.3 %, 0.091 mg/kg, day 14) and the olefine (VI) the major metabolite (2.7 %, 0.048 mg/kg, day 0; 3.6 %, 0.052 mg/kg, day 14).

The amount of radioactivity in the water phase derived from the pulp was very low. The total residue for day 0 was only 0.018 mg/kg, 1.0 % and that for day 14 only 0.027 mg/kg ,1.9 % and

each residue contained 21 metabolites. The only 2 metabolites identified were the guanidine NTN 33823 (II) (0.1 %, 0.001 mg/kg, day 0; 0.2 %, 0.003 mg/kg, day 14) and the glucoside RBN 1114 (X) (0.1 %, 0.002 mg/kg, day 0; 0.3 %, 0.004 mg/kg day 14). As there was so little radioactivity in these water phases no further attempts were made to identify the metabolites.

Identification of all metabolites in the pulp fractions was achieved by co-chromatography of the phases with reference compounds. For the ethyl acetate phase two 2-dimensional TLC systems were used (figures 15 and 16) and for the water phase one 2-dimensional system (figure 17). The identification of the metabolites in the ethyl acetate phase was further confirmed by HPLC (figure 18).

The raw data for the quantitative determination of the metabolites are given in appendix 13.

B. Translocation Experiment

During the course of these two experiments nearly half (44.06 and 47.77 %) of the applied radioactivity was lost by volatilisation (see table 7). In both experiments all but 0.06 % of the recovered radioactivity (55.90 and 52.22 %) was found in the leaves. This indicated that a negligible amount (0.06 % or less) of the applied radioactivty had translocated from the leaves into the peel or pulp of the fruit.

In this experiment the leaves were partly protected with plastic to try to ensure that any radioactivity detected in the apples was present as a direct result of translocation from the leaves. The possibility of transportation of radioactivity from the treated leaves to the target apple, via the air, was not totally eliminated. However, this is not considered a major factor since the distance between the target apple and the nearest treated leaf was > 10 cm. If such movement of radioactivity did occur then the % of radioactivity recovered in the apples (i.e. 0.06 %) arising as a direct consequence of translocation would have been even lower.

The raw data for the translocation experiment, including weights and volumes of solvents used, are given in appendix 14.

C. Storage Stability of Parent Compound and Main Metabolites

The results of the storage stability study are given in tables 8 (%) and 9 (mg/kg) and indicated that no change had occurred in the proportions of the parent compound and its main metabolite during storage at -20^{o}C over a period of 728 days. During storage the amount of radioactivity in each fraction (n-hexane, ethyl acetate, water and residue) was monitored and these values (see Tables 8 and 9) remained relatively constant. Since over 85% of the radioactivity was present in the ethyl acetate fraction at all sampling dates (days 1, 192, 274, 332, 590, 728) only this fraction was further analysed by TLC (figure 19). The amounts of the parent compound (I), the main olefine metabolite (VI) and the combined remaining metabolites were determined (Tables 8 and 9). These results ranged, for the parent compound, from 1.23 mg/kg (71.2 %) to 1.42 mg/kg (79.1 %) and for the main olefine metabolite from 0.10 mg/kg (5.59 %) to 0.13 mg/kg (6.88 %). No significant change in the amounts of these components was observed.

The raw data for the distribution of the radioactivity between the different phases are given in appendix 15 and that for the quantitative determination of the metabolites in appendix 16.

VI. CONCLUSIONS

The metabolism of NTN 33893 in apples proceeded along the same three basic pathways as previously reported for other plant studies, namely:-

a) reduction and loss of the nitro group

b) hydroxylation of the dihydroimidazole ring followed by loss of water

c) metabolism to 6-chloropicolyl alcohol followed by rapid glucoside formation.

All identified metabolites contained the pyridinyl moiety. The comparison of the radioactivity determined by the 6-CNA method (Weber) and the sum of the metabolites of this study showed good agreement (83 % 6-CNA method and ca. 85 % sum of the identified metabolites). Therefore, the residue method of Weber, based on 6-chloronicotinic acid, covers all relevant metabolites.

All metabolites, except the 6-chloropicolyl alcohol conjugate, contained both rings and accounted for ca. 83 % of the recovered radioactivity in the day 14 sample. Therefore, a single metabolite derived from the dihydroimidazole ring after cleavage of the two rings is not expected to occur in significant amounts. Thus, a further study on apples with a second label in the dihydroimidazole ring is not necessary.

In the metabolism experiment the main part (64.9 %) of the total residue (1.45 mg/kg) of the harvest sample (day 14) was in the surface wash solution. This result indicated that up-take and translocation of NTN 33893 were slow. Of the total recovered radioactivity in the day 14 sample nearly 70 % was unchanged parent compound and this showed that NTN 33893 was not rapidly metabolised in and on apples. A total of 85.5 % of the recovered radioactivity (day 14), comprising the parent compound and seven major metabolites, was identified. A further 26 metabolites were detected, totaling 11.1 % of the recovered radioactivity, but owing to their low concentrations were not identified.

In a separate translocation experiment less than 0.1 % of the radioactivity applied to the surface of five leaves had translocated into the peel and pulp of apples. This supported the finding of the metabolism study that translocation of NTN 33893 was slow.

The storage stability study (-20°C) of the active ingredient and main olefine metabolite in the fruit samples showed that both components were stable under the storage conditions used during the course of the experimental work (728 days).

A proposed degradation pathway for NTN 33893 in apples is given in figure 20.

VII. SIGNATURES

[redacted]

27/2/1992

Date

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Date

27/2/1992

Date

27/2/1992

Date

14/5/1992

Date

Acknowledgement

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

VIII. QAU Statement

[redacted]

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X. TABLES

Table 1: Structures and R_f values of reference compounds

Solvent Systems

SS I :	ethyl acetate/propan-2-ol/water	65:23:12
SS II :	ethyl acetate/toluene/methanol/acetic acid	80:20:20:1
SS III:	butan-1-ol/acetic acid/water	80:20:20
SS IV :	chloroform/methanol/acetic acid/water	65:25:3.5:3.5

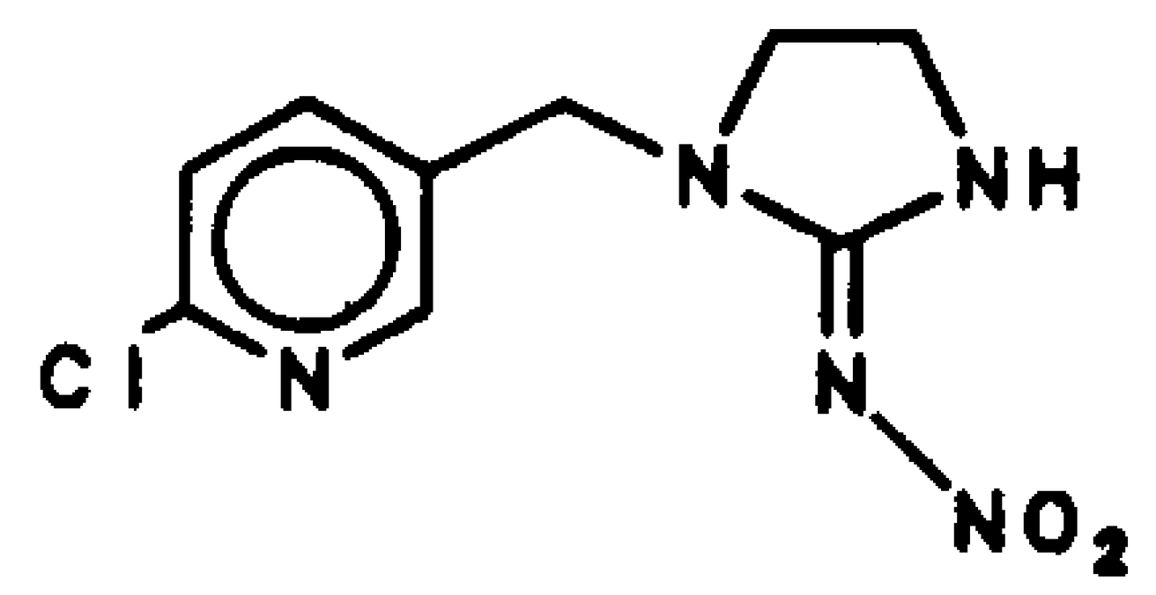
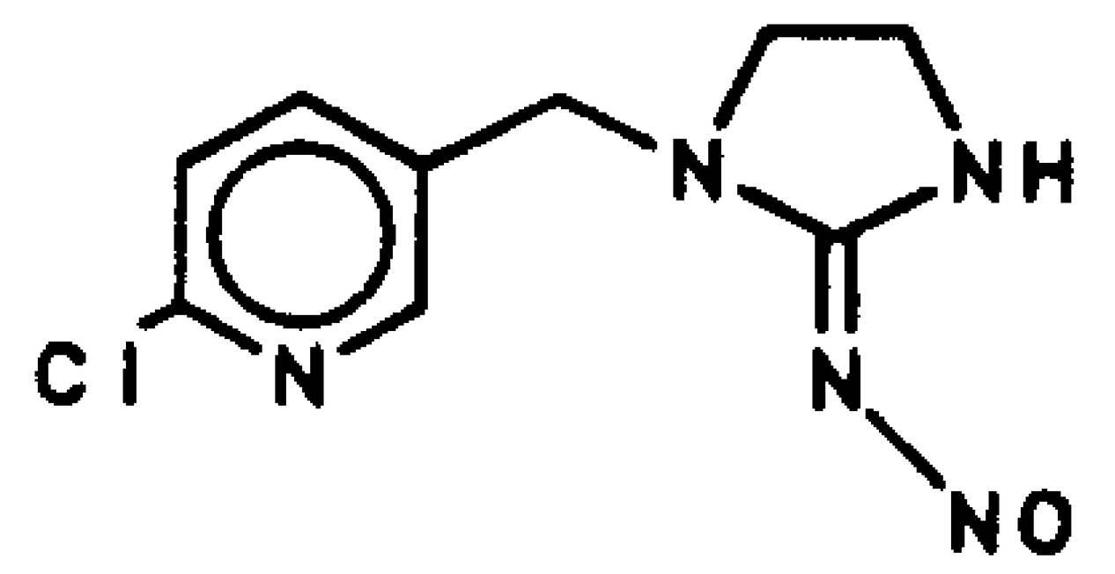
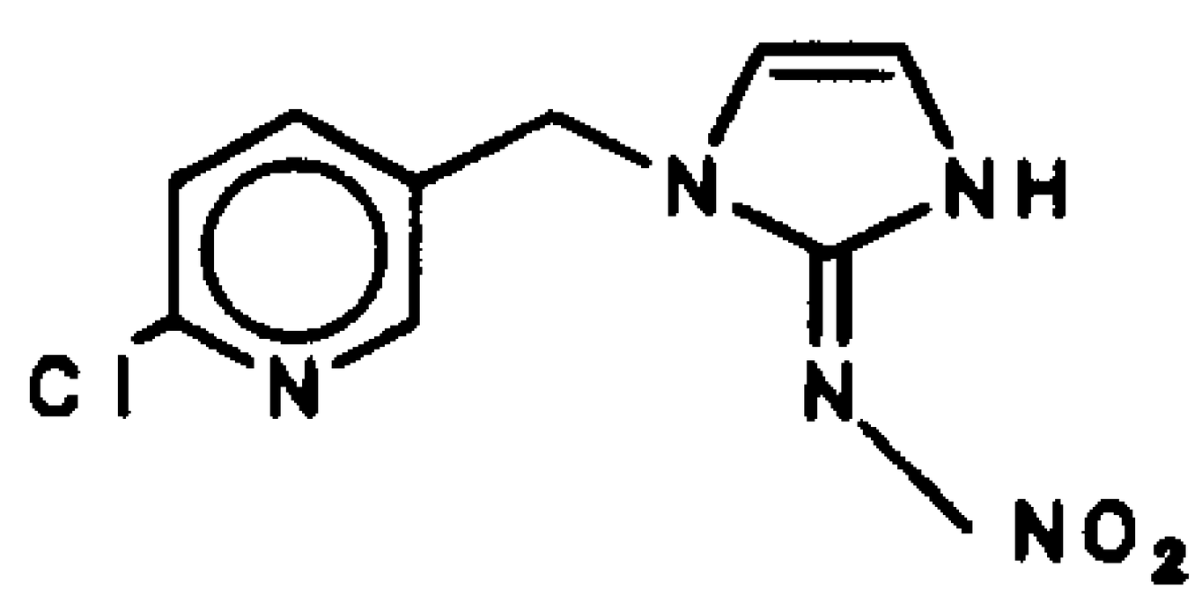
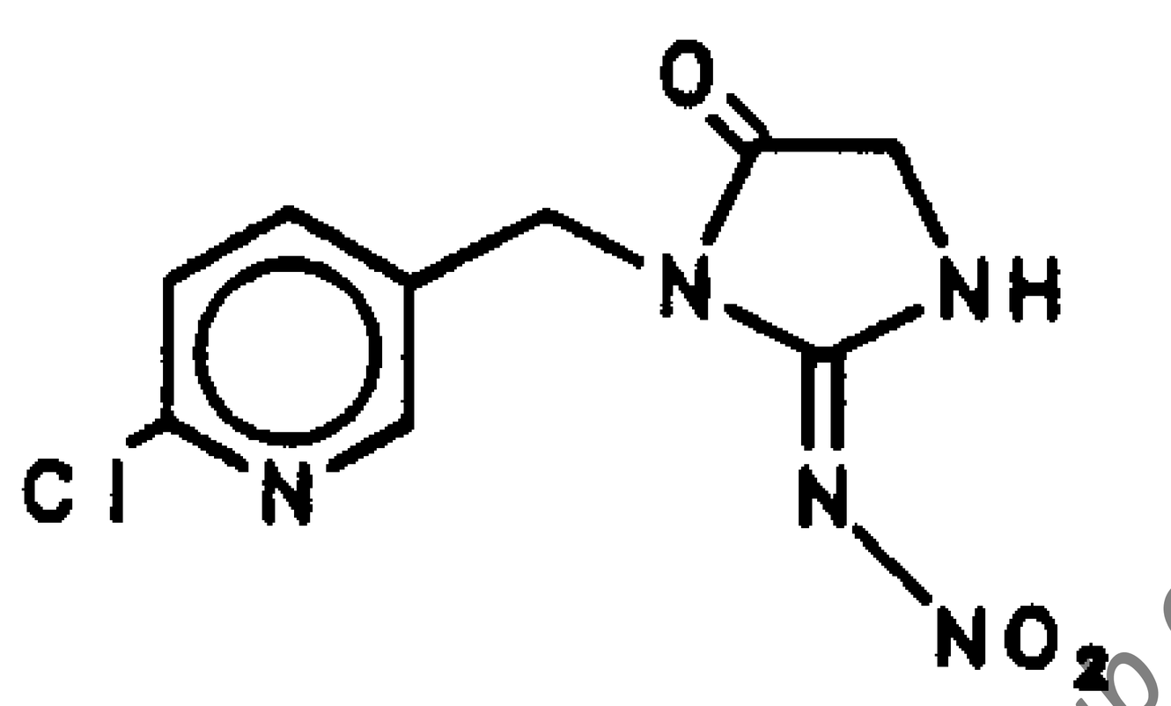
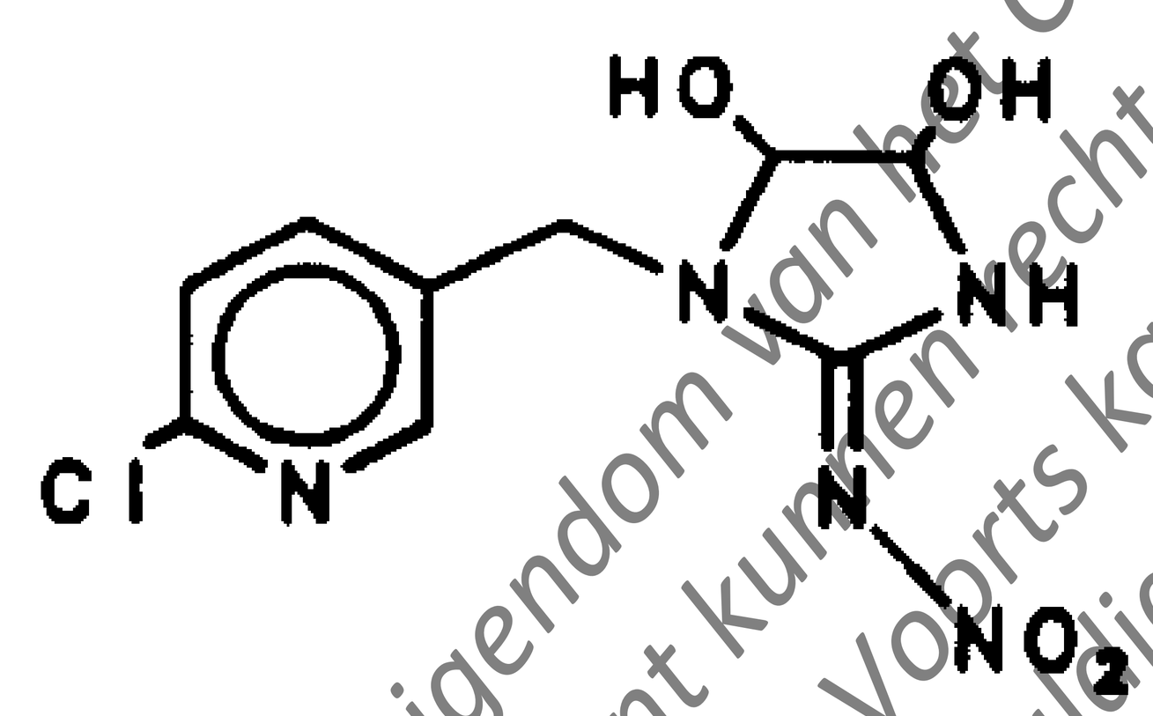
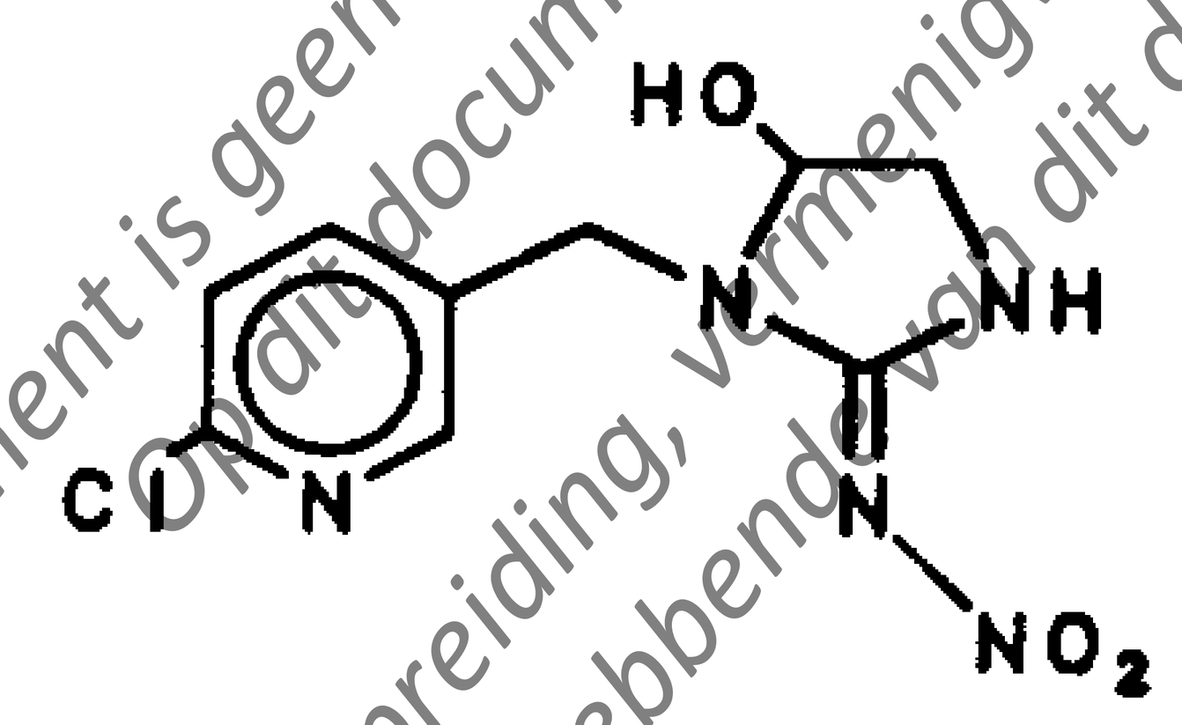
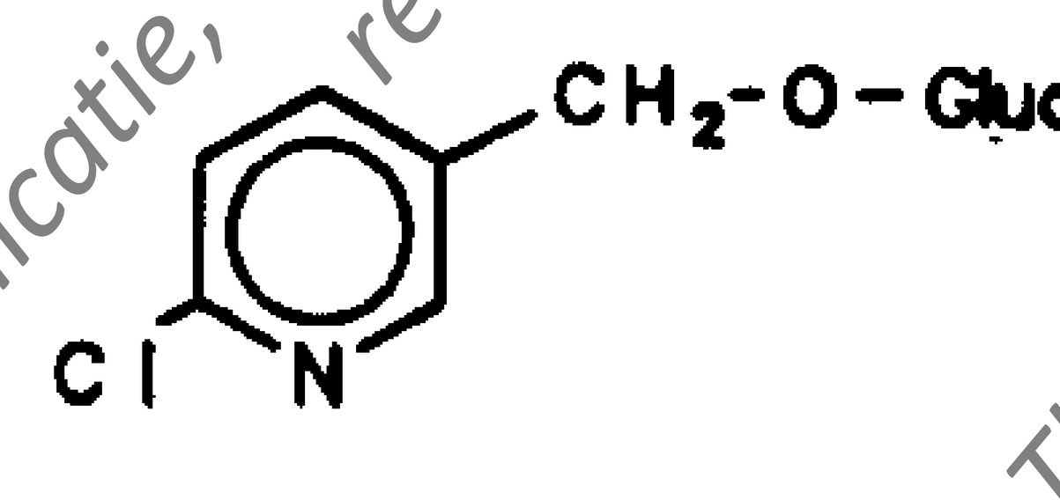
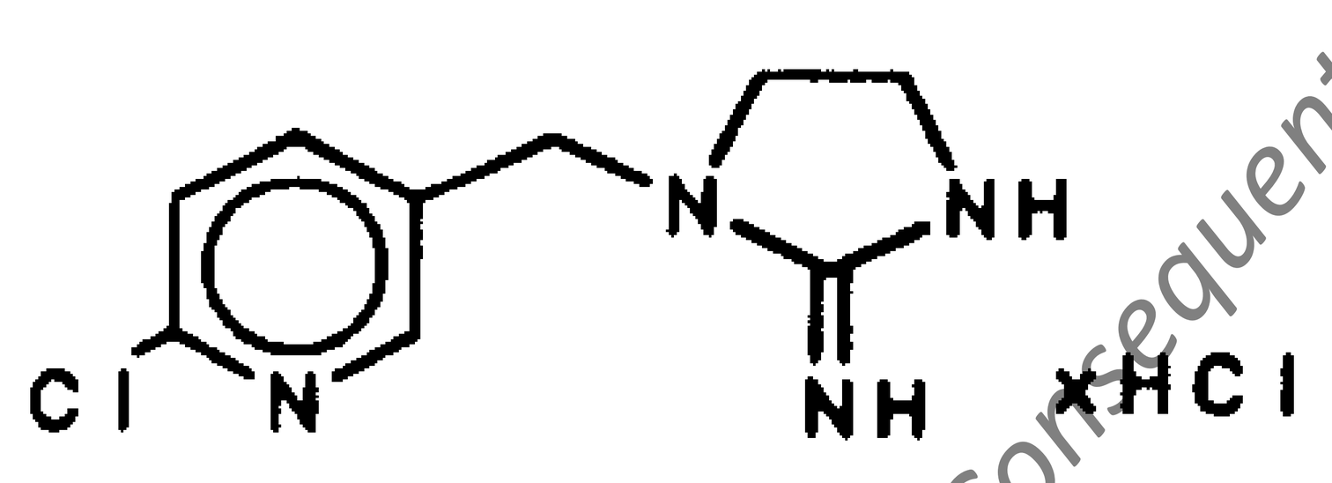
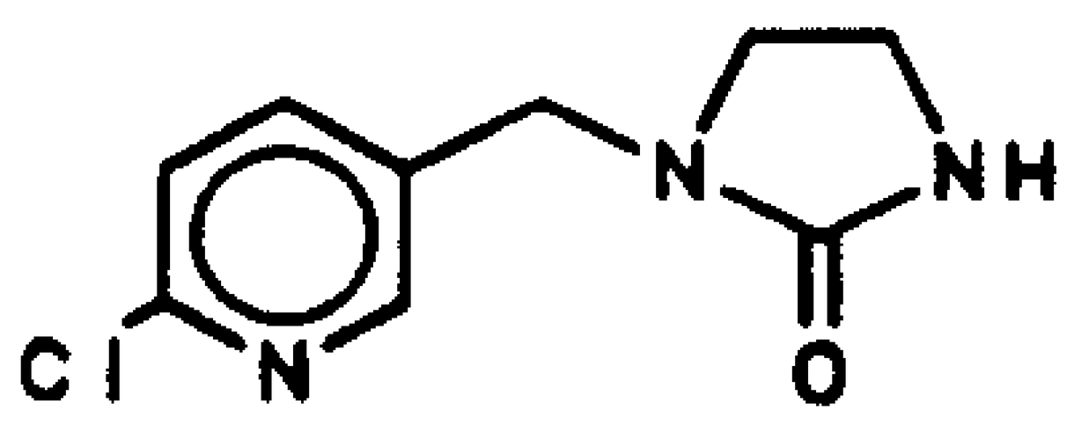
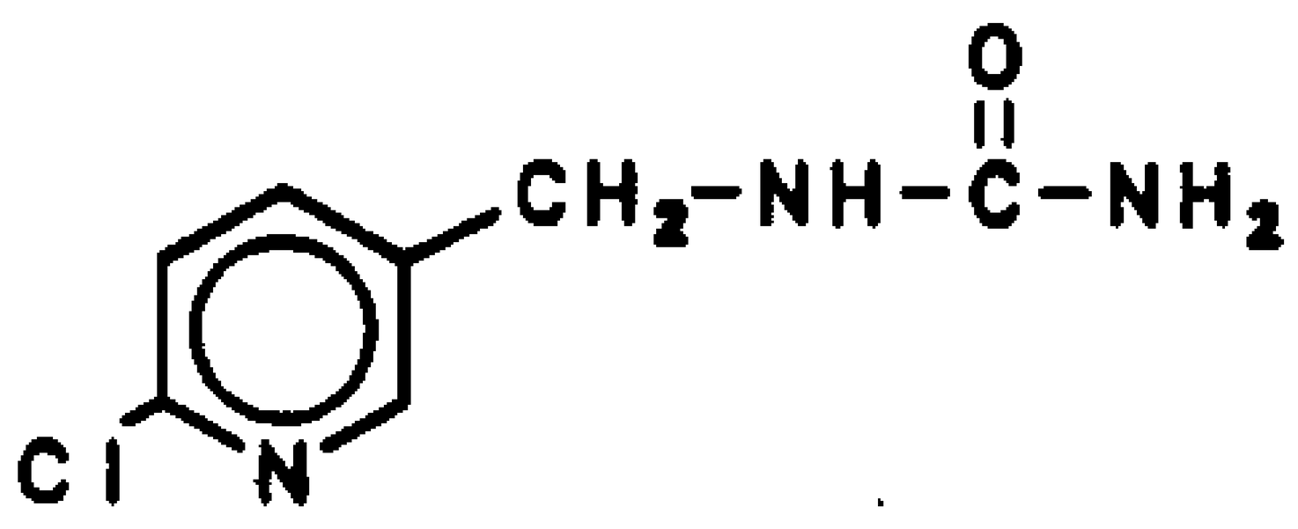
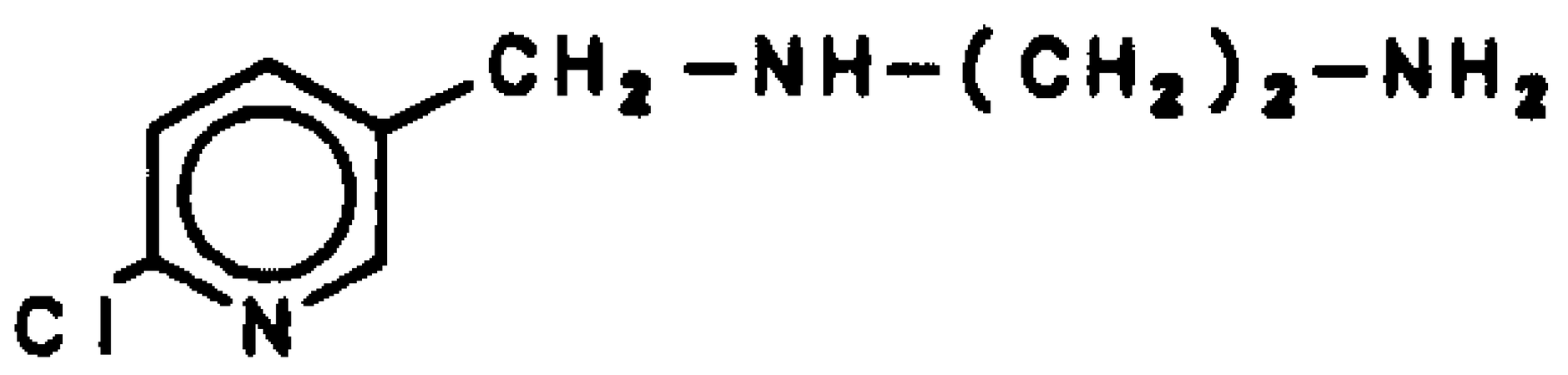
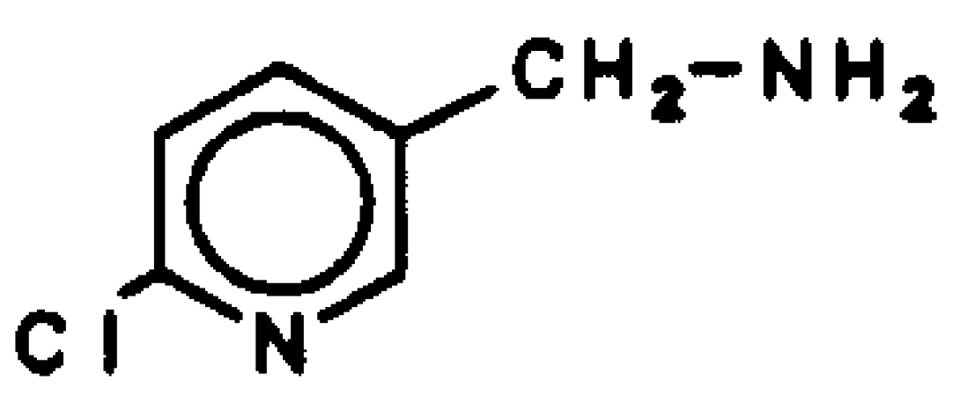
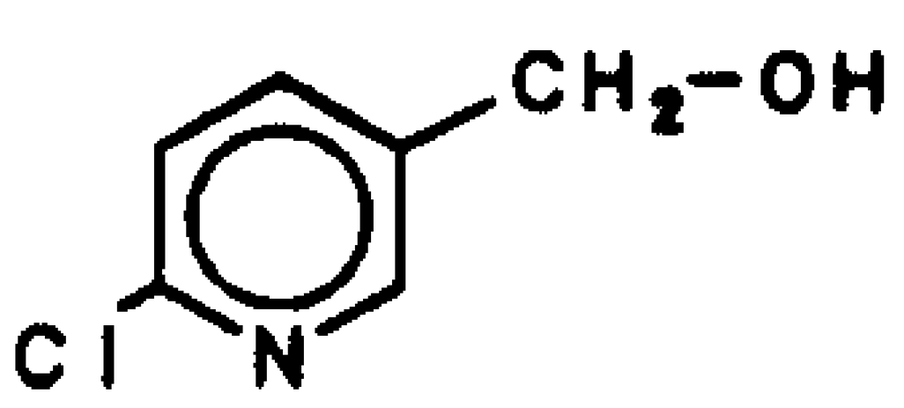
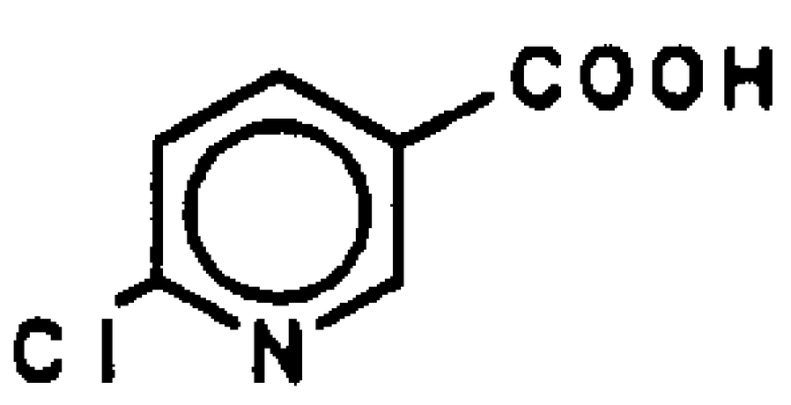
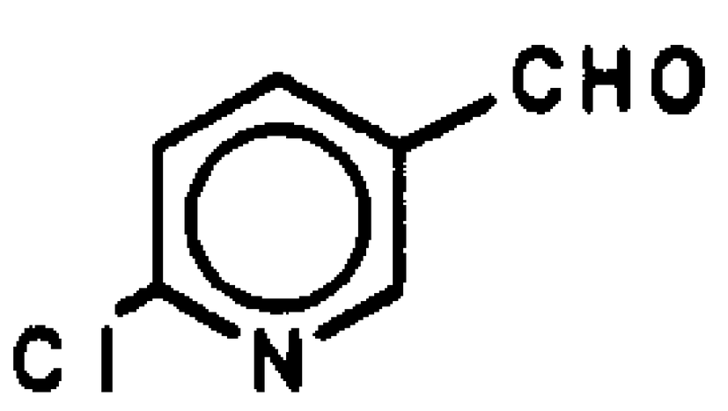
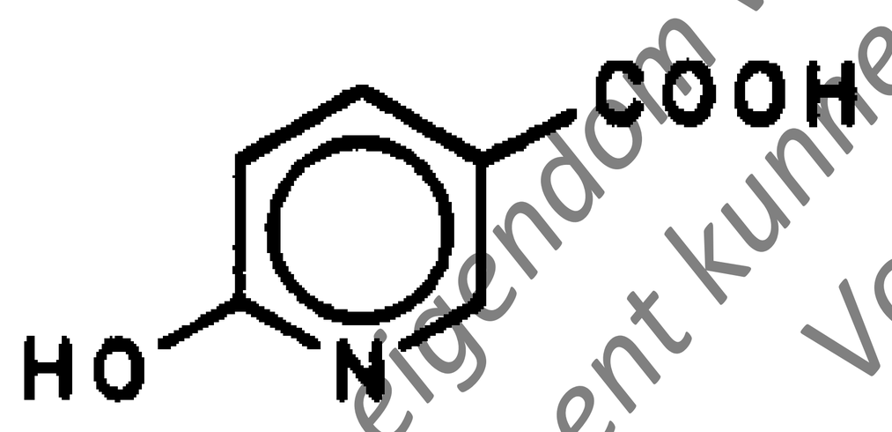
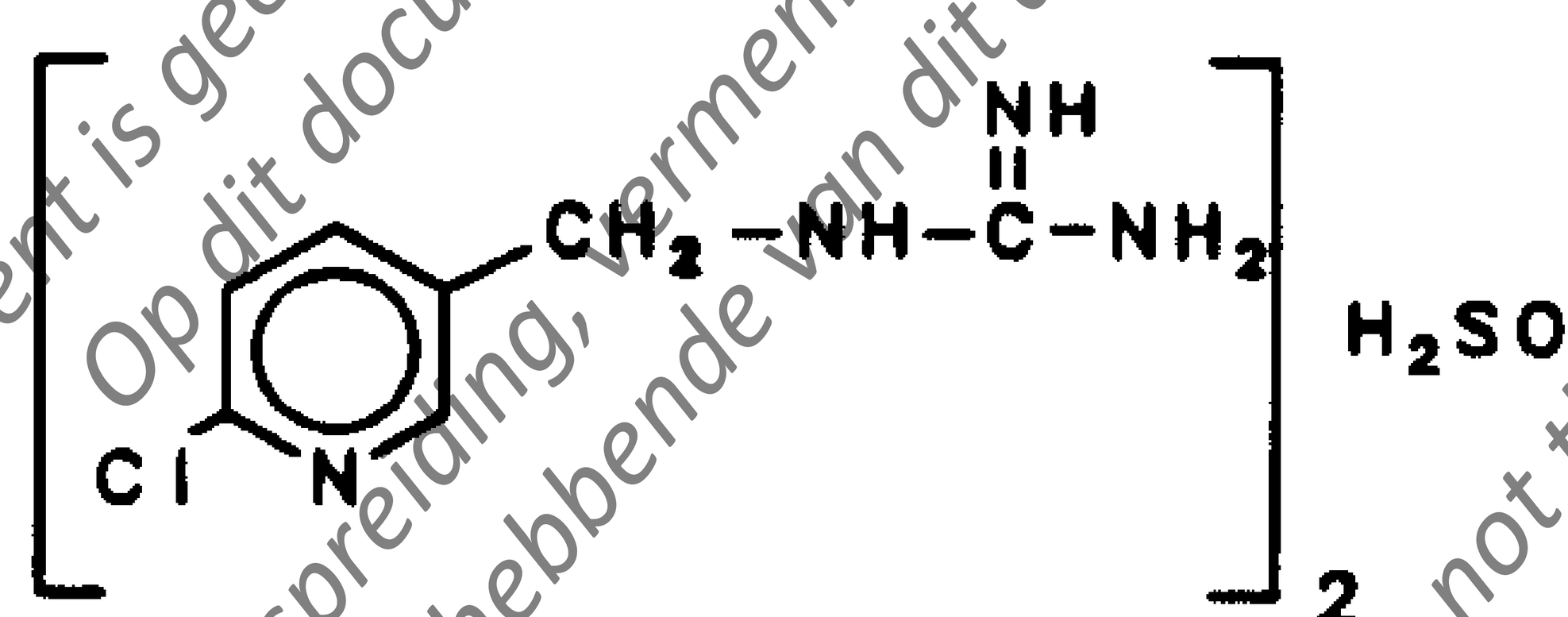
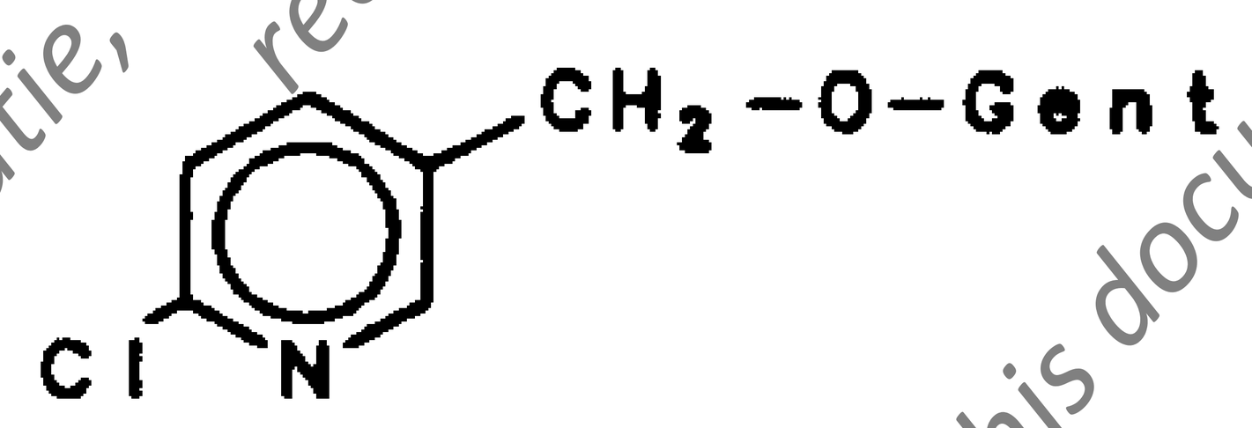
Compound		SS I	SS II	SS III	SS IV
	NTN 33893 (I) parent compound	0.80	0.70	0.64	0.91
	WAK 3839 (VIII) nitrosimine comp.	0.59	0.43	0.53	0.86
	NTN 35884 (VI) olefin compound	0.75	0.59	0.62	0.83
	WAK 3738 (XVI) 5-keto compound	0.94	0.88	0.83	0.90
	WAK 3772 (VII) dihydroxy compound	0.93	0.79	0.89	0.75
	WAK 4103 (IV) 5-hydroxy compound	0.89	0.75	0.78	0.85
	RBN 1114 (X) glucoside	0.60	0.39	0.54	0.56
	BEG 5322 (II) guanidine compound	0.05	0.02	0.24	0.53

Table 1 continued:

Compound		SS I	SS II	SS III	SS IV
	DIJ 9817 (III) urea compound	0.71	0.58	0.66	0.89
	NTN 36749	0.11	0.03	0.34	0.42
	DIJ 9646-2	0.02	0.00	0.11	0.13
	GSE 1478	0.13	0.05	0.37	0.44
	DIJ 9805 (XIII) 6-chloropicolyl alcohol	0.91	0.81	0.88	0.88
	6-chloro- (XII) nicotinic acid	0.39	0.41	0.81	0.82
	MAT 10249-D	0.95	0.89	0.94	0.95
	GBH 4315	0.22	0.18	0.71	0.54
	WAK 4126(XV) ring opened guanidine	0.05	0.00	0.42[2] 0.44	0.31
	gentiobioside[3]	nd[1]	nd[1]	0.33	0.18

1) nd = not determined
2) Two spots were observed under UV light (254 nm)
3) Radioactive reference compound isolated from tomato stem injection experiment (Dräger et al., 1989)

Table 2:

Quantitative distribution, in % of radioactivity recovered and in mg/kg NTN 33893 equivalents, of metabolites in apples (surface wash+peel+pulp)

Component			Day 0		Day 14	
			%	mg/kg	%	mg/kg
1+20	= NTN 33893	I	77.0	1.356	69.0	0.996
2+21	= 5-Hydroxy, WAK 4103	IV	2.2	0.038	2.7	0.039
3+22	= Dihydroxy WAK 3772	VII	0.9	0.014	1.1	0.016
4	= Olefine NTN 35884	VI	4.3	0.077	5.7	0.082
5+7+25	= Guanidine NTN 33823	II	2.6	0.045	2.4	0.038
8	= Glucoside RBN 1114	X	1.2	0.021	2.2	0.031
23	= Urea DIJ 9817	III	1.3	0.024	1.7	0.024
24	= Nitrosimine WAK 3839	VIII	0.6	0.011	0.7	0.010
Total identified			90.1	1.586	85.5	1.236
Components 6, 9-19, 26-29 = 26 unknown components			7.4	0.130	11.1	0.160
Unextractable residue			2.1	0.037	3.0	0.044
n-Hexane phase+Water phases XAD 4 of peel and pulp			0.4	0.006	0.4	0.005
Total			100	1.759	100	1.445

Table 3:

^{14}C-Residues in the different fractions of apples after surface washing and extraction of peel and pulp

Radioactivity recovered in the apple = 100 %
mg/kg in NTN 33893 equivalents

Fraction	day 0 10 apples = 1.36 kg %	 mg/kg	 MBq*	day 14 20 apples = 3.28 kg %	 mg/kg	 MBq*
Surface wash solution	**74.2**	**1.31**	**6.08**	**64.9**	**0.94**	**10.49**
Peel	**15.9**	**0.28**	**1.30**	**21.1**	**0.31**	**3.42**
n-hexane phase	<0.01	<0.001	<0.01	0.1	0.002	0.02
water eluate from XAD 4	0.2	0.003	0.02	0.2	0.002	0.02
methanol eluate from XAD 4	13.7	0.242	1.12	18.1	0.262	2.94
ethyl acetate phase	10.4	0.183	0.85	10.8	0.157	1.75
water phase	3.3	0.059	0.27	7.3	0.105	1.19
non-extractable residue	2.0	0.035	0.16	2.7	0.040	0.44
Pulp	**9.9**	**0.17**	**0.81**	**14.0**	**0.20**	**2.26**
n-hexane phase	0.1	0.002	0.01	<0.1	<0.001	<0.01
water eluate from XAD 4	0.1	0.001	<0.01	0.1	0.001	0.01
methanol eluate from XAD 4	9.6	0.169	0.79	13.6	0.197	2.20
ethyl acetate phase	8.6	0.151	0.71	11.7	0.170	1.90
water phase	1.0	0.018	0.08	1.9	0.027	0.30
non-extractable residue	0.1	0.002	0.01	0.3	0.004	0.05
Total	**100**	**1.76**	**8.19**	**100**	**1.45**	**16.17**

* amount applied = 10.21 MBq/10 apples, 20.42 MBq/20 apples

Table 4:

Quantitative distribution of metabolites in the surface wash solution

Component		Day 0		Day 14	
		%	mg/kg	%	mg/kg
20 = NTN 33893	I	66.1	1.163	55.8	0.805
21 = 5-Hydroxy,WAK 4103	IV	1.7	0.030	1.7	0.025
22 = Dihydroxy,WAK 3772	VII	0.5	0.008	0.4	0.006
23 = Urea,DIJ 9817	III	1.3	0.024	1.7	0.024
24 = Nitrosimine,WAK 3839	VIII	0.6	0.011	0.7	0.010
25 = Guanidine,NTN 33823	II	1.3	0.023	0.7	0.011
26 = 1 unknown metabolite		0.9	0.015	0.8	0.012
27 = 1 unknown metabolite		0.9	0.015	1.0	0.014
28 = 1 unknown metabolite		0.1	0.003	1.3	0.018
29 = Start activity		0.8	0.013	0.8	0.012
Sum of components 20-29		74.2	1.305	64.9	0.937

Components 20-24 and 26-29 were determined after one dimensional TLC in solvent system I

Component 25 was determined after one dimensional TLC in solvent system IV

Table 5:

Distribution of metabolites (mg/kg) in the methanol eluates from XAD-4 columns of the methanol/water extracts of peel and pulp

		Day 0		Day 14	
		Peel	Pulp	Peel	Pulp
		mg/kg		mg/kg	
Ethyl acetate phase					
1 = NTN 33893	I	0.106	0.087	0.100	0.091
2 = 5-Hydroxy WAK 4103	IV	0.003	0.005	0.008	0.006
3 = Dihydroxy WAK 3772	VII	0.003	0.003	0.004	0.006
4 = Olefine, NTN 35884	VI	0.029	0.048	0.030	0.052
5 = Guanidine, NTN 33823	II	0.015	<0.001	0.001	0.001
6 = 3 Unknown metabolites		0.027	0.008	0.014	0.014
Sum of components 1-6		0.183	0.151	0.157	0.170
Water phase					
7 = Guanidine NTN 33823	II	0.006	0.001	0.022	0.003
8 = Glucoside RBN 1114	X	0.019	0.002	0.027	0.004
9 = Start activity		0.004	0.001	0.003	0.001
10 = 1 Unknown metabolite		0.005	0.002	0.003	0.003
11 = 3 Unknown metabolites				0.004	
12 = 1 Unknown metabolite		0.004	0.003	0.006	0.005
13 = 1 Unknown metabolite		0.005	0.003	0.006	0.002
14 = 2 Unknown metabolites		0.010		0.013	
15 = 2 Unknown metabolites		0.005	0.001	0.002	0.002
16 = 4 Unknown metabolites			<0.001	0.005	0.002
17 = 1 Unknown metabolite			0.001	0.002	0.003
18 = 2 Unknown metabolites			0.002	0.004	0.001
19 = 1 Unknown metabolite		0.001	0.001	0.002	0.001
Sum of components 7-19		0.059	0.018	0.105	0.027
Total sum of components 1-19		0.242	0.169	0.262	0.197

Components were quantified using 1-dimensional TLC in the following systems:-components 1-4 and 6 in SS II, 5 in SS III and 7-19 in SS IV

Table 6:

Distribution of metabolites (%) in the methanol eluates from XAD 4 columns of the methanol/water extracts of peel and pulp

		Day 0 Peel %	Day 0 Pulp	Day 14 Peel %	Day 14 Pulp
Ethyl acetate phase					
1 = NTN 33893	I	6.0	4.9	6.9	6.3
2 = 5-Hydroxy WAK 4103	IV	0.2	0.3	0.6	0.4
3 = Dihydroxy WAK 3772	VII	0.2	0.2	0.3	0.4
4 = Olefine, NTN 35884	VI	1.6	2.7	2.1	3.6
5 = Guanidine, NTN 33823	II	0.9	<0.1	<0.1	<0.1
6 = 3 Unknown metabolites		1.5	0.5	0.9	1.0
Sum of components 1-6		10.4	8.6	10.8	11.7
Water phase					
7 = Guanidine NTN 33823	II	0.3	0.1	1.5	0.2
8 = Glucoside RBN 1114	X	1.1	0.1	1.9	0.3
9 = Start activity		0.3	<0.1	0.2	0.1
10 = 1 Unknown metabolite		}	}	0.2	}
11 = 3 Unknown metabolites		} 0.3	} 0.1	0.3	} 0.2
12 = 1 Unknown metabolite		0.3	0.2	0.4	0.3
13 = 1 Unknown metabolite		0.2	}	0.4	}
14 = 2 Unknown metabolites		0.5	} 0.1	0.9	} 0.2
15 = 2 Unknown metabolites		}	0.1	0.6	0.2
16 = 4 Unknown metabolites		}	<0.1	0.4	0.1
17 = 1 Unknown metabolite		} 0.3	0.1	0.1	0.2
18 = 2 Unknown metabolites		}	0.1	0.3	<0.1
19 = 1 Unknown metabolite		<0.1	0.1	0.1	0.1
Sum of components 7-19		3.3	1.0	7.3	1.9
Total sum of components 1-19		13.7	9.6	18.1	13.6

Components were quantified using 1-dimensional TLC in the following systems:-components 1-4 and 6 in SS II,5 in SS III and 7-19 in SS IV

Table 7:

Radioactivity content of the leaves, peel and pulp of apples following application of [^{14}C]NTN 33893 to apple leaves (translocation experiment)

100 % = Total radioactivity applied for each experiment (3.10 MBq for each experiment)

Plant Component	Experiment 1*		Experiment 2*	
	kBq	%	kBq	%
Leaves	1732.31	55.88	1617.07	52.16
Pulp	1.52	0.05	1.10	0.04
fraction				
methanol	1.30	0.04	0.75	0.03
residue	0.22	0.01	0.35	0.01
Peel	0.42	0.01	0.95	0.03
fraction				
methanol	0.32	0.01	0.75	0.02
residue	0.10	<0.005	0.20	0.01
Total	1734.25	55.94	1619.12	52.23

* for each experiment [^{14}C]NTN 33893 was applied to five leaves

Table 8. Distribution of radioactivity (in %) between the fractions and metabolites obtained from the storage stability study

Analysis	1st			2nd			3rd			4th			5th			6th		
Ext. Date	06/09/89			16/03/90			06/06/90			03/08/90			18/04/91			03/09/91		
Storage	1 day			192 days			274 days			332 days			590 days			728 days		
Run	No.1	No.2	Av.	No.1	No.2	Av.	No.1	No.2	Av.	No.1	No.2	Av.	No.1	No.2	Av.	No.1	No.2	Av.
n-Hexane phase	0.2	0.2	0.2	0.4	0.3	0.3	0.4	0.2	0.3	0.2	0.3	0.2	0.3	0.3	0.3	0.4	0.4	0.4
Ethyl acetate phase	86.1	85.5	85.8	89.8	91.2	90.5	85.4	90.2	87.8	90.6	89.6	90.1	85.9	86.0	86.0	87.6	87.5	87.5
NTN 33893(I)	71.9	71.2	71.5	77.0	77.3	77.2	73.2	74.7	73.9	79.1	76.8	78.0	71.6	74.8	73.2	69.7	75.7	72.7
Main metabol. Olefine(VI)	7.2	6.5	6.9	6.6	7.0	6.8	5.3	5.9	5.6	6.7	6.2	6.4	6.1	6.1	6.1	8.0	5.8	6.9
Other metabolites	7.0	7.8	7.4	6.2	6.9	6.5	6.9	9.6	8.3	4.8	6.6	5.7	8.2	5.1	6.7	9.9	6.0	7.9
Water phase	11.1	11.7	11.4	6.8	5.0	5.9	10.3	5.5	7.9	6.8	6.8	6.8	10.0	9.4	9.7	7.1	7.1	7.1
Not extractable	2.6	2.6	2.6	3.0	3.5	3.3	3.9	4.1	4.0	2.4	3.3	2.9	3.8	4.3	4.0	4.9	5.0	5.0
Total residue	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

Table 9. Distribution of radioactivity (in mg/kg) between the fractions and metabolites obtained from the storage stability study

Analysis	1st			2nd			3rd			4th			5th			6th		
Ext. Date	06/09/89			16/03/90			06/06/90			03/08/90			18/04/91			03/09/91		
Storage	1 day			192 days			274 days			332 days			590 days			728 days		
Run	No.1	No.2	Av.	No.1	No.2	Av.	No.1	No.2	Av.	No.1	No.2	Av.	No.1	No.2	Av.	No.1	No.2	Av.
n-Hexane phase	<0.01	<0.01	<0.01	0.01	<0.01	0.01	0.01	<0.01	0.01	<0.01	<0.01	<0.01	0.01	0.01	0.01	0.01	0.01	0.01
Ethyl acetate phase	1.56	1.48	1.52	1.52	1.47	1.49	1.55	1.58	1.56	1.63	1.62	1.62	1.51	1.58	1.54	1.56	1.65	1.60
NTN 33893(I)	1.30	1.23	1.27	1.30	1.25	1.27	1.32	1.30	1.31	1.42	1.39	1.41	1.25	1.37	1.31	1.24	1.42	1.33
Main metabol. Olefin(VI)	0.13	0.11	0.12	0.12	0.11	0.11	0.10	0.11	0.10	0.12	0.11	0.11	0.11	0.11	0.11	0.14	0.11	0.12
Other metabolites	0.13	0.14	0.13	0.10	0.11	0.11	0.13	0.17	0.15	0.09	0.12	0.10	0.15	0.10	0.12	0.18	0.12	0.15
Water phase	0.20	0.20	0.20	0.11	0.08	0.10	0.19	0.10	0.14	0.13	0.13	0.13	0.18	0.18	0.18	0.13	0.14	0.13
Not Extractable	0.05	0.05	0.05	0.05	0.06	0.05	0.07	0.07	0.07	0.04	0.06	0.05	0.07	0.08	0.07	0.09	0.10	0.10
Total residue	1.81	1.73	1.77	1.69	1.61	1.65	1.82	1.75	1.78	1.80	1.81	1.80	1.77	1.85	1.80	1.79	1.90	1.84

XI. FIGURES

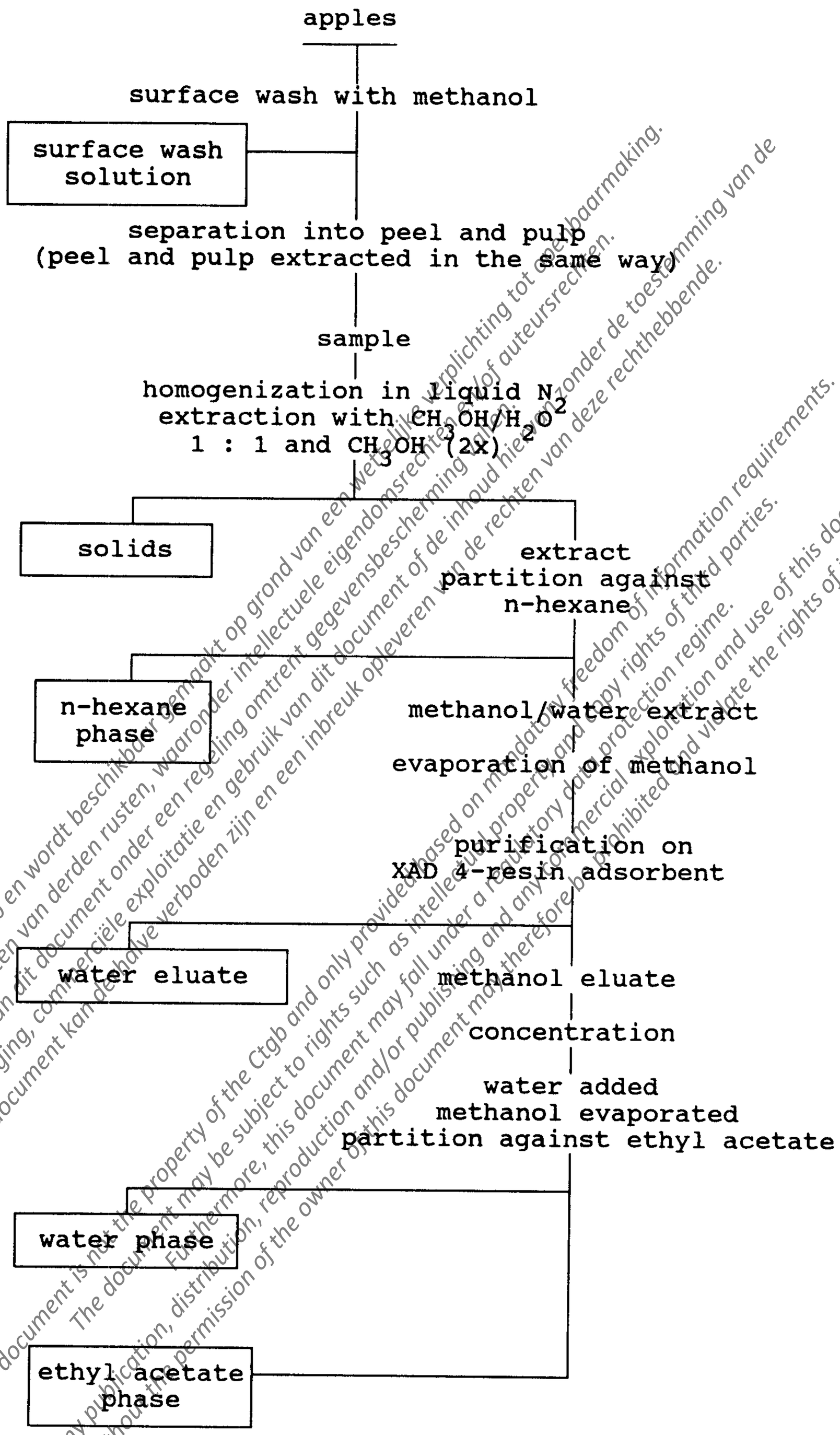


Figure 1: Scheme of the extraction procedure used for [^{14}C]NTN 33893 treated apples

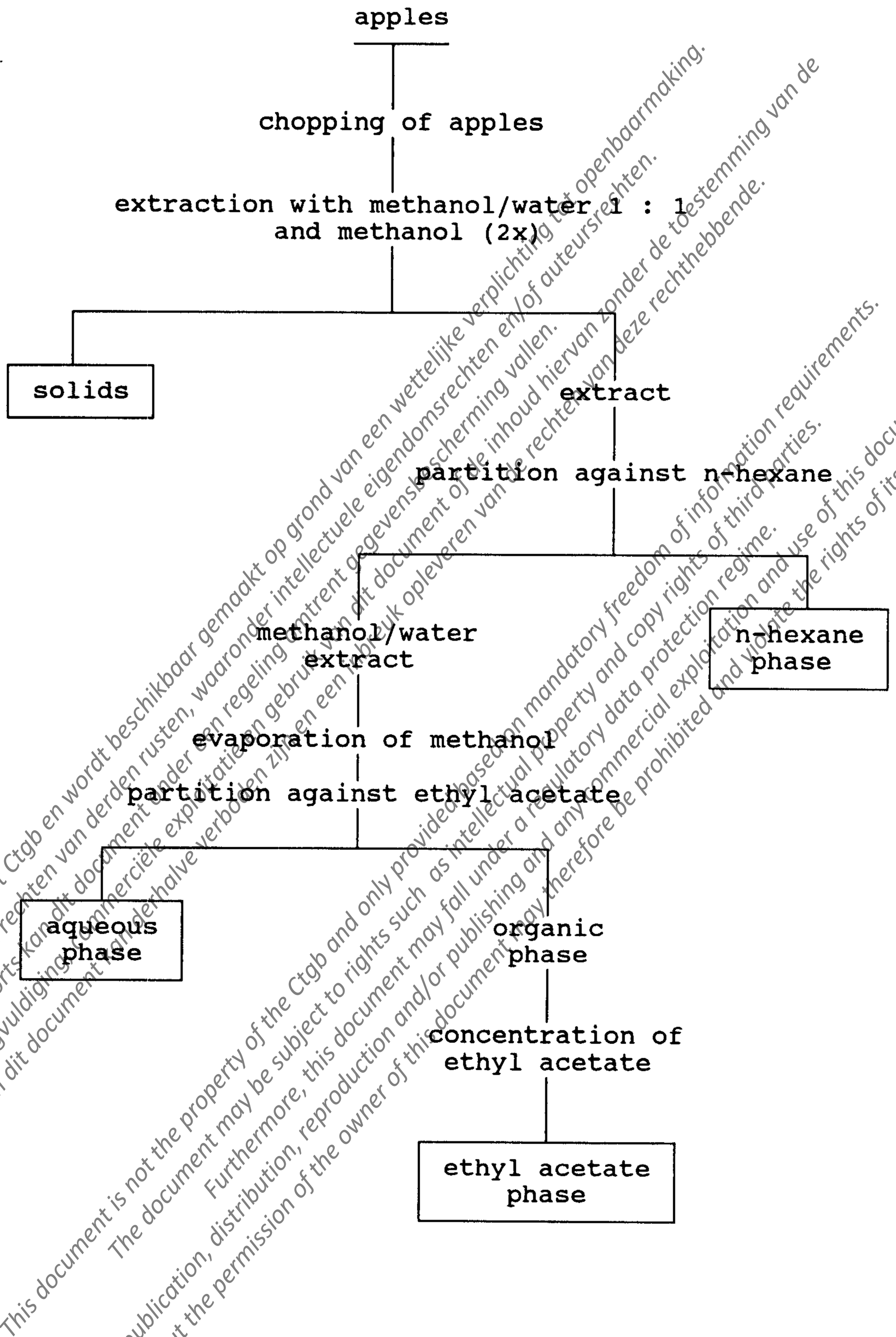


Figure 2: Scheme of the extraction procedure used for the storage stability study

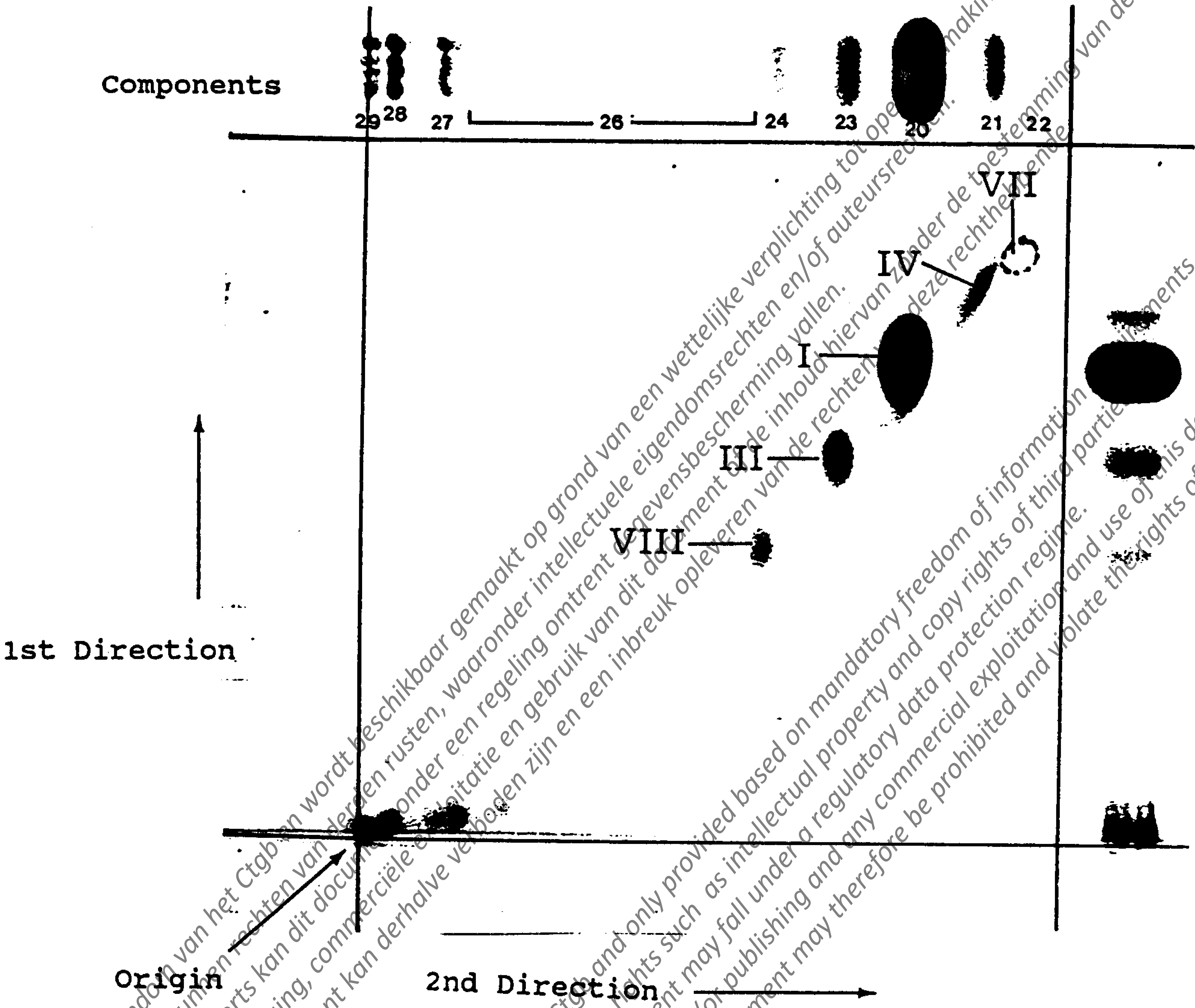


Figure 3: Autoradiogram of the surface wash solution, day 14, SS II/SS I
Arabic numbers refer to metabolites, Roman numbers refer to reference compounds

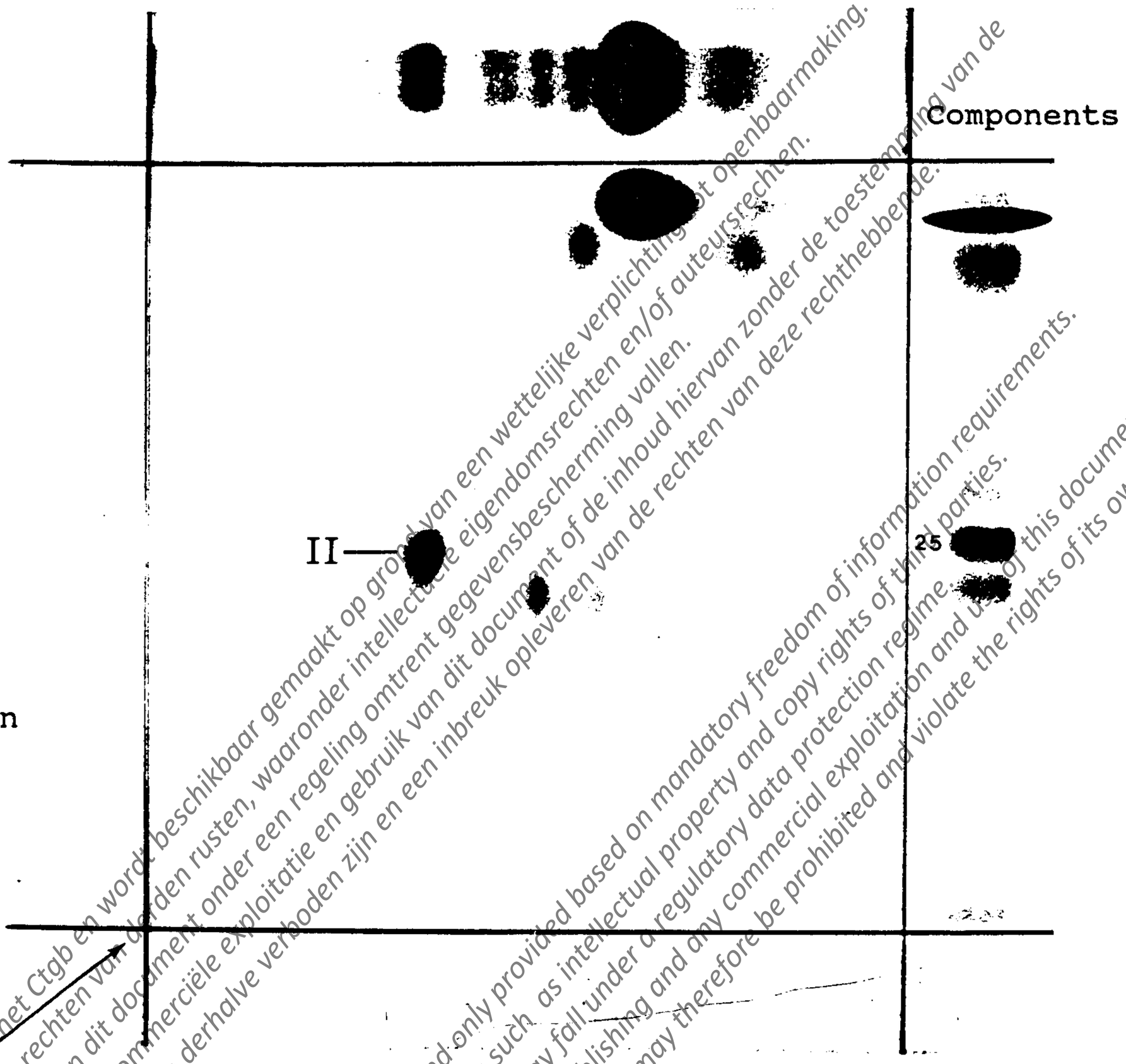


Figure 4: Autoradiogram of the surface wash solution, day 14, SS IV/SS III
Arabic numbers refer to metabolites, Roman numbers refer to reference compounds

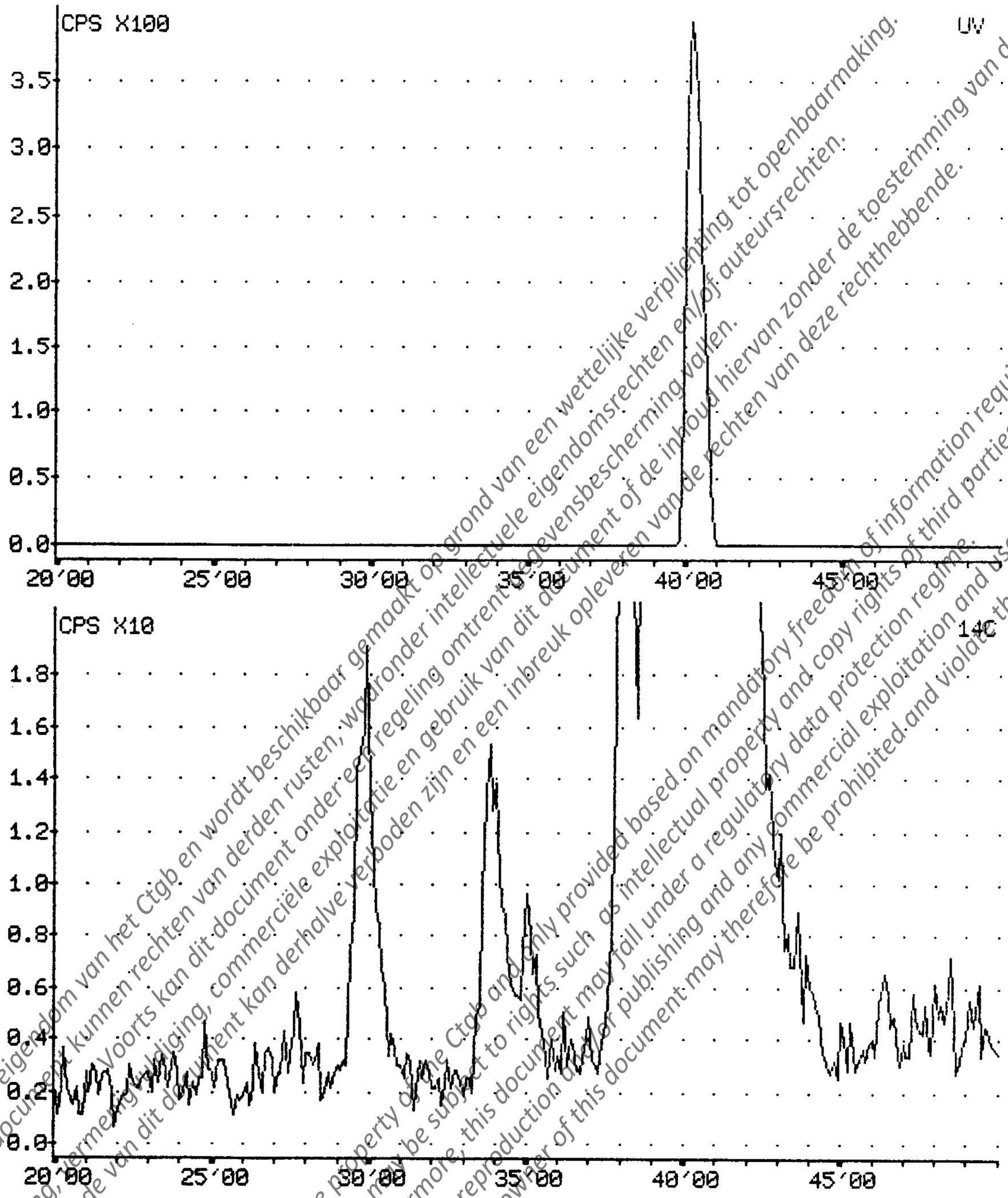


Figure 5: HPLC chromatogram obtained when isolating NTN 33893 from the surface wash solution (day 14)

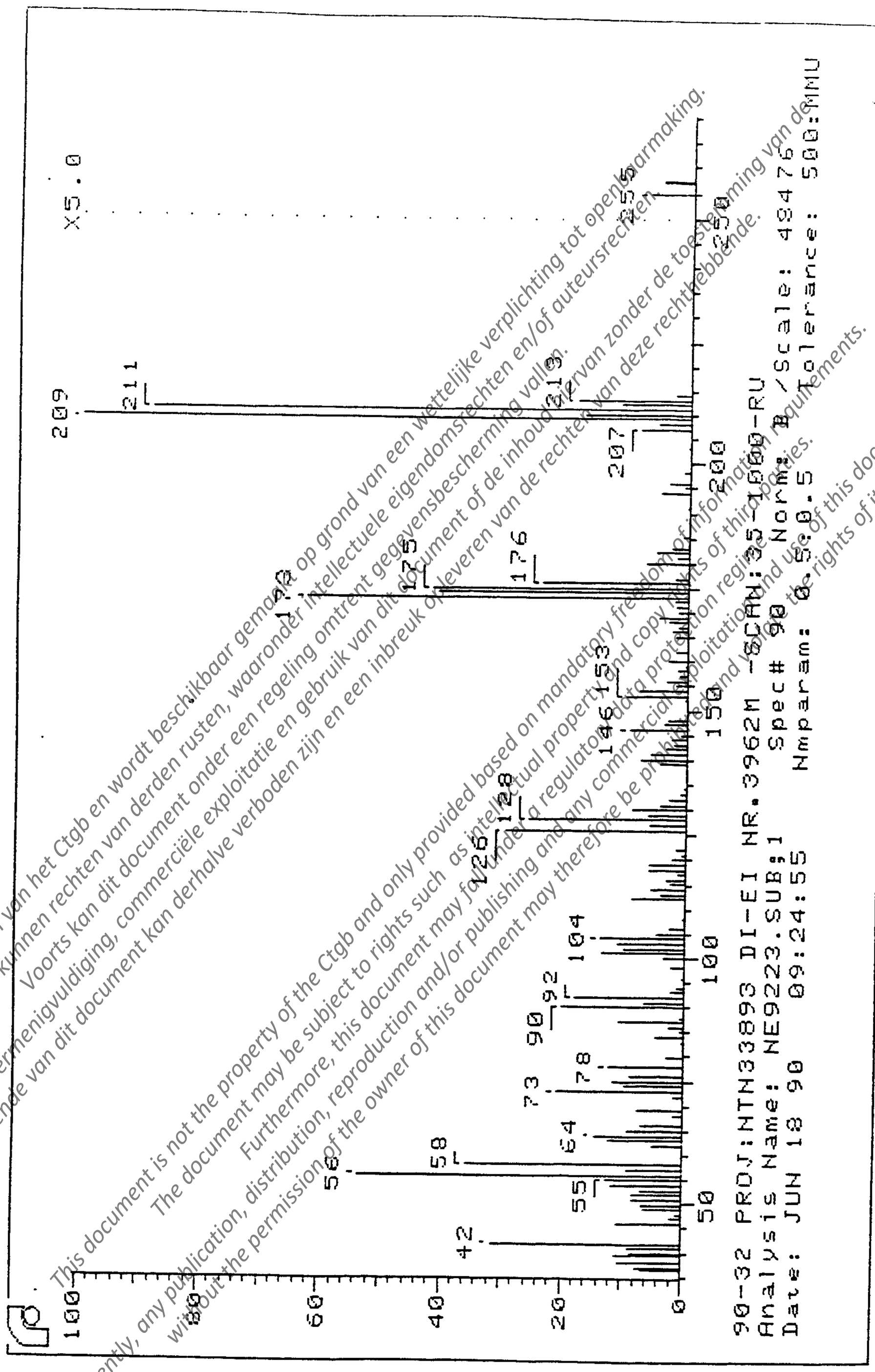


Figure 6: EI-MS Spectrum of NTN 33893 isolated from the surface wash solution

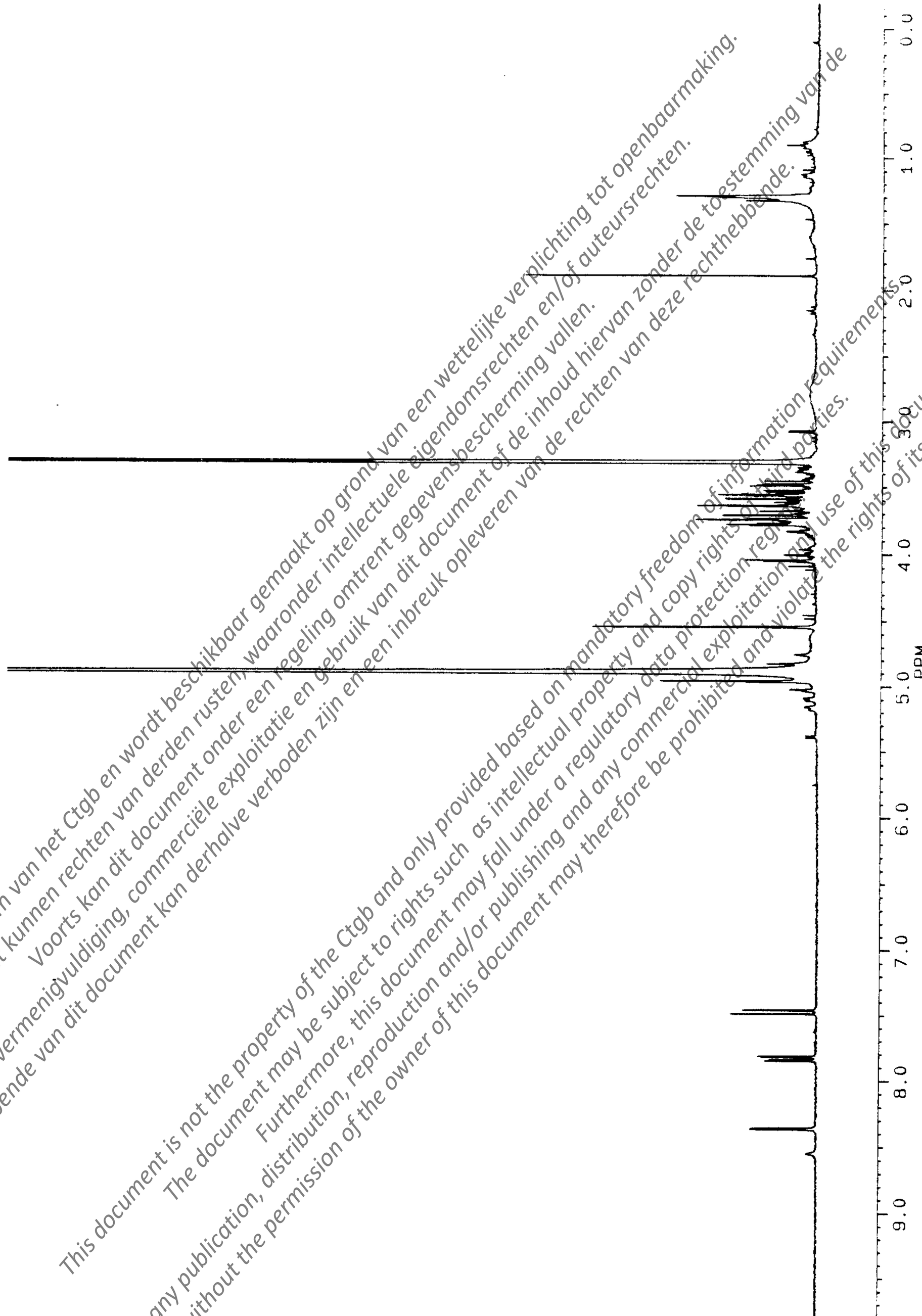


Figure 7: ^{1}H-NMR Spectrum of NTN 33893 isolated from the surface wash solution

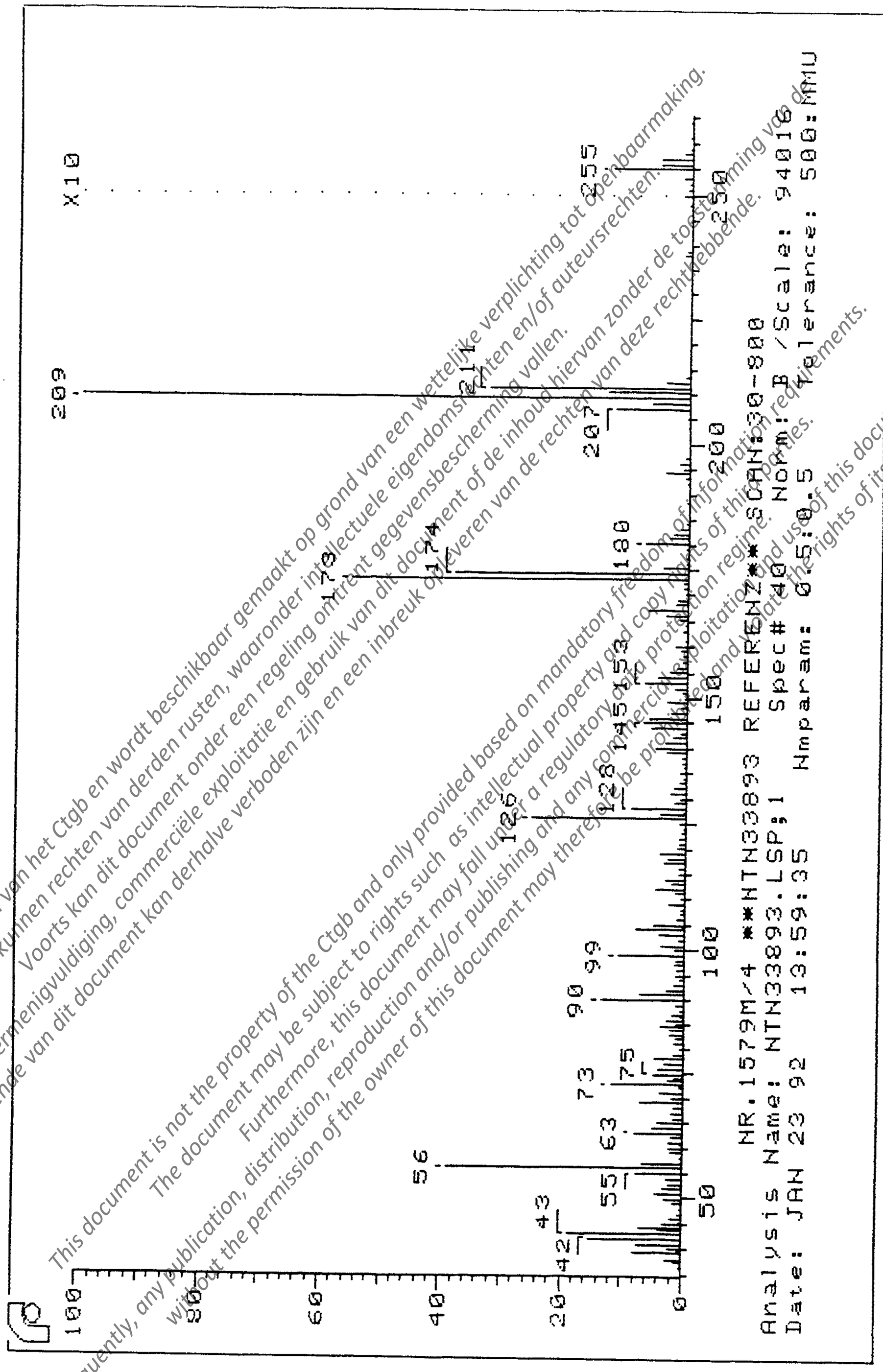


Figure 8: EI-MS spectrum of non-radiolabelled reference parent compound

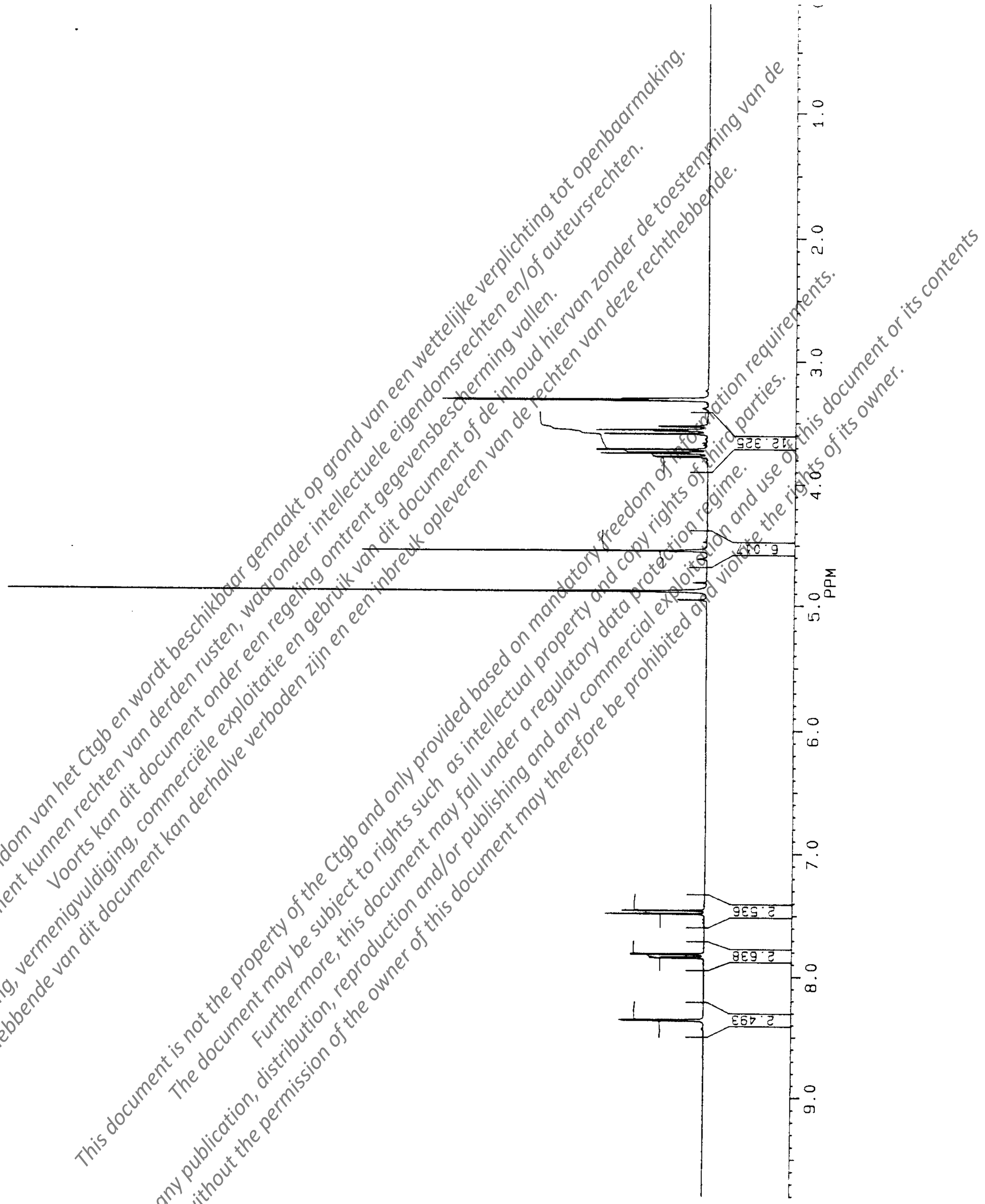


Figure 9: ^{1}H-NMR spectrum of non-radiolabelled reference parent compound

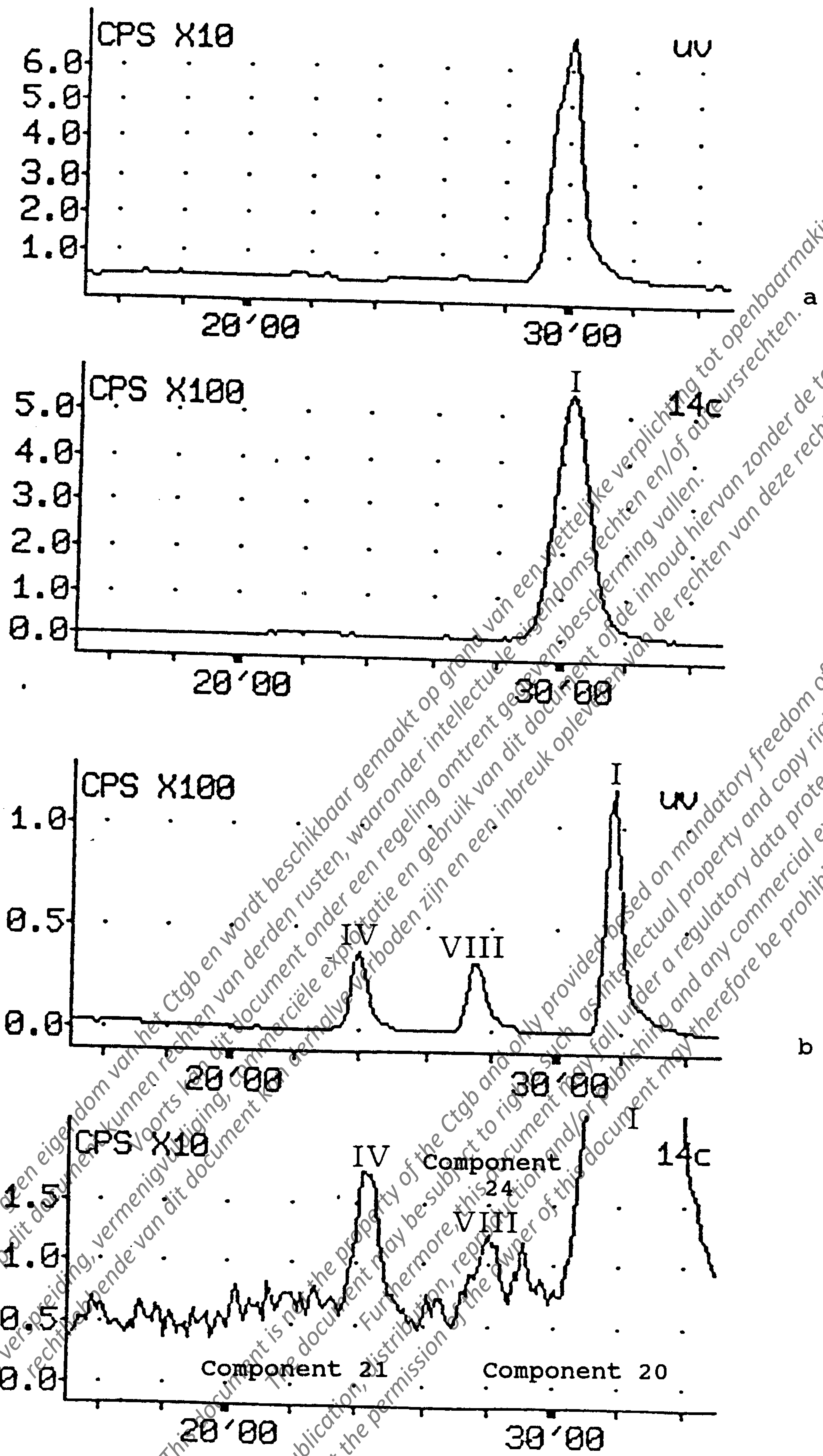


Figure 10: HPLC chromatograms of the surface wash solution day 14
(a) direct and (b) after co-injection with NTN 33893
(I), WAK 4103 (IV) and WAK 3839 (VIII)

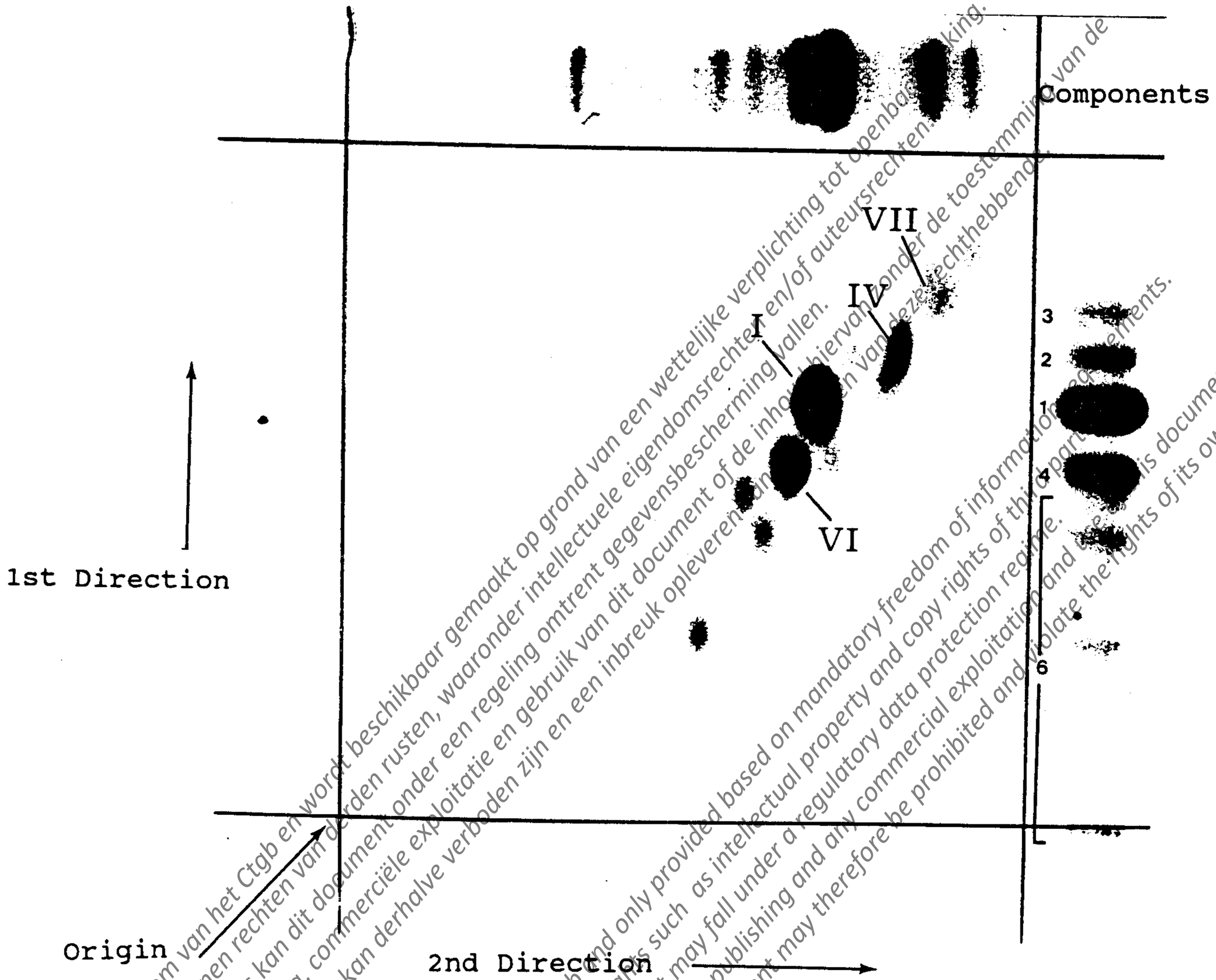


Figure 11: Autoradiogram of the ethyl acetate phase of peel, day 14, SS II/SS I
Arabic numbers refer to metabolites, Roman numbers refer to reference compounds

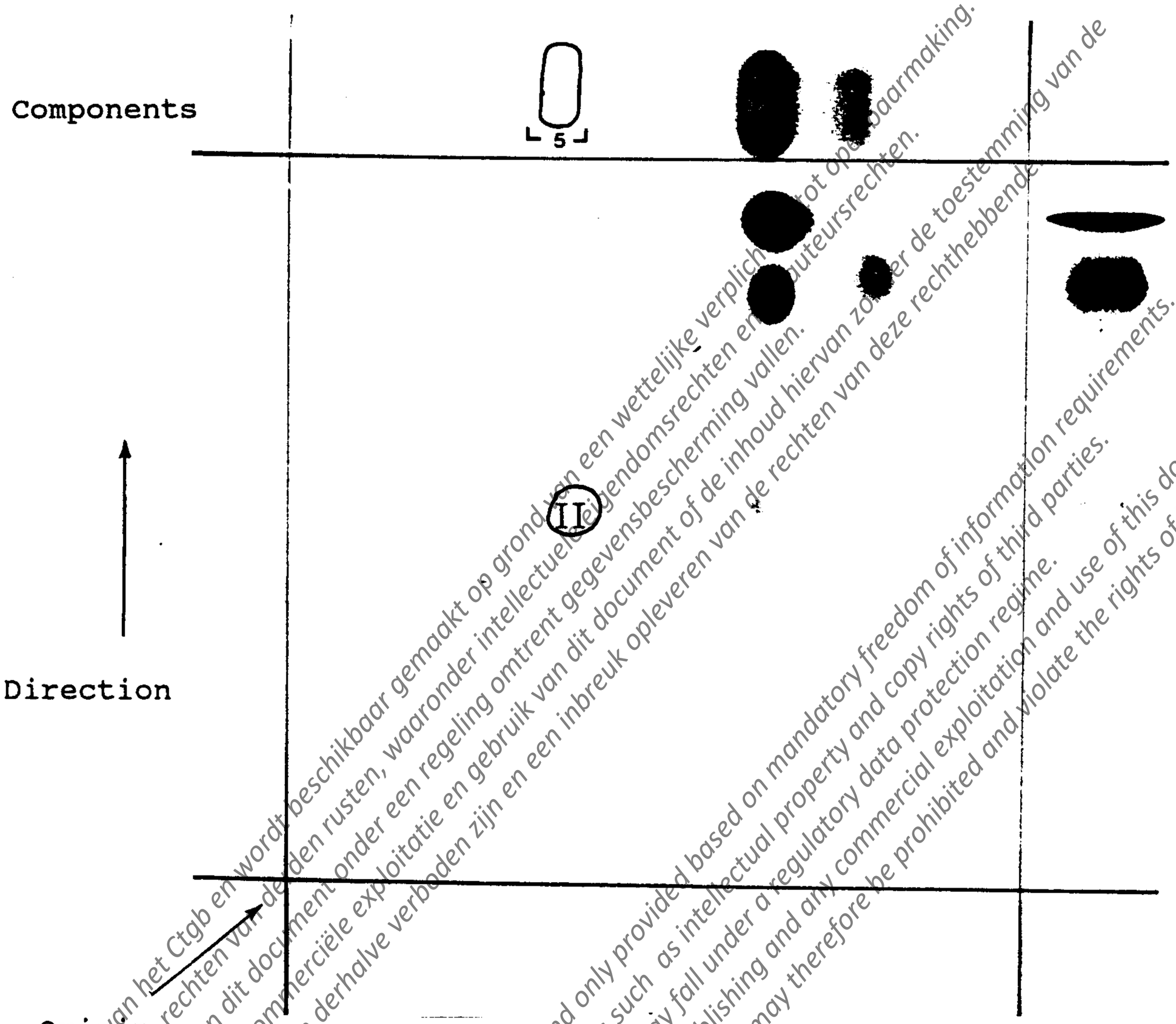


Figure 12: Autoradiogram of the ethyl acetate phase of peel, day 14, SS IV/SS III
Arabic numbers refer to metabolites, Roman numbers refer to reference compounds

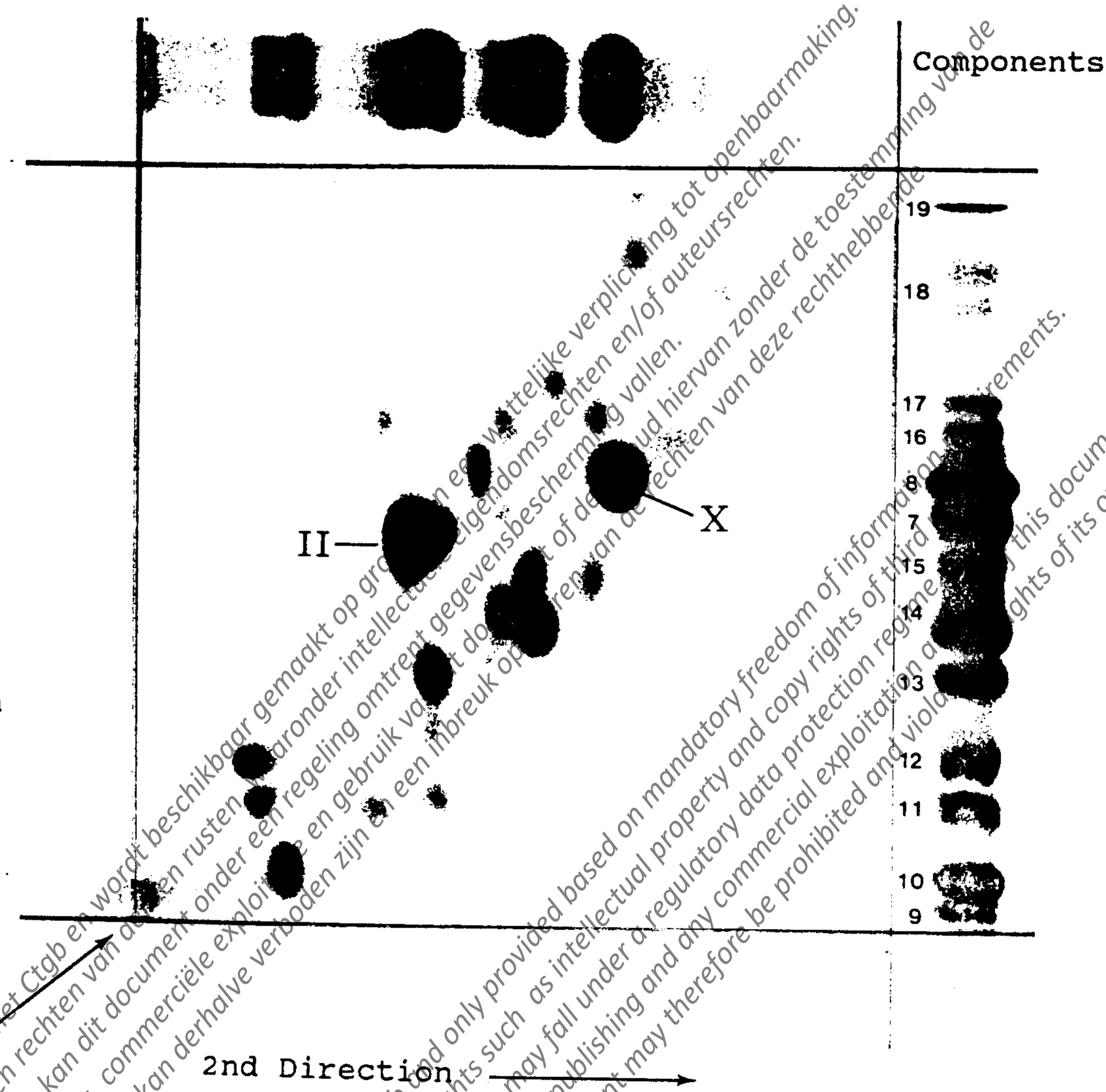


Figure 13: Autoradiogram of the water phase of peel, day 14, SS IV/SS III

Arabic numbers refer to metabolites, Roman numbers refer to reference compounds

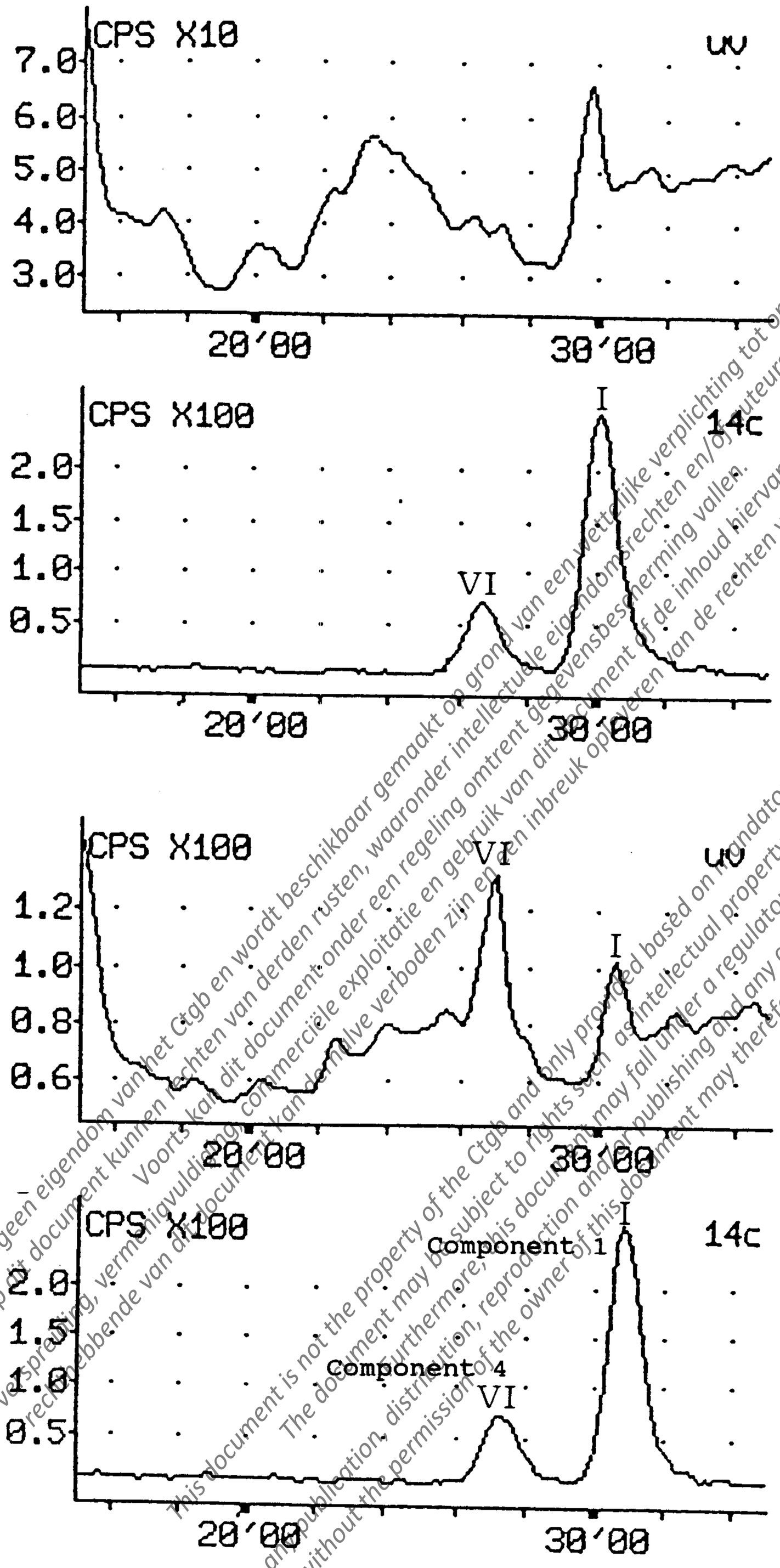


Figure 14: HPLC chromatograms of the ethyl acetate phase of peel day 0 (a) direct and (b) after co-injection with NTN 33893 (I) and NTN 35884 (VI)

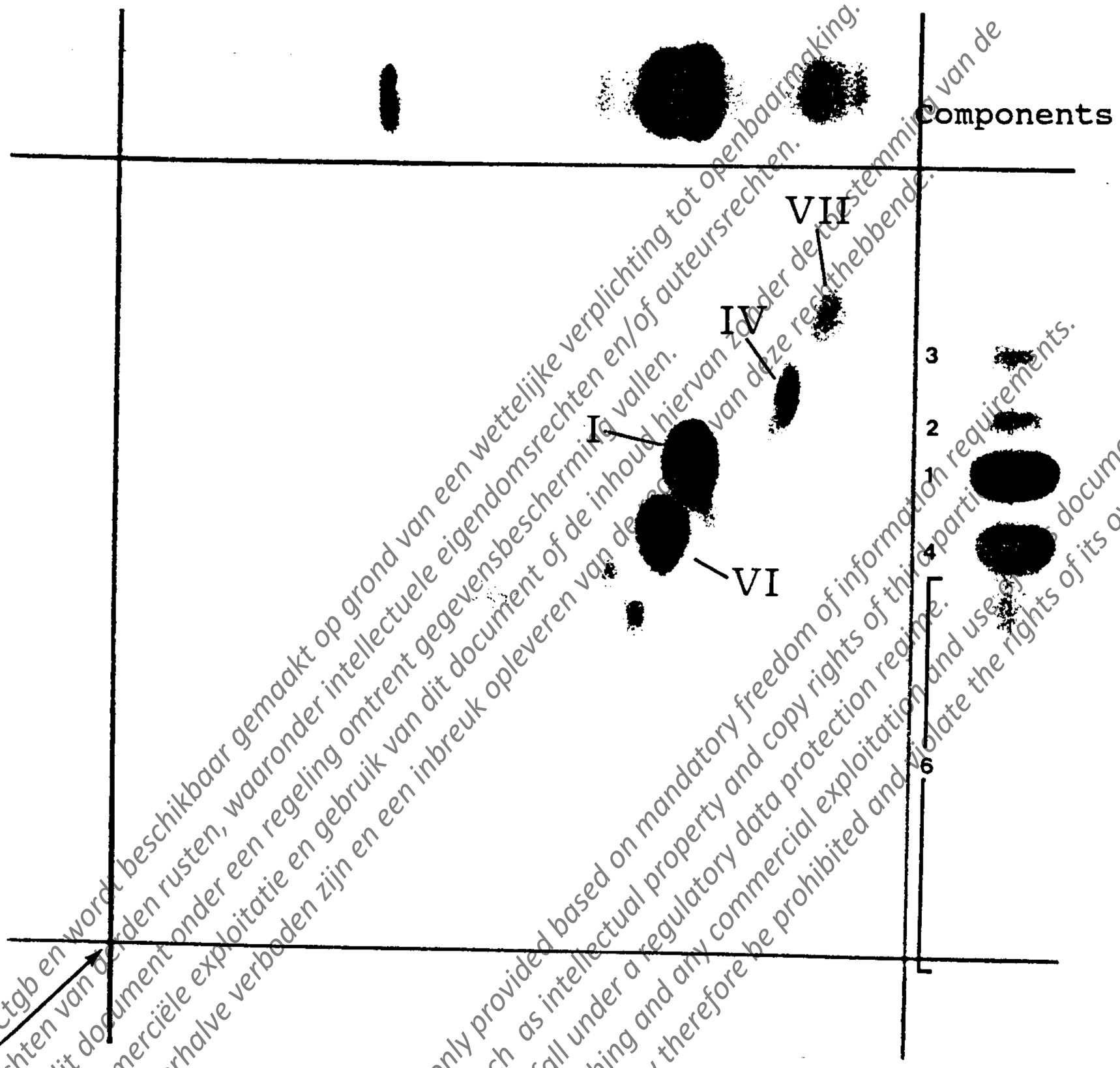


Figure 15: Autoradiogram of the ethyl acetate phase of pulp, day 14, SS II/SS I
Arabic numbers refer to metabolites, Roman numbers refer to reference compounds

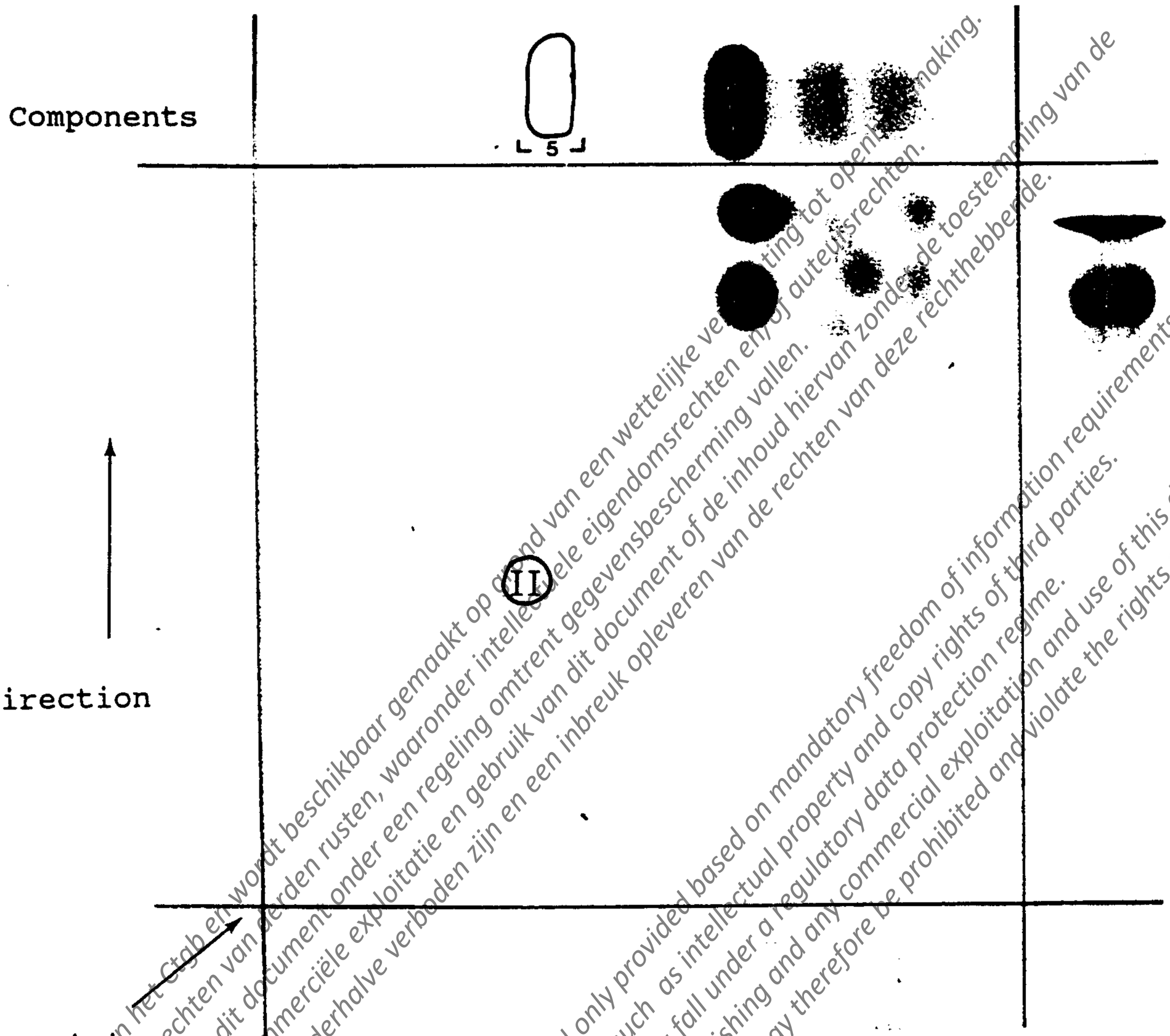


Figure 16: Autoradiogram of the ethyl acetate phase of pulp, day 14, SS IV/SS III
Arabic numbers refer to metabolites, Roman numbers refer to reference compounds

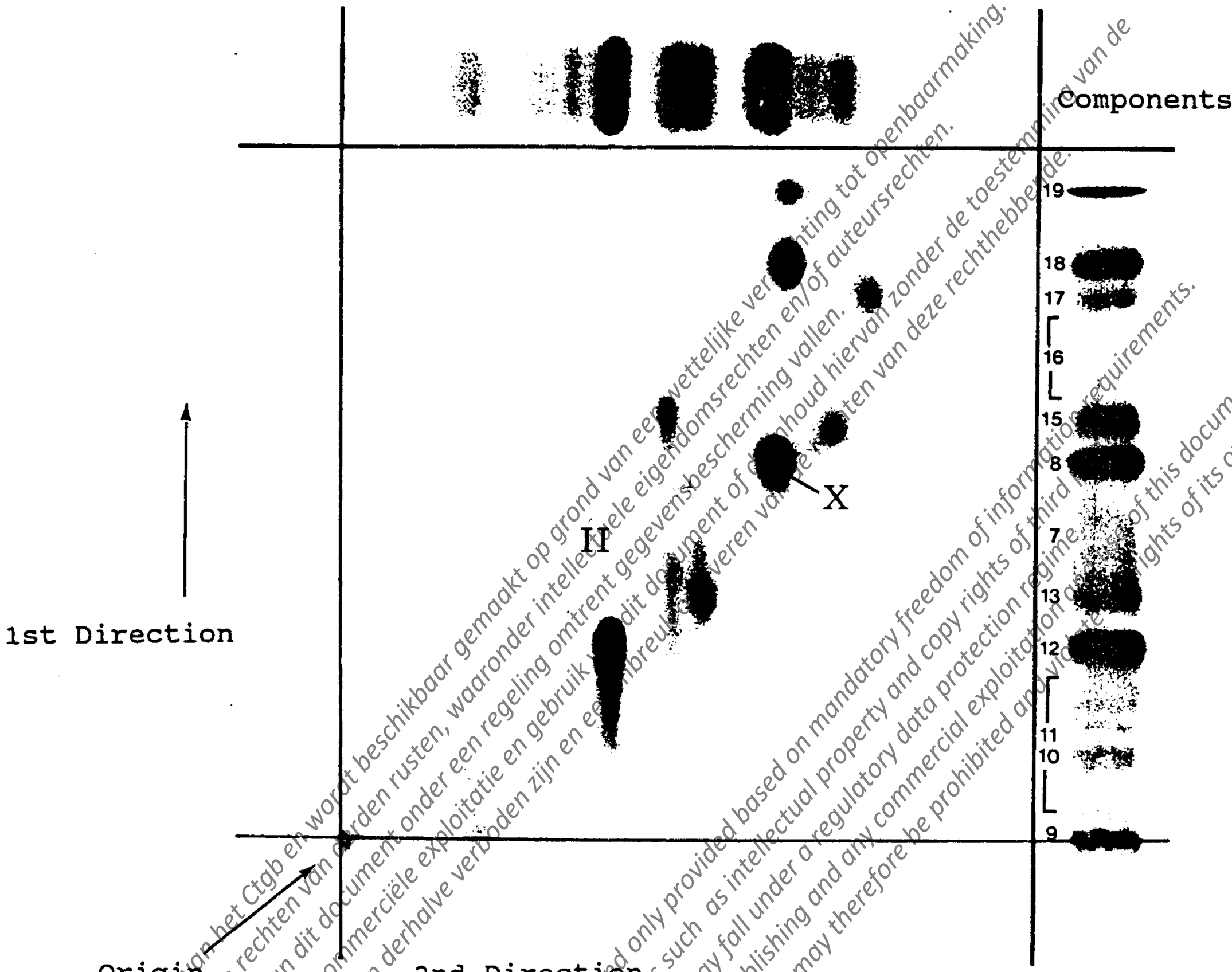


Figure 17: Autoradiogram of the water phase of pulp, day 14, SS IV/SS III

Arabic numbers refer to metabolites, Roman numbers refer to reference compounds

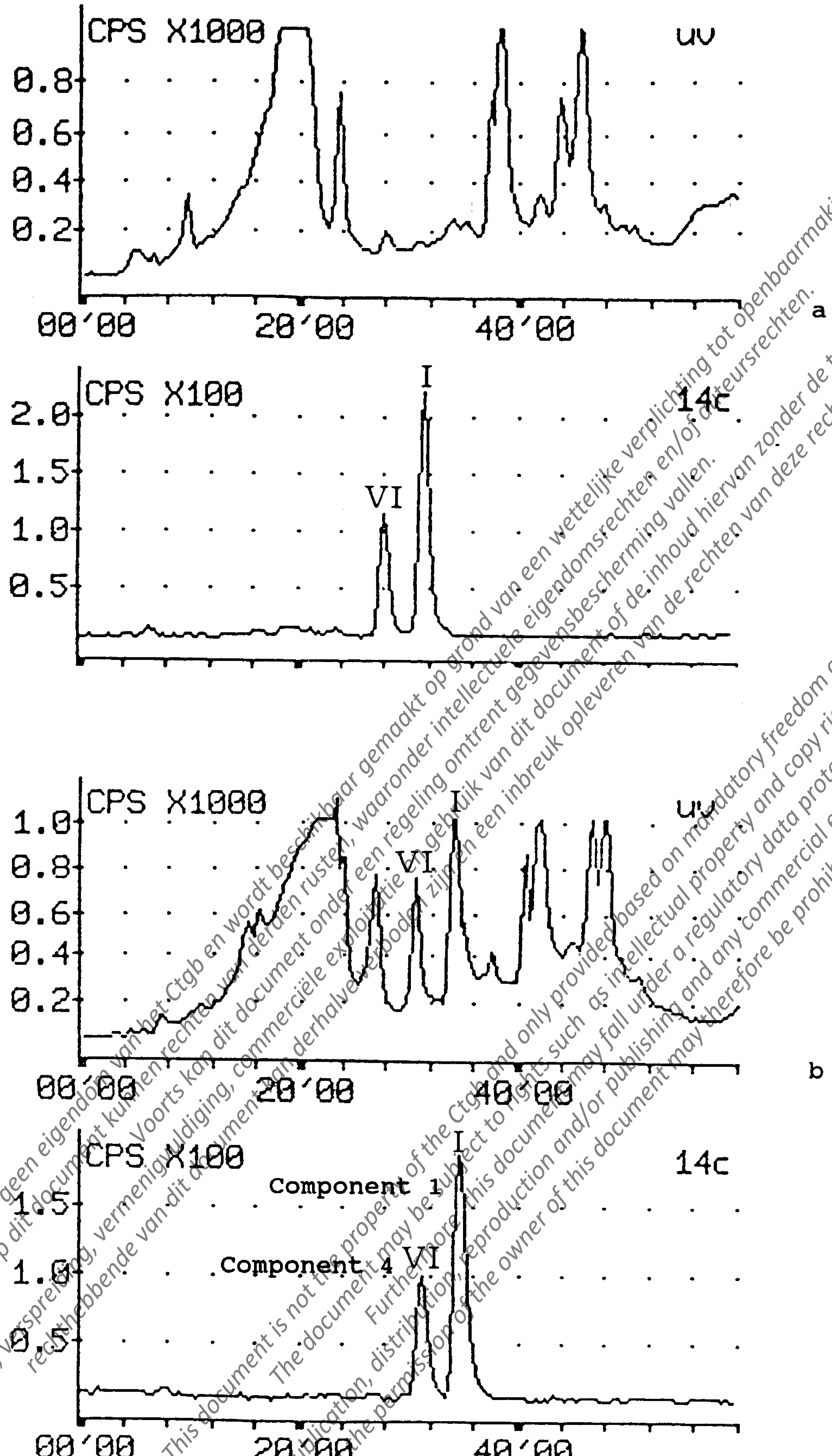


Figure 18: HPLC chromatogram of the ethyl acetate phase of pulp day 0 (a) direct and (b) after co-injection with NTN 33893 (I) and NTN 35884 (VI)

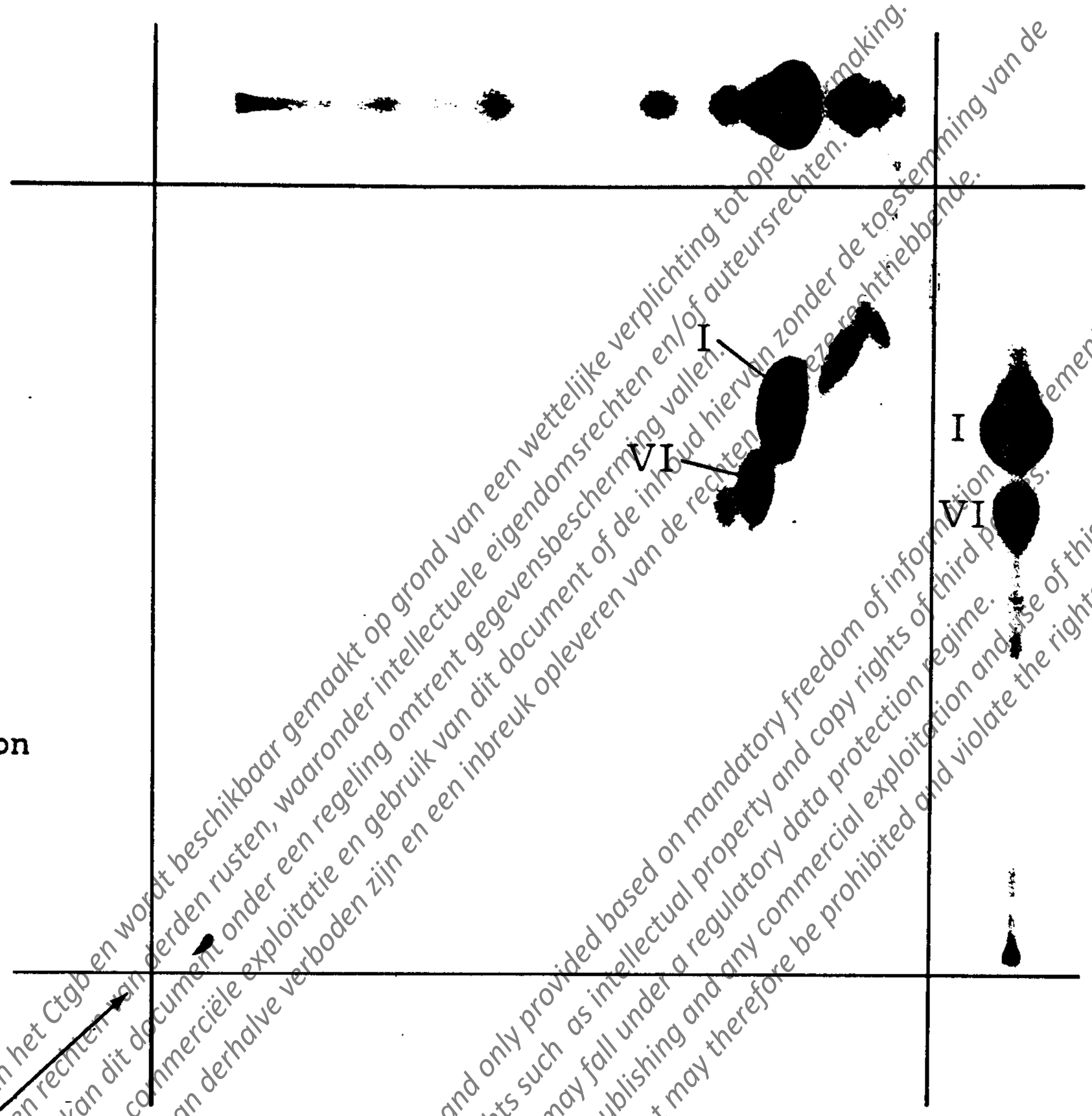


Figure 19: Representative autoradiogram of the ethyl acetate phase from the storage stability study, SS II/SS I
Roman numbers refer to reference compounds

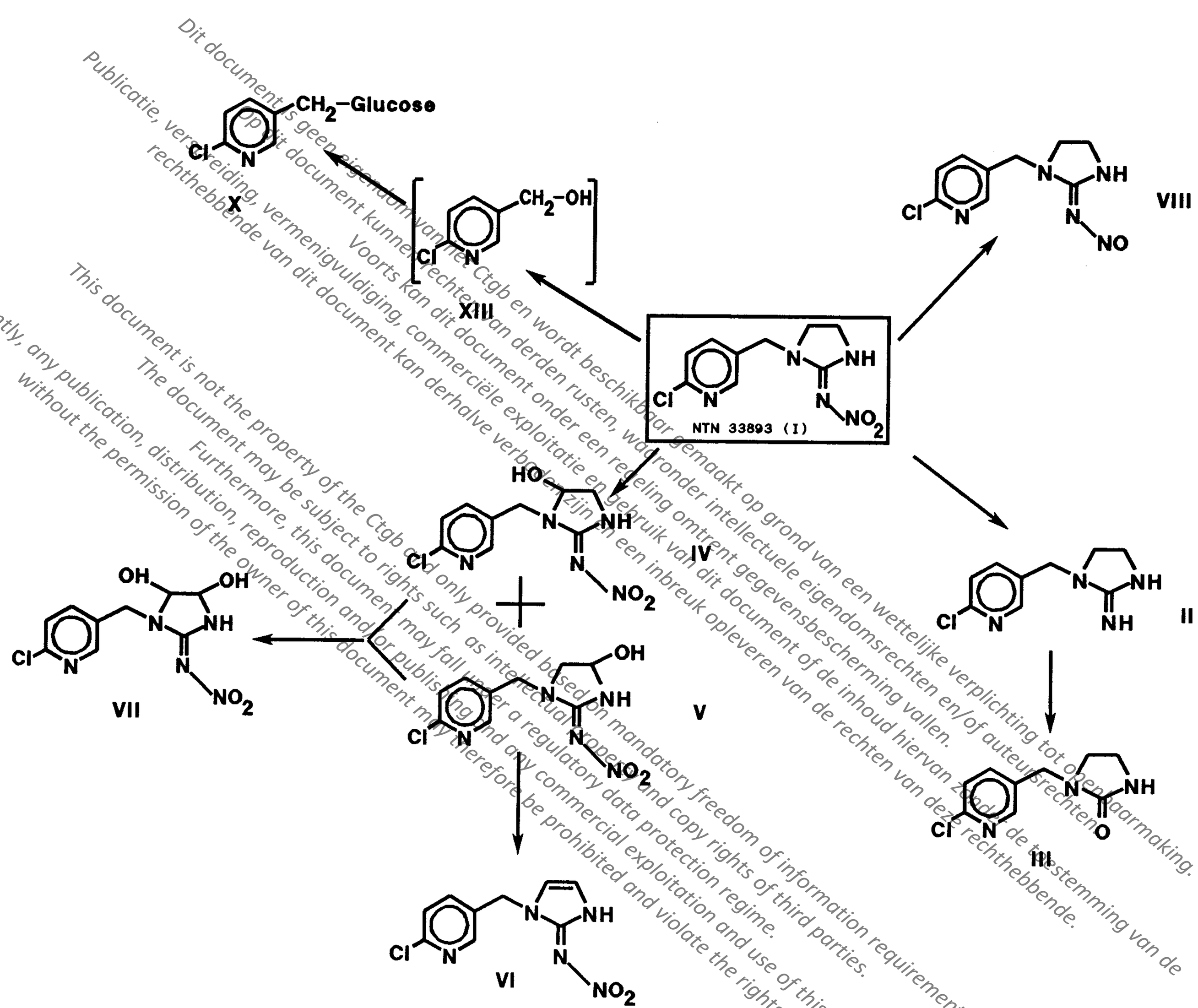


Figure 20: Degradation pathway of NTN 33893 in apples

XII. APPENDICES

Appendix 1: Details of the formulation of radiolabelled NTN 33893 for the metabolism experiment

S A M P L E C E R T I F I C A T I O N

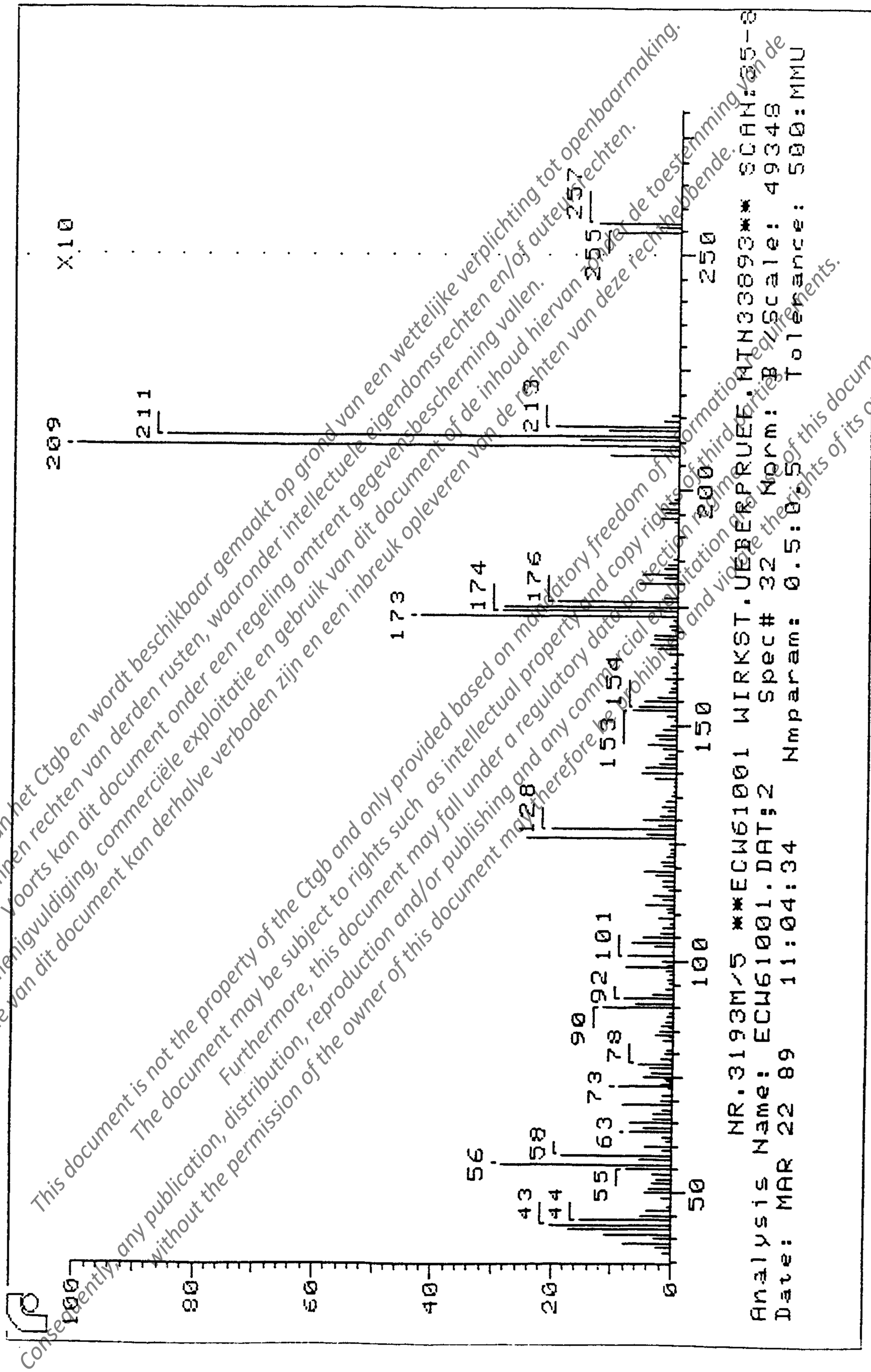
[redacted]

Appendix 2: Details of the formulation of radiolabelled NTN 33893 for the translocation experiment

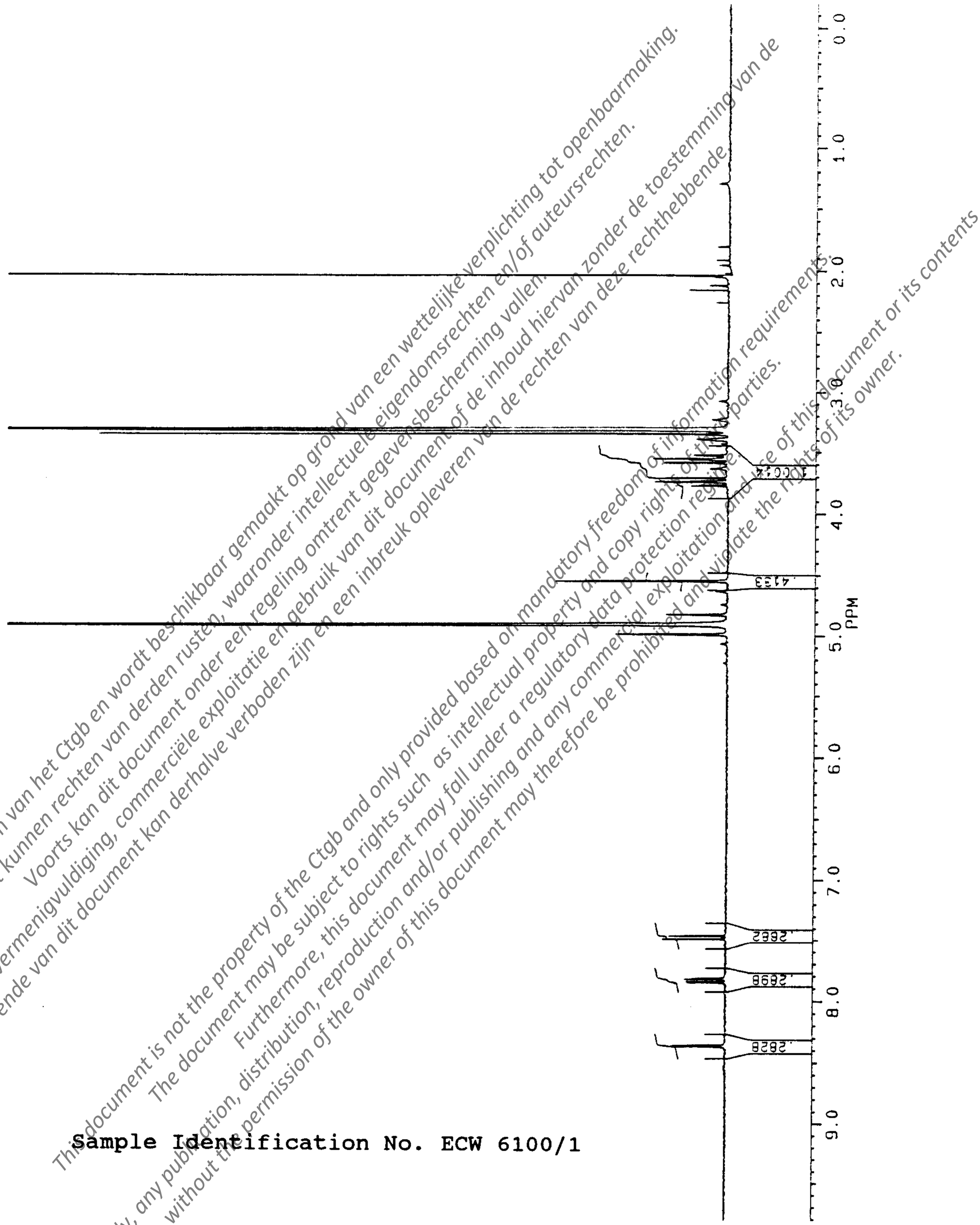
S A M P L E C E R T I F I C A T I O N

[redacted]

Appendix 3. EI-MS spectrum of NTN 33893 in the formulation



Appendix 4: ^{1}H-NMR spectrum of NTN 33893 in the formulation



Sample Identification No. ECW 6100/1

Appendix 5:

Environmental and growth conditions of the apples

Crop	:	Apple
Variety	:	Golden Delicious
Soil type	:	Frimmersdorf (see appendix 6)
Container size	:	0.5 m^2
Planting date	:	05.02.1986 4 trees for metabolism
		08.03.1988 1 tree for translocation
Fertilisation measures	:	14.01.1989 1000 kg/ha "Thomasmehl"
		27.01.1989 400 kg/ha Calcium carbonate
		From 02.02.1989 two applications a week alternating between "Wuchsal" and "Fertisal", 2-4 liters of a 2.5 g/l solution applied per week

Plant protection measures :

Date	Chemical	% a.i.
07.03.1989	Folidol-Öl	0.50
25.03.1989	Antracol	0.20
	Baycor	0.05
	Ecombi	0.10
	Netzschwefel	0.50
13.04.1989	Euparen	0.20
	Bayleton sp.	0.05
	Cropotex	0.10
05.05.1989	Baycor	0.05
	Gusathion MS	0.20
	Netzschwefel	0.30
22.05.1989	Antracol	0.20
	Bayleton sp.	0.05
	Gusathion MS	0.20
05.06.1989	Baycor	0.05
	Cropotex	0.10
	Netzschwefel	0.25
08.06.1989	Shell Phosdrin	0.05
15.06.1989	Baycor	0.05
	Gusathion MS	0.20
	Netzschwefel	0.20
	Polyram Combi	0.15

Average monthly temperature (measured at ground level) and hours of sunshine :

Month	Sunshine(h)	Temperature(^{o}C)
June	231	19.1
July	214	21.4
August	209	19.3
September	132	17.3

Appendix 6:

Analysis* of the soil "Frimmersdorf"

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

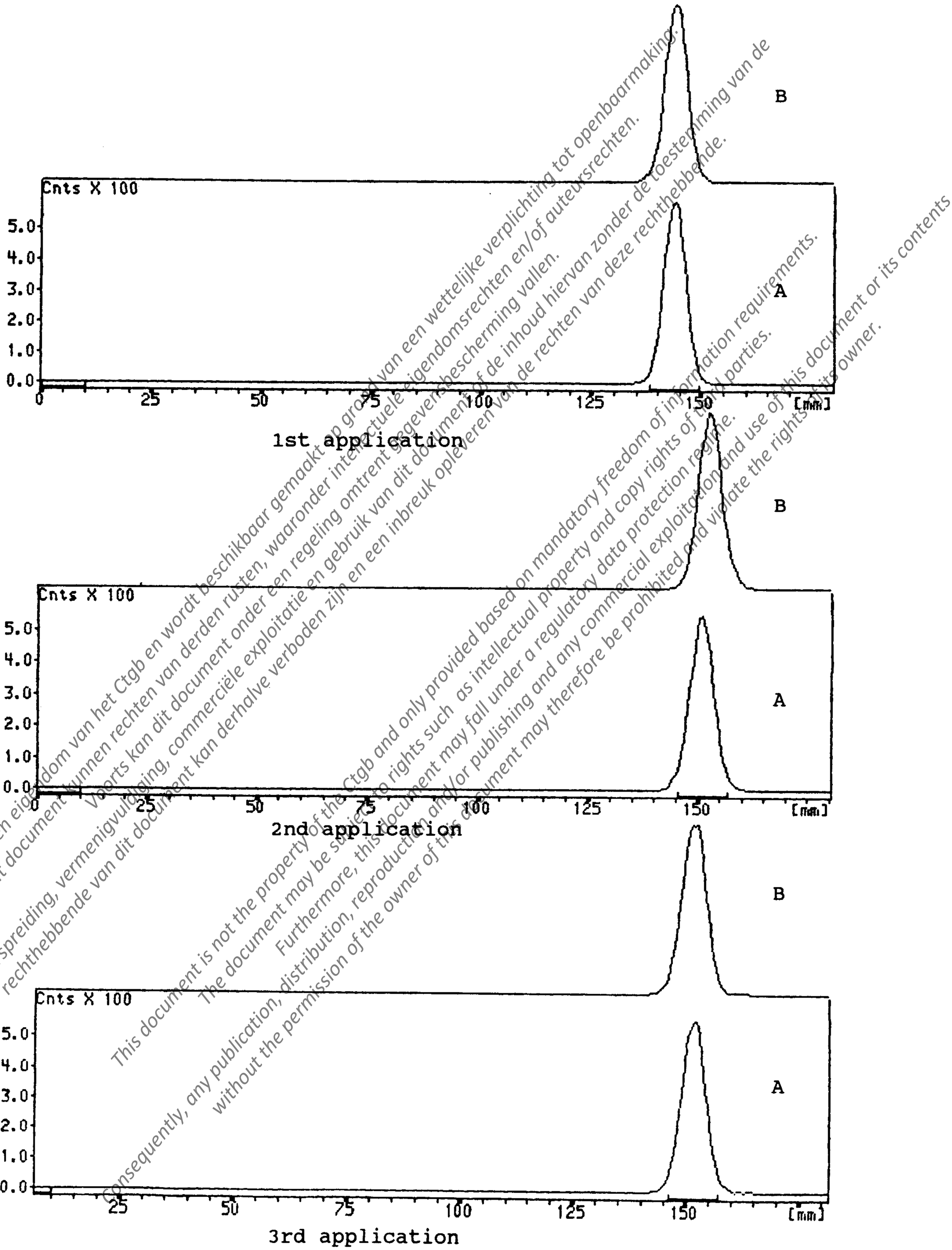
Texture analysis according to USDA soil diagram;

Sand	%	17.1
Silt	%	71.8 = silty loam
Clay	%	11.8

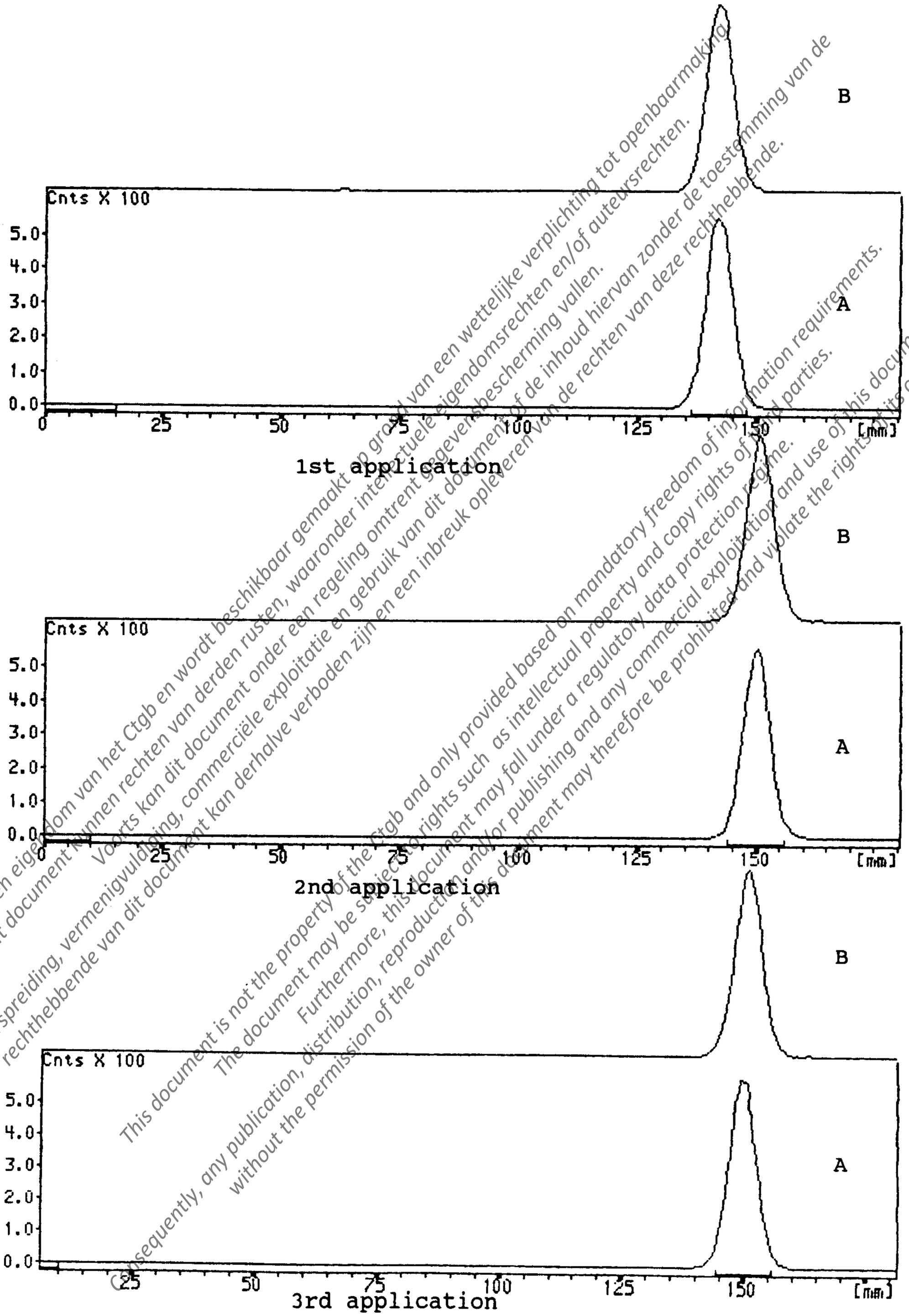
*

Laboratory [redacted] nstitute of Ecobiology, Bayer AG

Appendix 7: TLC chromatograms of the application solutions used for the metabolism experiment (A) before and (B) after each application



Appendix 8: TLC chromatograms of the application solutions used for the translocation experiment (A) before and (B) after each application



Appendix 9: Measurement of Radioactivty

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 306 Tri-carb (Packard) and
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)

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%

Appendix : 10

Raw data from the analysis of radioactivity in the different fractions of apples after surface washing and extraction of peel and pulp, day 0

Fraction	weight of sample extracted	total weight of sample	radioact. in extract/solution	radioact. in solids	total radioactivity MBq
Surface Wash Solution	10 apples/1362.55 g		2011.6 Bq/500 µl 6075.1 kBq/1510 ml		6.08
Peel	48.77 g	279.66 g	392.7 Bq/ml 197.5 kBq/503 ml	6910.1 Bq/g 28.7 kBq/4.16 g	1.30
Pulp	276.52 g	1081.11 g	151.8 Bq/ml 204.9 kBq/1350 ml	223.7 Bq/g 3.2 kBq/14.08 g	0.81

Appendix : 11

Raw data from the analysis of radioactivity in the different fractions of apples after surface washing and extraction of peel and pulp, day 14

Fraction	weight of sample extracted	total weight of sample	radioact. in extract/solution	radioact. in solids	total radioactivity MBq
Surface Wash Solution	20 apples/3279.16 g		3611.9 Bq/ml 10492.5 kBq/2905 ml		10.49
Peel	201.08 g	508.90 g	714.3 Bq/ml 1178.6 kBq/1650 ml	8815.5 Bq/g 173.3 kBq/19.66g	3.42
Pulp	839.71 g	2758.58 g	213.1 Bq/ml 674.5 kBq/3165 ml	352.3 Bq/g 13.9 kBq/39.52 g	2.26

Appendix 12:

Raw data (cpm), obtained by TLC, from the quantitative distribution of metabolites in the surface wash solution

Component		Day 0 cpm	Day 0 % in extract	Day 14 cpm	Day 14 % in extract
20 = NTN 33893	I	8268	89.1	6610	85.9
21 = 5-Hydroxy, WAK 4103	IV	216	2.3	207	2.7
22 = Dihydroxy, WAK 3772	VII	57	0.6	53	0.7
23 = Urea, DIJ 9817	III	168	1.8	199	2.6
24 = Nitrosimine	VIII	79	0.9	79	1.0
25 = Guanidine	II	161	1.7	93	1.2
26 = 1 unknown metabolite		108	1.2	98	1.3
27 = 1 unknown metabolite		108	1.2	118	1.5
28 = 1 unknown metabolite		18*	0.2	143*	1.9
		(179-161)		(236-93)	
29 = Start activity		96	1.0	94	1.2
Sum of components 20-29		9279	100	7694	100

Components 20-24 and 26-29 were determined after one dimensional TLC in solvent system I

Component 25 was determined after one dimensional TLC in solvent system IV

* the values for component 28 were determined by subtracting the amount of the guandine determined in SS IV from the total amount determined for component 28 determined in SS I since in this system the two components co-eluted

Appendix 13:

Raw data (cpm), obtained by TLC, from the distribution of metabolites in the methanol eluates from XAD-4 columns of the methanol/water extracts of peel and pulp after partitioning between ethyl acetate and water

		Day 0		Day 14	
Component		Peel	Pulp	Peel	Pulp
		cpm		cpm	
Ethyl acetate phase					
1 = NTN 33893	I	1249	1320	1950	1326
2 = 5-Hydroxy WAK 4103	IV	39	79	159	81
3 = Dihydroxy WAK 3772	VII	30	42	71	91
4 = Olefine NTN 35884	VI	341	725	589	760
5 = Guanidine NTN 33823	II	195	8	12	9
6 = 3 Unknown metabolites	*	307	126	259	204
		(494+8-195)	(129+5-8)	(256+15-12)	(207+6-9)
Sum of components 1-6		2161	2300	3040	2471
Water phase					
7 = Guanidine NTN 33823	II	358	185	1064	363
8 = Glucoside RBN 1114	X	1241	366	1331	476
9 = Start activity		286	159	163	161
10 = 1 Unknown metabolite		330	365	169	378
11 = 3 Unknown metabolites				212	
12 = 1 Unknown metabolite		279	633	287	614
13 = 1 Unknown metabolite		283	483	304	276
14 = 2 Unknown metabolites		611		637	
15 = 2 Unknown metabolites			296	405	284
16 = 4 Unknown metabolites			76	252	234
17 = 1 Unknown metabolite		305	202	96	341
18 = 2 Unknown metabolites			380	186	44
19 = 1 Unknown metabolite		75	216	73	137
Sum of components 7-19		3768	3361	5179	3308
Total sum of components 1-19		5929	5661	8219	5779

Components were quantified using 1-dimensional TLC in the following systems:-components 1-4 & 6 in SS II, 5 in SS III and 7-19 in SS IV

* in SS II three unknown metabolites, one of which contained the guanidine II, were summed together to give component 6. Therefore, to determine the actual value of component 6 the value for the guanidine determined in SS III was subtracted

Appendix 14: Raw data for the translocation experiment

	weight (g) of plant material extracted	weight (g) of solids after extraction	Bq/g measured	vol. of extract (ml)	Bq/5ml measured
Leaves not extracted, only combusted					
exp.1	2.73		634,545		
exp.2	2.15		752,126		
Peel					
exp.1	28.67	2.85	33.22	200	8.05
exp.2	29.64	2.90	70.13	200	18.80
Pulp					
exp.1	124.04	7.75	28.55	500	12.98
exp.2	106.33	7.81	45.70	500	7.45

Appendix 15. Raw data for the different phases of the storage stability study

All values given in Bq/100 µl taken from 10 ml samples unless otherwise stated

Analysis	1st		2nd		3rd		4th		5th		6th	
Ext. Date	06/09/89		16/03/90		06/06/90		03/08/90		18/04/91		03/09/91	
Storage	1 day		192 days		274 days		332 days		590 days		728 days	
Run	No.1	No.2	No.1	No.2	No.1	No.2	No.1	No.2	No.1	No.2	No.1	No.2
n-Hexane phase	5.48	5.79	12.32	8.53	13.92	7.87	6.07	8.50	10.03	8.52	25.37	27.24
Ethyl acetate phase	3081.09	2778.87	§1340.05	§1498.43	2898.80	3197.48	2783.77	2978.72	2597.85	2697.85	5581.35	5645.18
Water phase	#217.33	+210.61	202.32	165.10	348.67	194.78	209.83	226.82	303.82	297.70	$224.69	$229.99
Non-Extractable*	3585.18 (2.57g)	3508.91 (2.42g)	3684.40 (2.45g)	3860.27 (3.01g)	4991.72 (2.69g)	4669.62 (3.07g)	3454.25 (2.16g)	4313.66 (2.58g)	4719.93 (2.40g)	5655.53 (2.38g)	4618.17 (6.80g)	4913.11 (6.56g)
Weight of Sample (g)	57.68	54.94	51.79	59.55	54.79	59.06	50.20	54.12	50.54	50.19	104.33	100.45

§ 50 µl from 10 ml measured # 500 µl from 91.5 ml measured
+ 500 µl from 90.5 ml measured $ 100 µl from 20 ml measured
* Bq/gram, value in bracket = weight of sample

Appendix 16. Raw data (cpm), obtained by TLC, from the distribution of NTN 33893 and main metabolite in the ethyl acetate phase obtained from the storage stability study

Analysis	1st		2nd		3rd		4th		5th		6th	
Ext. Date	06/09/89		16/03/90		06/06/90		03/08/90		18/04/91		03/09/91	
Storage	1 day		192 days		274 days		332 days		590 days		728 days	
Run	No.1	No.2	No.1	No.2	No.1	No.2	No.1	No.2	No.1	No.2	No.1	No.2
NTN 33893(I)	13535	9911	12634	13485	13216	15543	13404	13062	11910	12697	13320	13316
Main metabol. Olefine(VI)	1350	903	1079	1215	961	1219	1126	1044	1009	1027	1527	1016
Other metabolites	1324	1082	1015	1210	1247	2011	807	1119	1360	872	1902	1051
Total cpm	16209	11896	14728	15910	15424	18773	15337	15225	14279	14596	16749	15383