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Residues of ^{14}C-NTN 33893 (Imidacloprid) in Blossoms of Sunflower *(Helianthus annuus)* after Seed Dressing

Data Requirement

Supplementary Study

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Performing Laboratory

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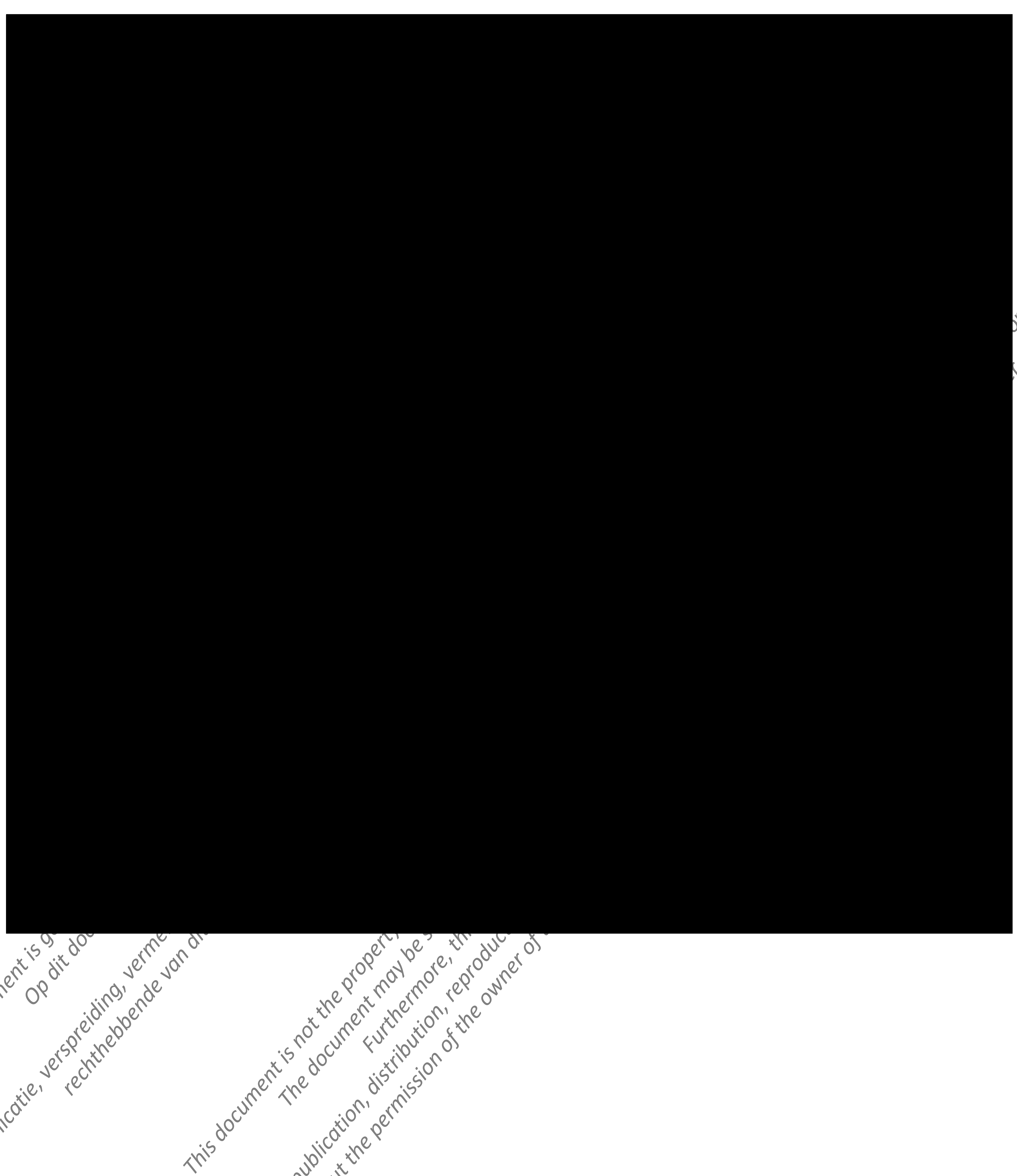

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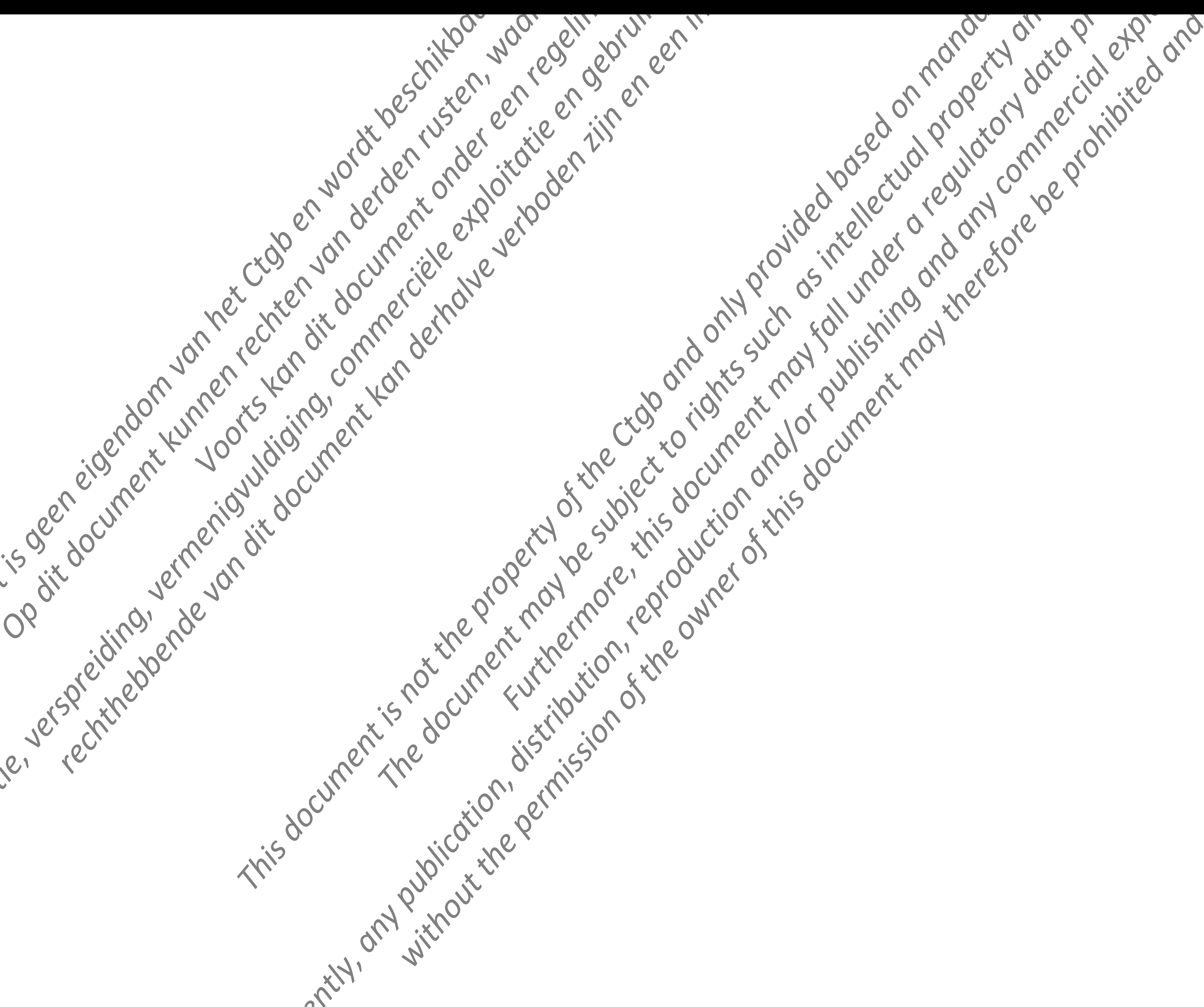

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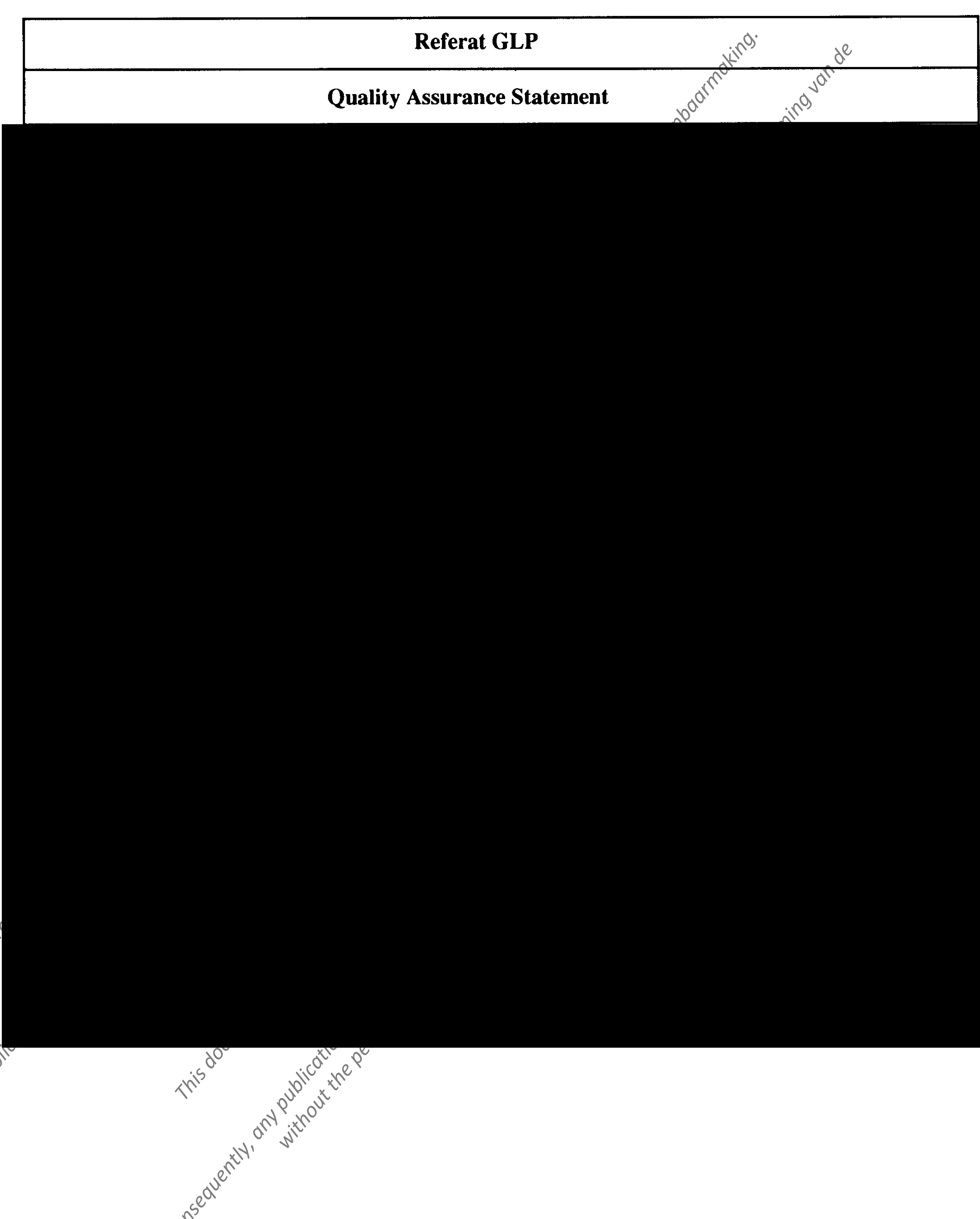

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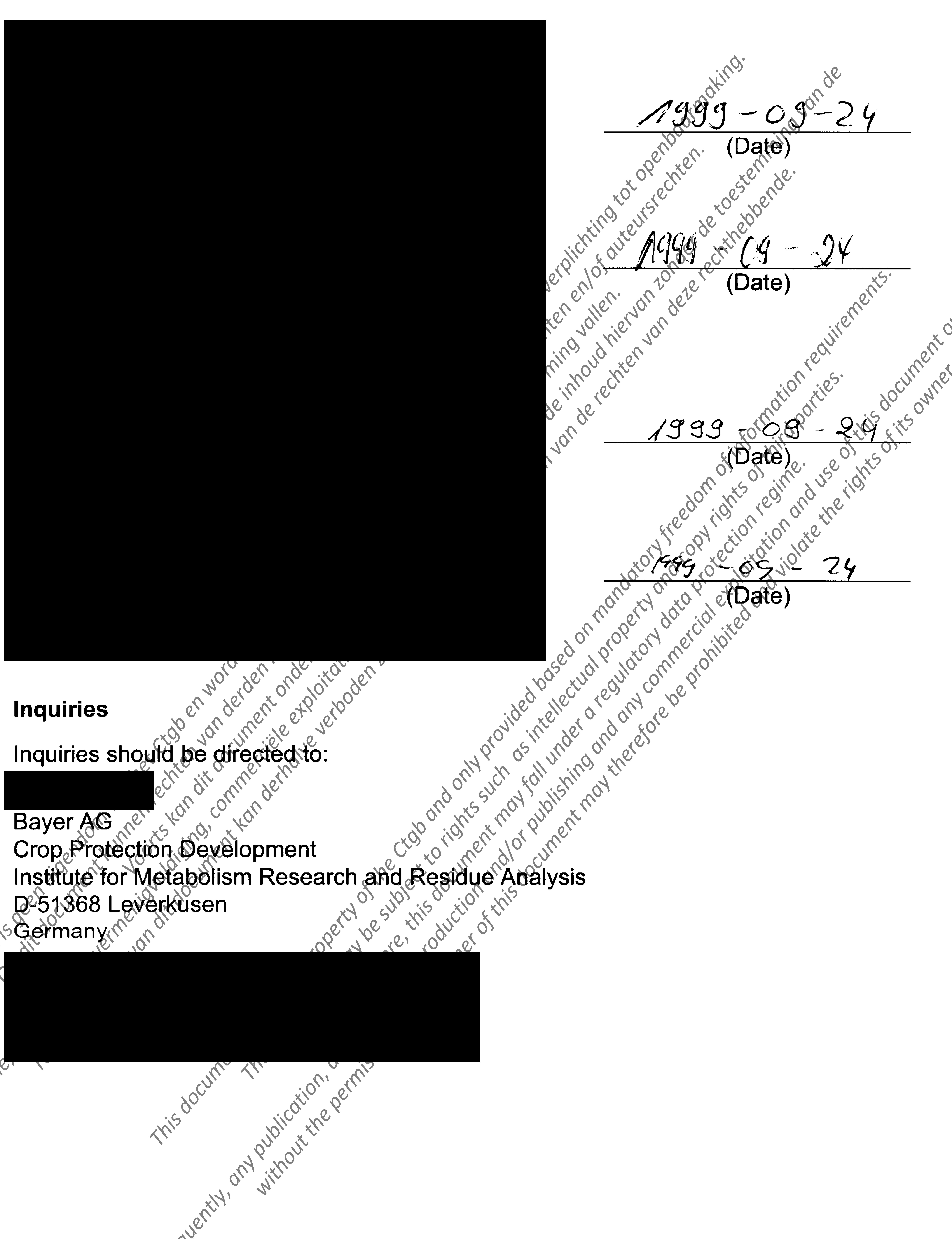

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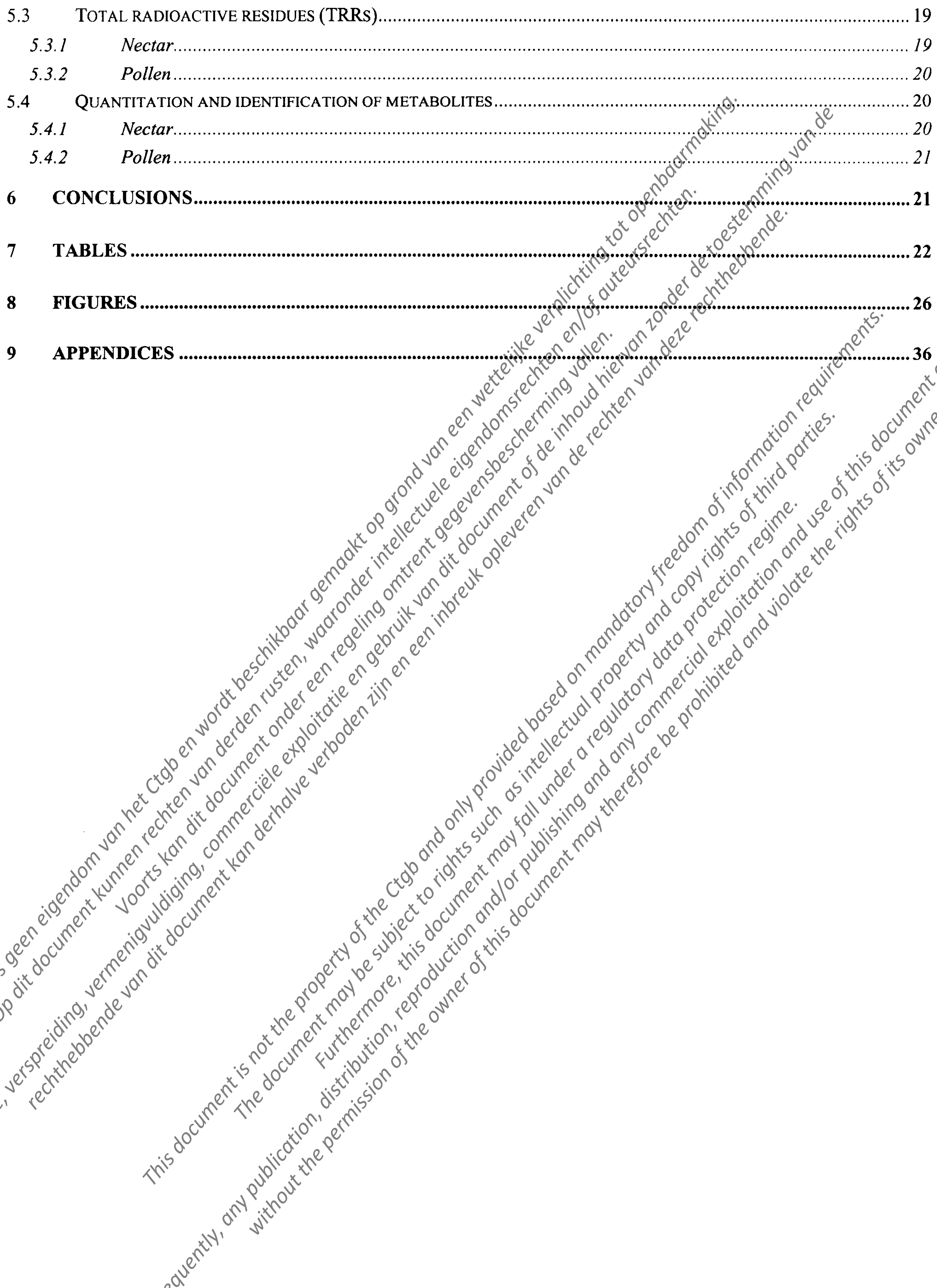

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ABBREVIATIONS

AMD	Automated multiple development
HPLC	High performance liquid chromatography
LSC	Liquid scintillation counter / counting
SPE	Solid phase extraction
TLC	Thin-layer chromatography
TRR	Total radioactive residue

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1 SUMMARY

The occurrence of residues of the insecticide NTN 33893 (imidacloprid) and its metabolites in nectar and pollen of sunflower was investigated after seed dressing in a greenhouse experiment. [Methylene-^{14}C]imidacloprid was formulated as a WS 70 (equivalent to "Gaucho"). The application conditions projected for this experiment simulated the practice conditions of 150 g WS 70/unit sunflower seeds (1 unit = 150,000 grains), equivalent to 105 g a.i./unit. In the experiment, each sunflower seed was coated with ca. 1.0 mg of formulation, equivalent to ca. 0.7 mg a.i. A total of 22 sunflower plants (variety „Fleury") were separately grown in 34-L pots (ca. 40 cm diameter) in the greenhouse, subdivided into two rows of 11 plants each.

During flowering, nectar was collected every day with a capillary from the florets that were in the female stage. In total, ca. 1.7 g nectar/row was collected during a period of 2 weeks. Pollen was collected with the aid of plastic boxes that were installed underneath the inflorescence. The pollen freely trickled into the plastic boxes. In total, ca. 4.8 g pollen/row was collected.

The total radioactive residues (TRR) of both rows (nectar and pollen) were almost identical and averages are presented. On average, the TRR in nectar amounted to 0.0019 mg/kg and 0.0039 mg/kg in pollen. In total, 85.8 % of the TRR in the pollen was extractable with methanol/water (3:1, v/v) and methanol. Only 14.2 % of the TRR (0.0006 mg/kg) was not extractable and remained in the solids. Due to the very low radioactivity content, the solids were not further investigated.

The nectar and the pollen extracts were purified using an Oasis® resin SPE cartridge (Waters) and analyzed by 2-dimensional thin-layer chromatography as well as AMD co-chromatography. Imidacloprid was the only residue observed in the nectar and pollen extracts (0.0019 mg/kg and 0.0033 mg/kg, respectively). No metabolites of imidacloprid were observed in either nectar or pollen of sunflower.

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2 INTRODUCTION

NTN 33893 (common name: imidacloprid) is the first commercial product of a new class of insecticides, the chloronicotinyl insecticides. Due to its systemic properties, a main use pattern for NTN 33893 is seed dressing application (trademark e.g. Gaucho®) in a large number of crops. The occurrence of residues of the insecticide NTN 33893 and its metabolites in sunflower nectar and pollen was investigated after seed dressing in a greenhouse experiment. Nectar and pollen samples were taken during flowering (growth stage 61-67 of the BBCH code).

The active ingredient (a.i.) was ^{14}C-radiolabelled in the methylene-^{14}C position. The application used for this experiment simulated the typical agricultural practice of 150 g WS 70/unit sunflower seeds (1 unit = 150,000 grains), equivalent to 105 g a.i./unit. The seed density was assumed to be 0.5 units/ha (75,000 grains/ha). Thus, each sunflower seed was coated with ca. 1.0 mg of formulation that was equivalent to ca. 0.7 mg a.i.

The study began with the seed dressing and sowing of the sunflower seeds on February 23, 1999 and was finished experimentally on September 24, 1999. The raw data, the original report and all documents pertaining to the study will be archived in the central GLP-Archive at Monheim, building 6500.

3 MATERIALS

3.1 Test compound

Common name:	Imidacloprid
Code name:	NTN 33893
CAS name:	1-[(6-chloro-3-pyridinyl)methyl]-N-nitro-2-imidazolidinimine
CAS number:	138261-41-3
Empirical formula:	$C_9H_{10}ClN_5O_2$
Structural formula:	Cl, N, *, N, NH, N, NO_2
Radiolabelling position:	[methylene-^{14}C]imidacloprid, asterisk (*) denotes radiolabelling position
Molar mass:	255.7 g/mole

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Specific radioactivity: 3.77 MBq/mg (ca. 102 µCi/mg)

Source: Isotope Laboratory Elberfeld, Bayer AG, Wuppertal

Radiochemical purity (protocol THS 4918): > 99 % (radio-HPLC)
> 99 % (radio-TLC)

The radiolabelled NTN 33893 used for formulation was sythesised in the Isotope Laboratory Elberfeld, Bayer AG, Wuppertal, where its specific radioactivity and radiochemical purity were determined. The identity of the parent compound was confirmed by ^{1}H-NMR (**Appendix I**) and LC-MS/MS (**Appendix II** and **Appendix III**) in the laboratory of Dr. Bornatsch.

3.2 Formulation

The radiolabelled NTN 33893 was formulated as a WS 70 (70 % a.i., laboratory Dr. Ecker) by finely mixing the radiolabelled active ingredient with the blank formulation ("WS 70 rot") using a ball mill. The conditions needed to obtain a formulation equivalent to the commercial product were determined in pre-experiments. The content of the active ingredient in the formulation was determined by weighing. Details of the formulation are given in **Appendix IV**.

3.3 Reference compounds

The structural formulae of the reference compounds, used for identification of metabolites, are given in **Table I**. The stability of the reference compounds was regularly checked during the course of the study.

3.4 Test facilities and test system

The experiment was conducted in the greenhouse (Building 6681) of the Institute for Metabolism Research and Residue Analysis, Monheim, Bayer AG. Twentytwo plants (variety „Fleury") were grown in 34-L pots (ca. 40 cm diameter, surface area of ca. 1300 cm^2, 1 plant per pot), filled with soil "Langenfeld". Details of the soil analysis are given in **Appendix V**. The sunflower plants were subdivided into two rows of 11 pots each.

Conditions were maintained so as to assure good plant growth. Plant protection and fertilization measures were carried out and documented as were climatic details (**Appendix VI**).

Each planting container was marked with the study number and a radioactivity symbol.

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4 METHODS

4.1 Application and sowing

On February 23, 1999, 35 sunflower seeds were treated with 40.02 mg of the formulation (**3.2**), containing 28.01 mg (105.61 MBq) of [methylene-^{14}C]imidacloprid. The seed dressing was performed in a test tube on a laboratory shaker. At first, dry formulation (powder) was added to the seeds and evenly distributed during shaking. Subsequently, a small amount of water was added during shaking. This procedure was optimized in pre-experiments.

The losses during application (contamination of the test tube) amounted to 1.71 MBq (1.62 %). Thus, the net applied amount per 35 seeds was 103.90 MBq (27.56 mg a.i.) or 0.787 mg a.i./seed. This is slightly exaggerated (+ 12.5 %) compared to the projected application rate of 0.7 mg a.i./seed (see section **2**).

The radiochemical purity and stability of the parent compound in the formulation was determined before and after application by radio-TLC (**Appendix VII**) and radio-HPLC (**Appendix VIII**).

After application, each of the seeds was sown in a separate soil pot (ca. 6 x 6 x 6 cm), made of soil „Langenfeld". On March 04, 1999 (9 days after application), 22 strong plants, including the whole soil pot, were transferred into the final 34-L pot (**3.4**). The surface area per plant (ca. 1300 cm^2) was comparable to the field situation (ca. 1300 cm^2, section **2**). However, due to the limited depth of the pots, the soil volume of the root zone represents a worst case situation in this study.

4.2 Sampling and storage

Sunflower inflorescences generally start flowering in circles from the outermost florets to the inner florets. Each floret is in the male phase (shedding of pollen) on the first day of flowering and in the female phase (stigma visible) during the next day. After the female stage, the floret fades and the seed starts to grow. In pre-experiments it had been established, that considerable amounts of nectar were only present in female florets after noon.

Flowering started between April 26, 1999 (first plant row B) and April 30, 1999 (first plant row A) and was completed on May 09, 1999 (**Appendix IX**). During the flowering period, nectar and pollen was sampled. One day after flowering, the plants were harvested and fractionated.

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The plant fractions of row A and row B were sampled and stored separately in order to have two independent samples.

All samples were labelled using laboratory journal numbers and stored deep frozen at ca. -20°C until processed and analyzed. During the analytical work, the extracts were stored at ca. +4°C in a refrigerator.

4.2.1 Nectar

During flowering, the nectar was collected every day. The procedure was as follows:

Every day, all female florets were plucked from the inflorescence after 1 p.m. using tweezers. The florets were stored in a refrigerator (ca. +4°C) for some hours until nectar sampling. In the laboratory, the nectar was pipetted out of the florets using glass capillaries (1.3-1.4 mm o.d., Merck). At the end of each day, the filled capillaries were emptied using a pipette bulb into a weighed glass vial. The daily nectar harvests were pooled in this vial, resulting in two nectar samples (row A and row B). The amount of nectar collected each day was determined by weighing. The nectar was stored frozen without further processing.

4.2.2 Pollen

Plastic boxes were installed underneath the inflorescences before flowering (**Figure 1**). During flowering, the pollen freely trickled into these boxes. At the end of the flowering, the content of the boxes was pooled for each row. The pollen was cleaned using a 0.5-mm sieve and stored frozen without further processing.

4.2.3 Plant

On May 10, 1999, the plants were harvested and fractionated as follows:

- The petals (big yellow leaves) of the inflorescences were collected and stored frozen.
- The remaining florets on the inflorescences were collected and stored frozen.
- The inflorescences (disks) were cut off and stored frozen.
- All leaves were harvested and pooled according to their developmental age: a) bottom of plant (oldest leaves, lower third), b) middle of plant (mid third) and c) top of plant (youngest leaves, upper third). The leaf samples were cut in pieces and macerated in liquid nitrogen using a Polytron (Kinematica). The macerated leaves were stored frozen.
- The stems were cut above the ground, cut in pieces and stored frozen.

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The above mentioned fractions were kept in reserve, e.g. for metabolite identification, etc.

4.3 Extraction and purification

4.3.1 Nectar

The extraction scheme used for nectar (rows A and B) is shown in **Figure 2**. Due to its high viscosity, the nectar was diluted with H_2O (**Appendix X**) in order to facilitate pipetting etc. for further measurements. The density of the diluted nectar was determined by weighing 100-µL aliquots (**Appendix XI**). The TRR was determined by LSC, measuring 100-µL aliquots of the diluted nectar.

HPLC analysis of the nectar could not be done due to the limited amount of radioactivity. Therefore, TLC and AMD co-chromatography were utilized for compound identification. Due to the biological matrix (sugars etc.), the diluted nectar could not be chromatographed directly. Thus, the diluted nectar was analyzed after purification by SPE as follows:

A 6-g Oasis® cartridge (Waters) was pre-conditioned with 30 mL methanol and 30 mL of 10 % formic acid in H_2O. Approximately 50 % of the diluted nectar (corresponding to ca. 1.7 mL, ca. 7 Bq, average of row A (**Appendix XIV)** and row B (**Appendix XV**)) was pipetted onto the cartridge. The cartridge was washed with 30 mL H_2O and subsequently eluted with 30 mL methanol using gravity flow.

The aqueous wash solution contained minimal radioactivity and was discarded. The methanol extract was rotary evaporated (40° C) to ca. 500 µL, and the radioactivity content was determined by LSC. After further concentration under N_2, the methanol extract was analyzed by 2-dimensional TLC (**4.4**) and AMD (**4.5**).

From nectar of row A, two extractions with SPE purification were performed (**Appendix XIV**). The methanol extract of the first cleanup was used for 2-dimensional TLC analysis, and the methanol extract of the second cleanup was used for AMD analysis. From nectar row B, only one SPE procedure was necessary and the same methanol extract was used for both 2-dimensional TLC and AMD analysis (**Appendix XV**).

4.3.2 Pollen

The extraction scheme used for pollen (row A and B) is shown in **Figure 3**. Two grams of pollen were soaked in 30 mL methanol/water (3:1, v/v) for 30 min. Subsequently, the suspension was macerated with a Polytron (Kinematica) for ca. 3 min and vacuum filtered. The extraction was repeated twice with methanol. The extracts were combined (raw extract). No radioactivity measurement was performed on the raw extract due to the very

low amount of radioactivity. The raw extract was concentrated to the aqueous remainder on a rotary evaporator (40°C), and aliquots were analyzed by LSC.

HPLC analysis of the pollen extract could not be done due to the limited amount of radioactivity. Therefore, TLC and AMD co-chromatography were utilized for compound identification. Due to the biological matrix, the aqueous remainder of the pollen extract was purified using SPE in the same manner as for the diluted nectar (**4.3.1**).

4.4 Thin-layer chromatography (TLC)

The radioactive solutions were investigated by one or two dimensional TLC using glass backed silica gel plates (Kieselgel 60 F_{254}, 20 x 20 cm, 0.25 mm thickness, Merck no. 1.05715). For one dimensional TLC, bands of 15 mm were spotted on the TLC plates using an automatic plate spotter (Linomat IV, Camag) and the plates were developed over a distance of ca. 15 cm. For two dimensional TLC, aliquots were spotted manually on the origin and the plates were developed over a distance of ca. 10 cm. No chamber saturation was performed. The following solvent systems were used (ratios by volume):

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

The R_f-values of the reference compounds in the different solvent systems are listed in **Table I**.

The radioactive zones were detected using a BAS 2000 BioImaging Analyzer (Fuji) and quantified using the software "Tina" (Raytest, version 2.09g). The reference compounds used in co-chromatography were visualized by UV light (254 nm).

4.5 Automated multiple development (AMD)

AMD analysis was performed on an AMD-II instrument (Camag). The radioactive solutions were analyzed using glass backed silica gel HPTLC plates (Kieselgel 60 WR F_{254}s, 10 x 20 cm, 0.20 mm thickness, Merck no. 15552). Bands of 10-15 mm were spotted on the HPTLC plates using an automatic plate spotter (Linomat IV, Camag). The plates were developed over a distance of ca. 8 cm. For details of the AMD method see **Appendix XII**. The evaluation of the HPTLC plates was performed in the same manner as the TLC plates (**4.4**).

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4.6 High performance liquid chromatography (HPLC)

The HPLC method "MI3601H" was based on reversed phase chromatography with columns from NEOS Company Ltd (Japan). The solvents were of HPLC quality. The instrument set-up and chromatographic conditions are listed below:

HPLC:	HP 1050 (Hewlett Packard)
UV-detector:	Variable wavelength detector (HP 1050), set to 270 nm
^{14}C-detector:	Ramona 2000 (2"), connected in line with HPLC equipped with a 370 µL CaF_2 scintillator cell (Raytest, Straubenhardt, Germany)
Column:	Fluofix - 120E, 1EW425, 5 µm, 4.6 x 250 mm
Oven temperature:	40°C
Flow rate:	1 mL/min
Eluent A:	Buffer pH3 (1:10) + 5 mmol/L octane-1-sulfonic acid
Eluent B:	Buffer pH3 (1:10) + 5 mmol/L octane-1-sulfonic acid in acetonitrile / water 1:1
Gradient:	0-5 min. 0 % B, at 70 min. 40 % B, at 75 min. 95 % B, at 80 min 95 % B, at 81 min 0 % B until 95 min

The method was used to measure the radiochemical purity of the parent compound in the formulation (**Appendix VIII**). All other extracts contained not enough radioactivity for HPLC investigation.

4.7 Radioactivity measurement

The radioactivity in liquid samples was determined by LSC. Due to the very low level of radioactive residues in nectar and pollen, sample measuring times were up to 100 min. Technical details of the measurement of radioactivity are given in **Appendix XIII**.

Solid samples were combusted using an Oxidizer 387 (Canberra-Packard Instruments). The CO_2 produced by combustion was absorbed in a CO_2 absorbent / scintillation cocktail mixture (8 mL Carbosorb E + 10 mL Permafluor E$^+$, Canberra Packard) and the radioactivity measured by LSC (PW 4700, Philips).

4.8 Spectroscopy

4.8.1 MS-Spectroscopy

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The electro-spray ionisation MS spectra (ESI) were obtained with a TSQ 7000 (Finnigan). Sheath gas pressure: 52 psi, capillary temperature: 210 °C. For the MS/MS experiments, argon was used as the collision gas (pressure in the collision chamber: 2.7 mT).

The chromatographic conditions for the MS experiments are given below. After the HPLC instrument (HP 1050, Hewlett Packard) the flow is split. Approx. 20 % of the eluent went to the mass spectrometer and ca. 80 % went to a radioactivity detector (Ramona 90, Raytest).

Sample:	ECW 11683
HPLC:	HP 1050 (Hewlett Packard)
^{14}C-detector:	Ramona 90 (Raytest, Straubenhardt, Germany)
Column:	LiChrospher 60 RP select B (VDS Optilab), 5 µm, 250 x 2 mm
Oven temperature:	40°C
Flow rate:	0.2 mL/min
Eluent A:	1 % acetic acid in water
Eluent C:	acetonitrile
Gradient:	0-1 min. 5 % C, at 25 min. 95 % C, at 35 min. 95 % C
Split ratio:	40 µL/min : 160 µL/min (MS : ^{14}C)

4.8.2 NMR-Spectroscopy

The 600-MHz NMR-spectrum was recorded on a DMX 600 (Bruker). Sample ID and solvent (supplier: Merck, Wilmad or Aldrich) are given in the spectra header.

4.9 Data evaluation

Calculations were primarily performed on a personal computer using the software EXCEL 97 (Microsoft). In general, nonrounded numbers were used for computations. The results given in the tables are rounded to a certain number of decimal places. Thus, rounding errors may occur if recalculations are made using the rounded figures.

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5 RESULTS AND DISCUSSION

5.1 Stability check of Imidacloprid in the formulation

Before and after the seed dressing, an aliquot of the formulation was investigated for radiochemical purity of the parent compound by co-chromatography with non-labelled imidacloprid by radio-TLC (**Appendix VII**) and radio-HPLC (**Appendix VIII**). No degradation was detected by TLC or HPLC.

5.2 Yield of nectar and pollen

The total nectar yield was 1.72 g (row A) and 1.74 g (row B) (**Table II**). On average, ca. 0.16 g nectar/plant was harvested.

The total pollen yield was 4.74 g (row A) and 4.81 g (row B) (**Table II**). On average, ca. 0.4 g pollen/plant was harvested.

5.3 Total radioactive residues (TRRs)

The total radioactive residue (TRR) in nectar was determined by measuring the radioactivity in aliquots of the diluted nectar (**Figure 2**). The radiocativity concentration in the methanol/water raw extract was too low for direct LSC measurement. Therefore, the TRR in pollen was determined by summation of the radioactivity in the aqueous remainder of the raw extract and in the remaining solids after extraction (**Figure 3**). Generally, the TRR was expressed in mg a.i. equivalents per kg sample weight (on basis of the specific radioactivity of the test compound of 3.77 MBq/mg, **3.1**). Amounts of radioactive residues in different extracts were expressed as percentage of the TRR and also as mg a.i. equivalents per kg sample weight.

Since the TRRs and the extractability of radioactivity of both rows (nectar and pollen) were almost identical, averages were calculated and presented below. Single values of row A and row B can be obtained from the tables and appendices.

5.3.1 Nectar

The average TRR in nectar was 0.0019 mg/kg (**Table II**). The extraction procedure and the distribution of radioactivity in different extracts is shown in **Figure 2**. After SPE, the methanol extract contained on average 96.2 % of the radioactivity in the diluted nectar (average of 3 SPE purifications). The aqueous SPE wash contained insignificant amounts of radioactivity (≤3.5 % of the TRR) and was discarded. Despite the very low level of radioactive residues and the corresponding accuracy of the radioactivity measurement,

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significant losses of radioactivity during SPE purification and extracts concentration could be excluded.

Raw data and calculations are given in **Appendix XIV** and **Appendix XV**.

5.3.2 Pollen

The average TRR in pollen was 0.0039 mg/kg (**Table II**). The extraction procedure and the distribution of radioactivity in different extracts is shown in **Figure 3**. On average, 85.8 % (0.0033 mg/kg) of the TRR was extractable with methanol/water and methanol. After SPE purification, the methanol extract contained on average 85.7 % of the TRR (97.1 % column recovery). The aqueous wash solution contained insignificant amounts of radioactivity (≤0.3 % of the TRR) and was discarded. Despite the very low level of radioactive residues and the corresponding accuracy of the radioactivity measurement, significant losses of radioactivity during SPE purification and extracts concentration could be excluded. Only 0.0006 mg/kg (14.2 % of the TRR) remained in the solids and were not further investigated

Raw data and calculations are given in **Appendix XVI** and **Appendix XVII**.

5.4 Quantitation and identification of metabolites

Quantitation of the metabolites (profiling) was performed by 2-dimensional TLC (**4.4, Figure 4, Figure 5, Figure 7 and Figure 8**).

Identification of the radioactive residue (i.e. imidacloprid) was achieved by co-chromatography of the different extracts with non-labelled reference compounds in two independent chromatographic systems, i.e. 2-dimensional TLC (**4.4**) and AMD (**4.5**).

Due to the limited amount of available extract / radioactivity, profiling and metabolite identification was performed on the same TLC plate. The maximum number of compounds for co-chromatography on 2-dimensional TLC was determined to be five. Thus, co-chromatography was performed with a selection of five reference compounds, i.e. imidacloprid (NTN 33893), 4-hydroxy-imidacloprid (FHW 0105D), dihydroxy-imidacloprid (WAK 3772), cyclic urea compound (DIJ 9817), and olefin compound (WAK 3745), which were assumed to be the most likely compounds. Subsequently, co-chromatography on AMD was performed with an imidacloprid standard as a confirmatory method.

5.4.1 Nectar

After SPE, the methanol extracts of each row have been investigated both by 2-dimensional TLC (**Figure 4** and **Figure 5**) and AMD (**Figure 6**). In all chromatograms, only

parent compound (imidacloprid) was detected. Thus, imidacloprid in nectar amounted to 0.0019 mg/kg, which is 100 % of the TRR (**Table III**).

5.4.2 Pollen

After SPE, the methanol extracts of each row was investigated both by 2-dimensional TLC (**Figure 7** and **Figure 8**) and AMD (**Figure 9**). In all chromatograms, only parent compound (imidacloprid) was detected. Thus, imidacloprid in pollen amounted to 0.0033 mg/kg, which is 100 % of the methanol extract and 85.8 % of the TRR (**Table III**).

6 CONCLUSIONS

After practice-like seed dressing of sunflower seeds with a WS 70 formulation of ^{14}C-labelled imidacloprid (equivalent to "Gaucho"), very low residues of imidacloprid were detected in nectar and pollen of blooming sunflowers in a greenhouse experiment. The TRR in nectar consisted just of the parent compound imidacloprid (0.0019 mg/kg). The TRR in pollen amounted to 0.0039 mg/kg of which 0.0033 mg/kg (85.8 %) were identified as parent compound imidacloprid.

Acknowledgements

The author gratefully acknowledges the horticultural assistance of [redacted] development of an AMD method of [redacted] and the help of many colleagues during nectar collection.

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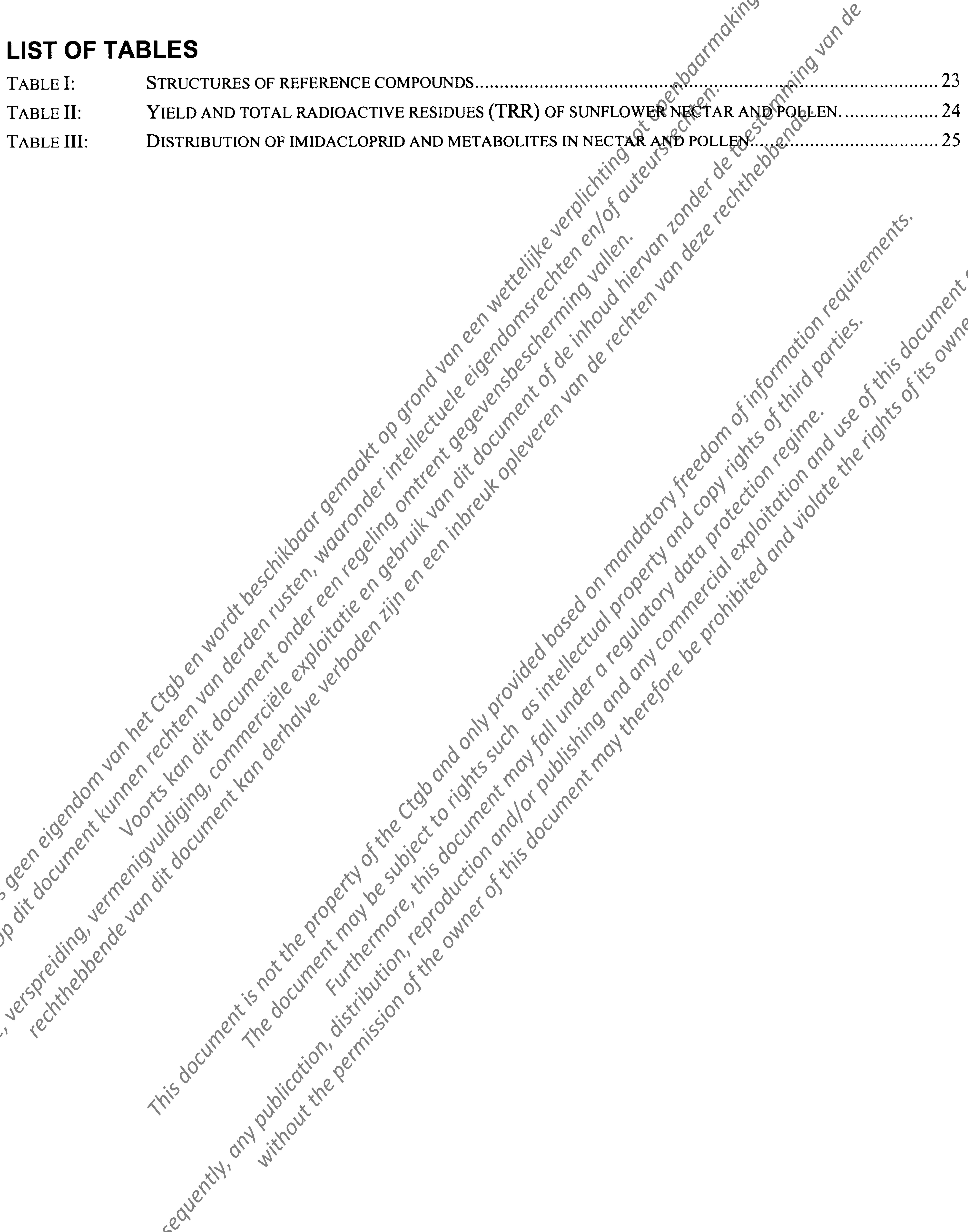

Table I: Structures of reference compounds.

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

Met. No.	Structural Formula	Compound / Chemist Code (former codes in brackets)	R_f-values SS I	SS II	SS III	SS IV
0	Cl, N, N, NH, N, NO_2	**NTN 33893** parent compound	0.75	0.67	0.66	0.88
2	OH, Cl, N, N, NH, N, NO_2	**FHW 0105D** (WAK 5839) 4-hydroxy-imidacloprid	0.83	0.65	0.74	0.80
3	HO, OH, Cl, N, N, NH, N, NO_2	**WAK 3772** dihydroxy-imidacloprid	0.90	0.74	0.96	0.68
6	Cl, N, N, NH, N, NO_2	**WAK 3745** (NTN 35884) olefin compound	0.71	0.59	0.66	0.84
12	Cl, N, N, NH, O	**DIJ 9817** (WAK 4740) cyclic urea compound	0.63	0.54	0.68	0.94

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Table II: **Yield and total radioactive residues (TRR) of sunflower nectar and pollen.**

Sample	Sample ID No.	Weight (yield)	TRR		Total radioactivity in whole sample	
		[g]	[Bq/g]	[mg/kg]	[Bq]	[ng]
Nectar						
Row A	MI3605B	1.72	7.7	0.0020	13	3.5
Row B	MI3606B	1.74	6.8	0.0018	12	3.2
Average				*0.0019*		
Pollen [①]						
Row A	MI3607D	4.74	15.1	0.0040	72	19
Row B	MI3607I	4.81	14.3	0.0038	69	18
Average				*0.0039*		

[①] TRR was determined by summation of combined raw extracts and solids (**Figure 3**).

Corresponding raw data see **Appendix XIV - Appendix XVII**.

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Table III: **Distribution of imidacloprid and metabolites in nectar and pollen.**

Radioactivity recovered in nectar/pollen = 100 %.

mg/kg values expressed as active ingredient equivalents.

A)

Nectar	%	mg/kg
Subtotal identified	**100.0**	**0.0019**
Parent compound (imidacloprid)	100.0	0.0019
Total	**100.0**	**0.0019**

Corresponding raw data see **Appendix XIV** and **Appendix XV**.

B)

Pollen	%	mg/kg
Subtotal identified	**85.8**	**0.0033**
Parent compound (imidacloprid)	85.8	0.0033
Solids	**14.2**	**0.0006**
Total	**100.0**	**0.0039**

Corresponding raw data see **Appendix XVI** and **Appendix XVII**.

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Figure 1: Plastic box underneath a sunflower inflorescence for pollen collection.

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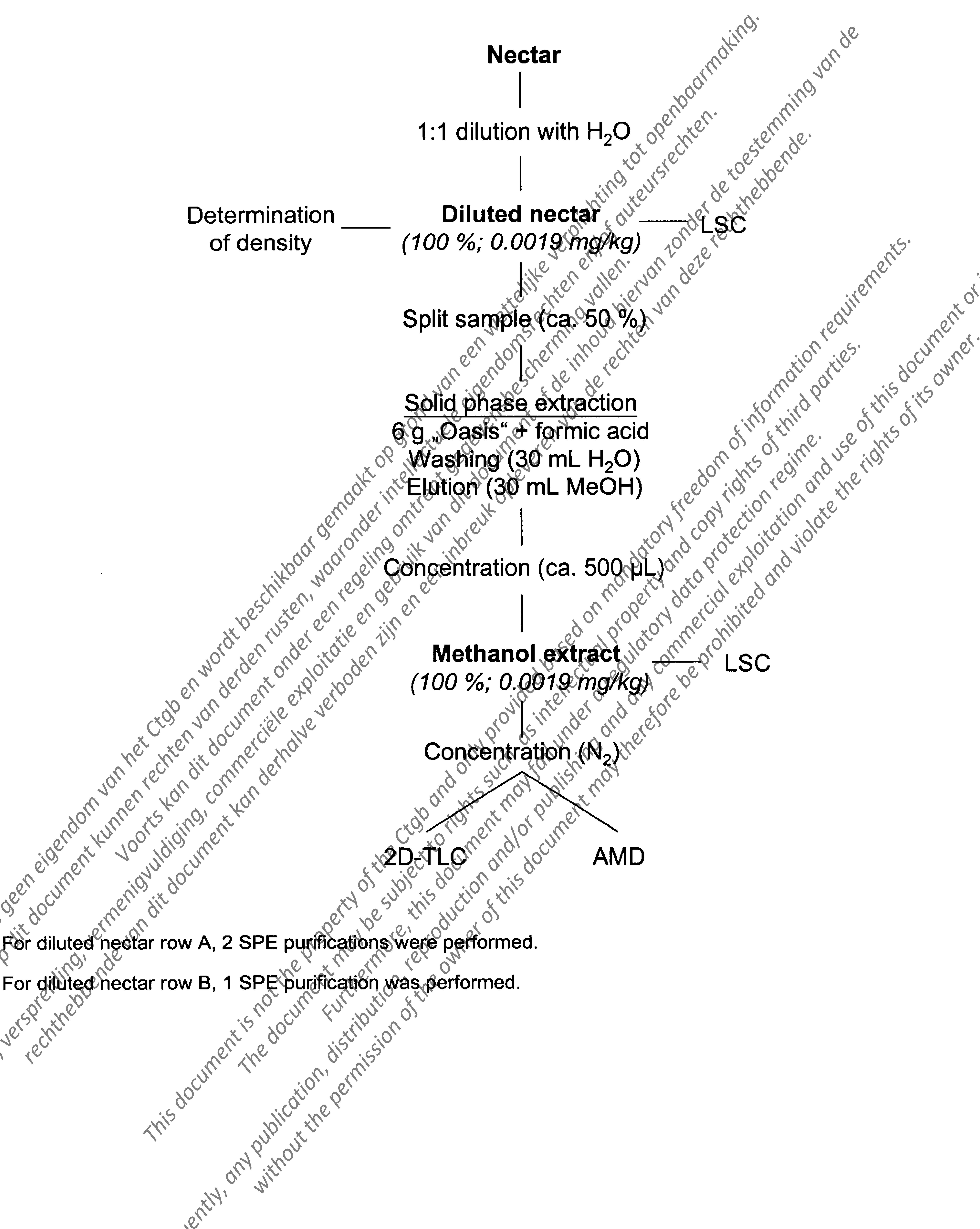
Figure 2: Scheme of the extraction procedure for sunflower nectar.



For diluted nectar row A, 2 SPE purifications were performed.

For diluted nectar row B, 1 SPE purification was performed.

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Figure 3: Scheme of the extraction procedure for sunflower pollen.

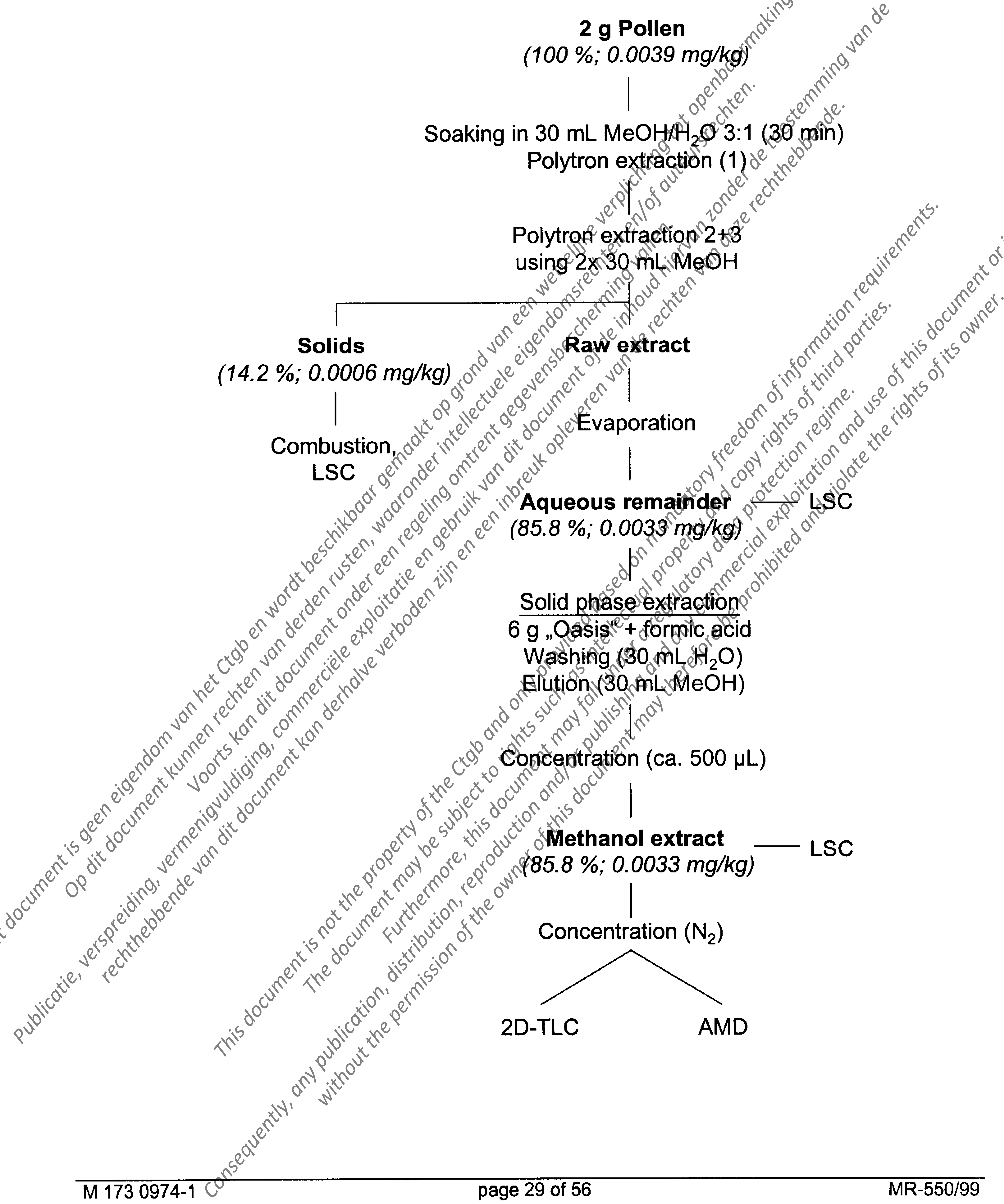

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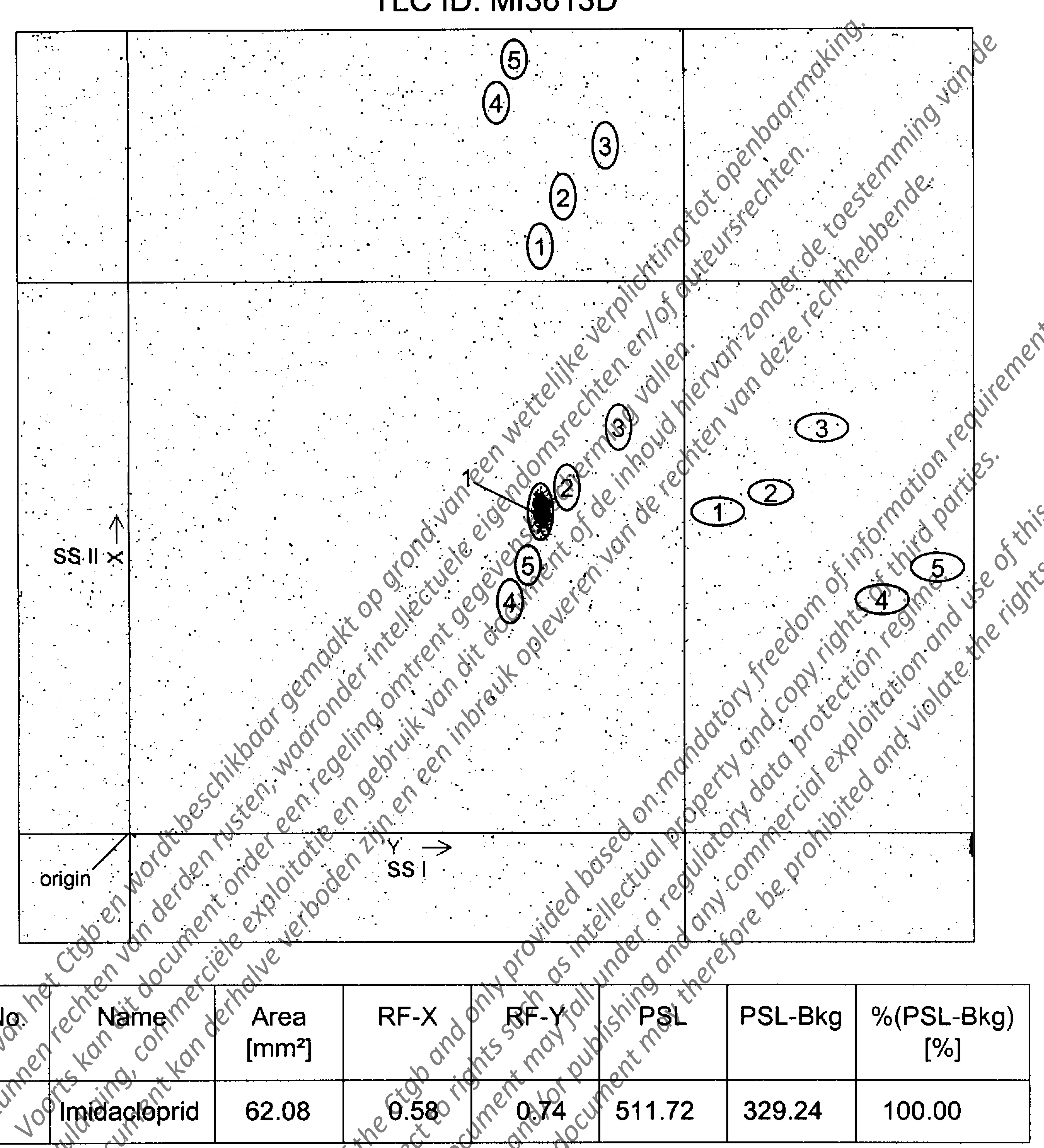
Figure 4: 2-dimensional TLC analysis of the methanol extract of nectar, row A.

TLC ID: MI3613D



No.	Name	Area [mm²]	RF-X	RF-Y	PSL	PSL-Bkg	%(PSL-Bkg) [%]
1	Imidacloprid	62.08	0.58	0.74	511.72	329.24	100.00

Sample: MI3613C plus all reference compounds listed below
1: reference compound NTN 33893 (imidacloprid)
2: reference compound FHW 0105D
3: reference compound WAK 3772
4: reference compound DIJ 9817
5: reference compound WAK 3745

The non-labelled reference compounds were visualized under UV light. They were labelled with circles on the TLC plate and in this figure.

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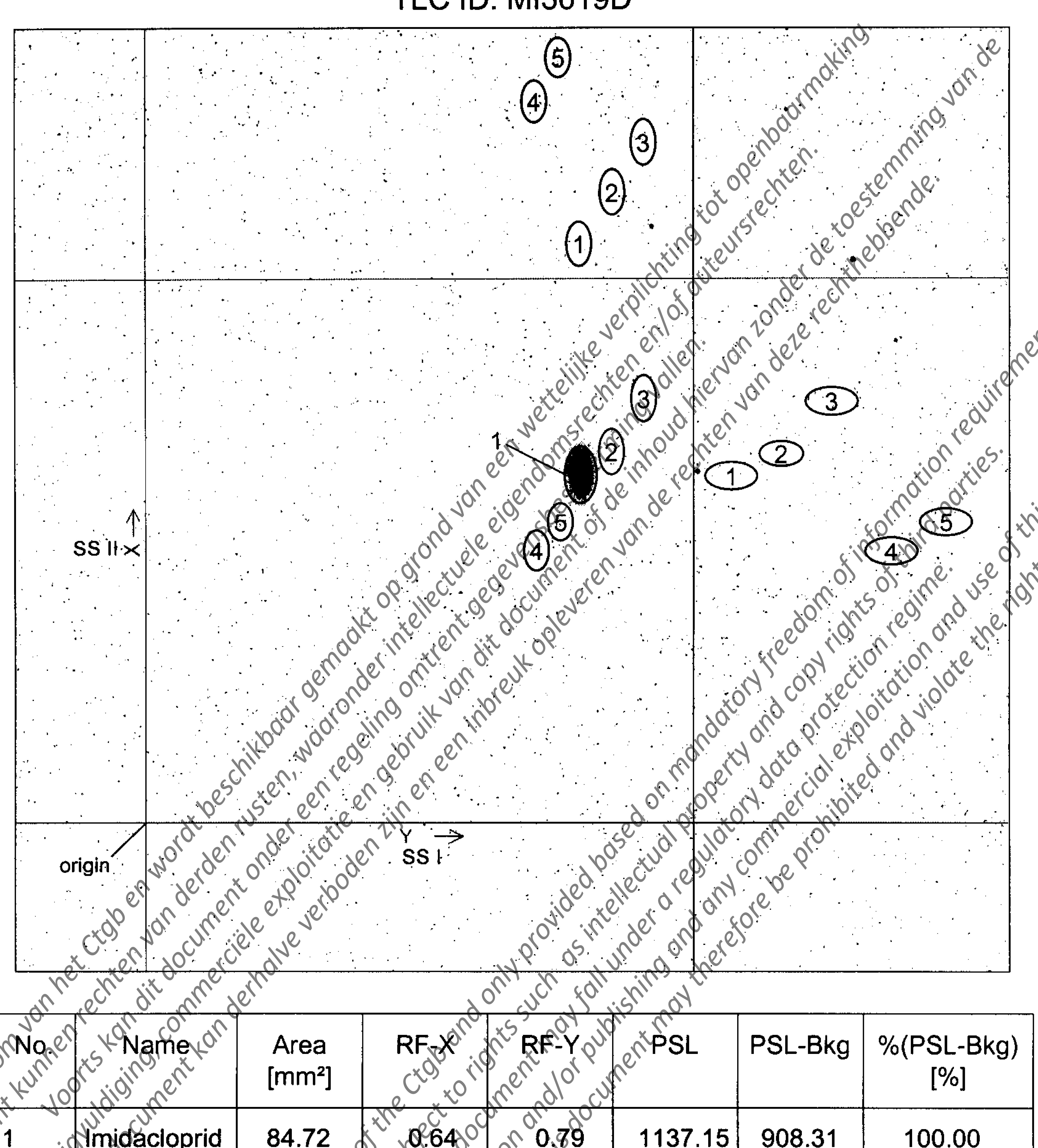
Figure 5: 2-dimensional TLC analysis of the methanol extract of nectar, row B.

TLC ID: MI3619D



No.	Name	Area [mm²]	RF-X	RF-Y	PSL	PSL-Bkg	%(PSL-Bkg) [%]
1	Imidacloprid	84.72	0.64	0.79	1137.15	908.31	100.00

Sample: MI3619C plus all reference compounds listed below
1: reference compound NTN 33893 (imidacloprid)
2: reference compound FHW 0105D
3: reference compound WAK 3772
4: reference compound DIJ 9817
5: reference compound WAK 3745

The non-labelled reference compounds were visualized under UV light. They were labelled with circles on the TLC plate and in this figure.

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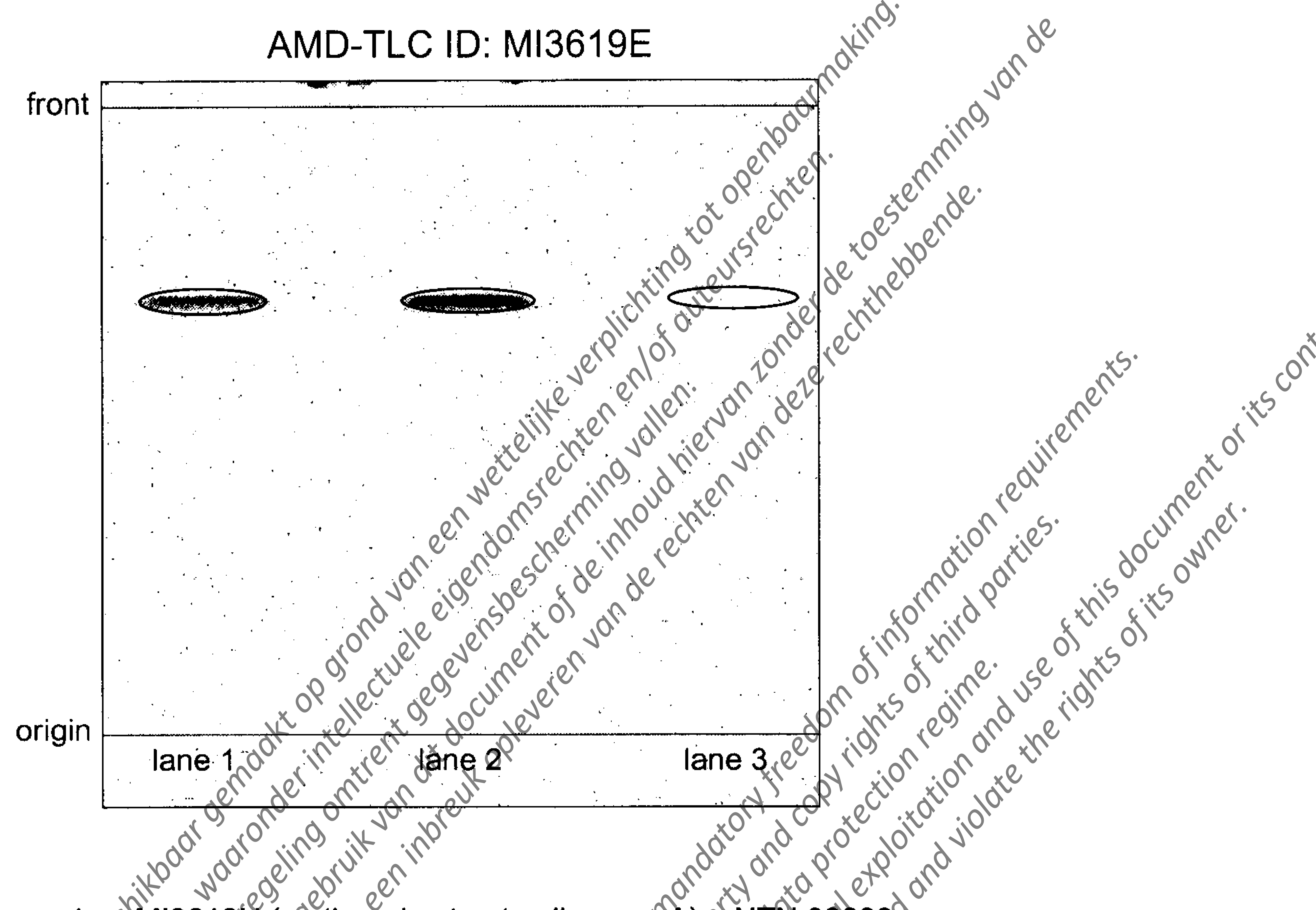
Figure 6: **AMD analysis of the methanol extract of nectar.**



Lane 1: MI3613H (methanol extract pollen row A) + NTN 33893
Lane 2: MI3619C (methanol extract pollen row B) + NTN 33893
Lane 3: reference compound NTN 33893

The non-labelled reference compounds were visualized under UV light. They were labelled with circles on the TLC plate and in this figure.

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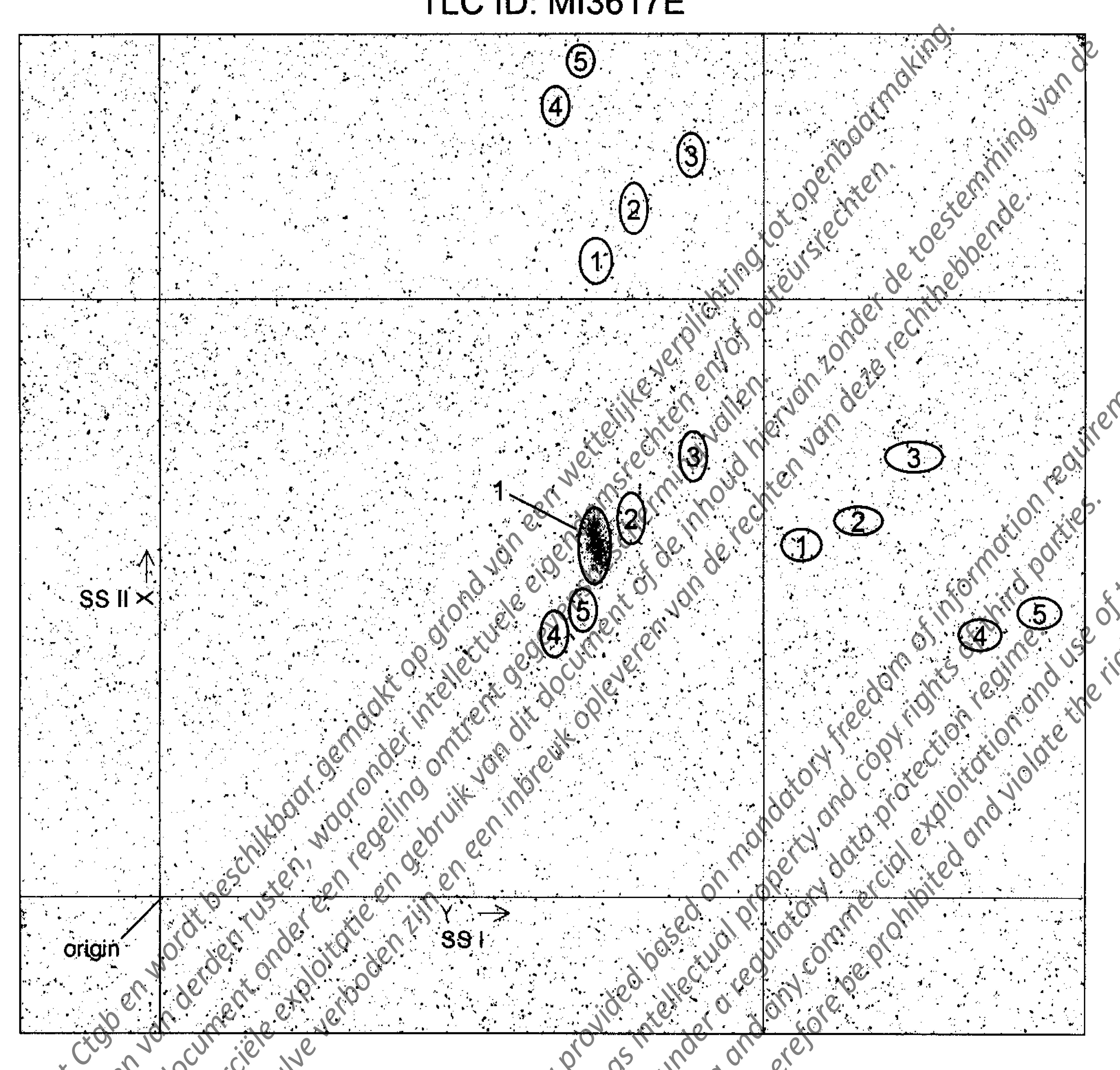
Figure 7: 2-dimensional TLC analysis of the methanol extract of pollen, row A.

TLC ID: MI3617E



No.	Name	Area [mm²]	RF-X	RF-Y	PSL	PSL-Bkg	%(PSL-Bkg) [%]
1	Imidacloprid	89.80	0.59	0.73	396.22	170.14	100.00

Sample: MI3617D plus all reference compounds listed below
1: reference compound NTN 33893 (imidacloprid)
2: reference compound FHW 0105D
3: reference compound WAK 3772
4: reference compound DIJ 9817
5: reference compound WAK 3745

The non-labelled reference compounds were visualized under UV light. They were labelled with circles on the TLC plate and in this figure.

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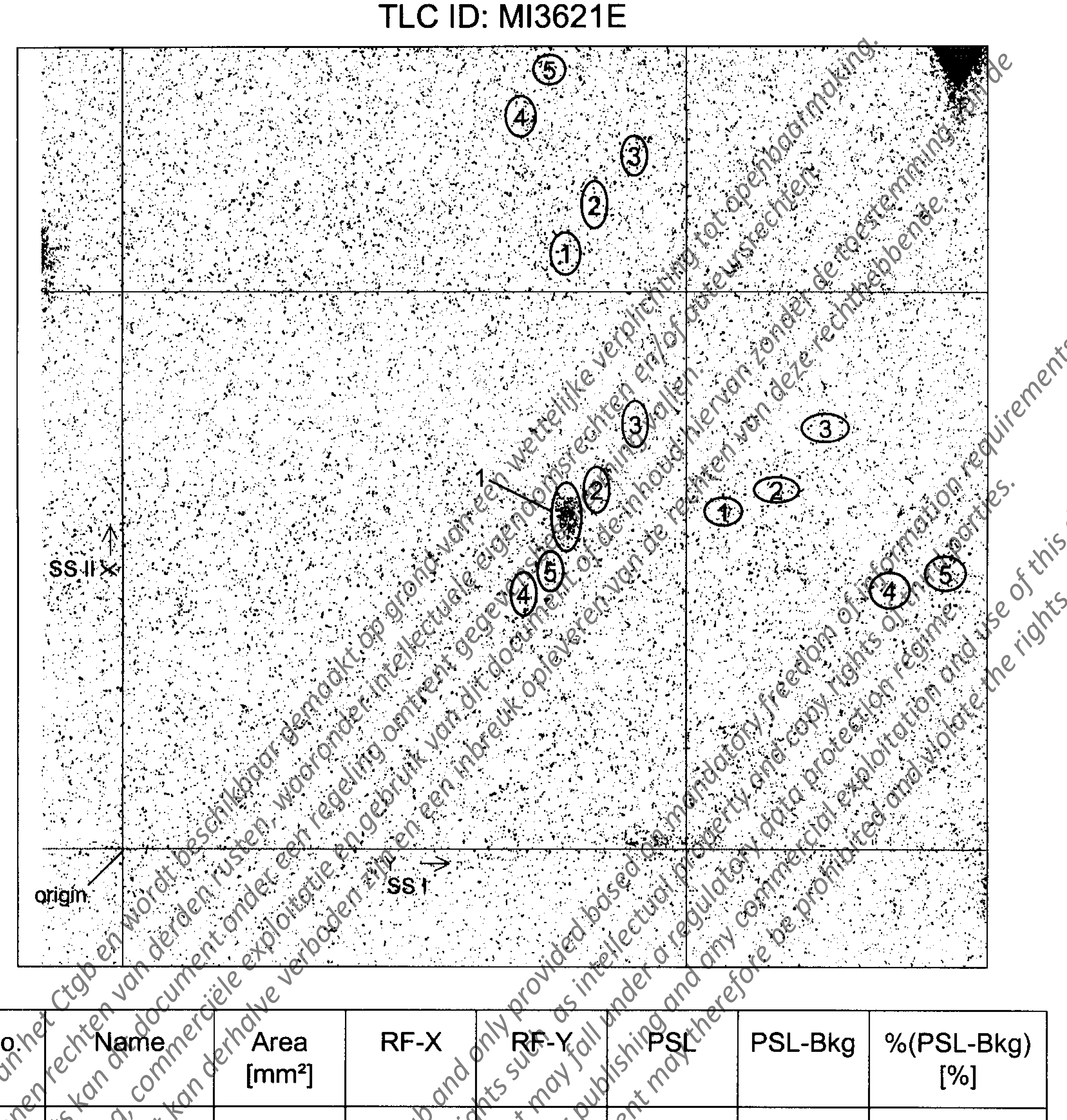
Figure 8: 2-dimensional TLC analysis of the methanol extract of pollen, row B.

TLC ID: MI3621E



No.	Name	Area [mm²]	RF-X	RF-Y	PSL	PSL-Bkg	%(PSL-Bkg) [%]
1	Imidacloprid	79.72	0.60	0.79	256.96	71.36	100.00

Sample: MI3621D plus all reference compounds listed below
1: reference compound NTN 33893 (imidacloprid)
2: reference compound FHW 0105D
3: reference compound WAK 3772
4: reference compound DIJ 9817
5: reference compound WAK 3745

The non-labelled reference compounds were visualized under UV light. They were labelled with circles on the TLC plate and in this figure.

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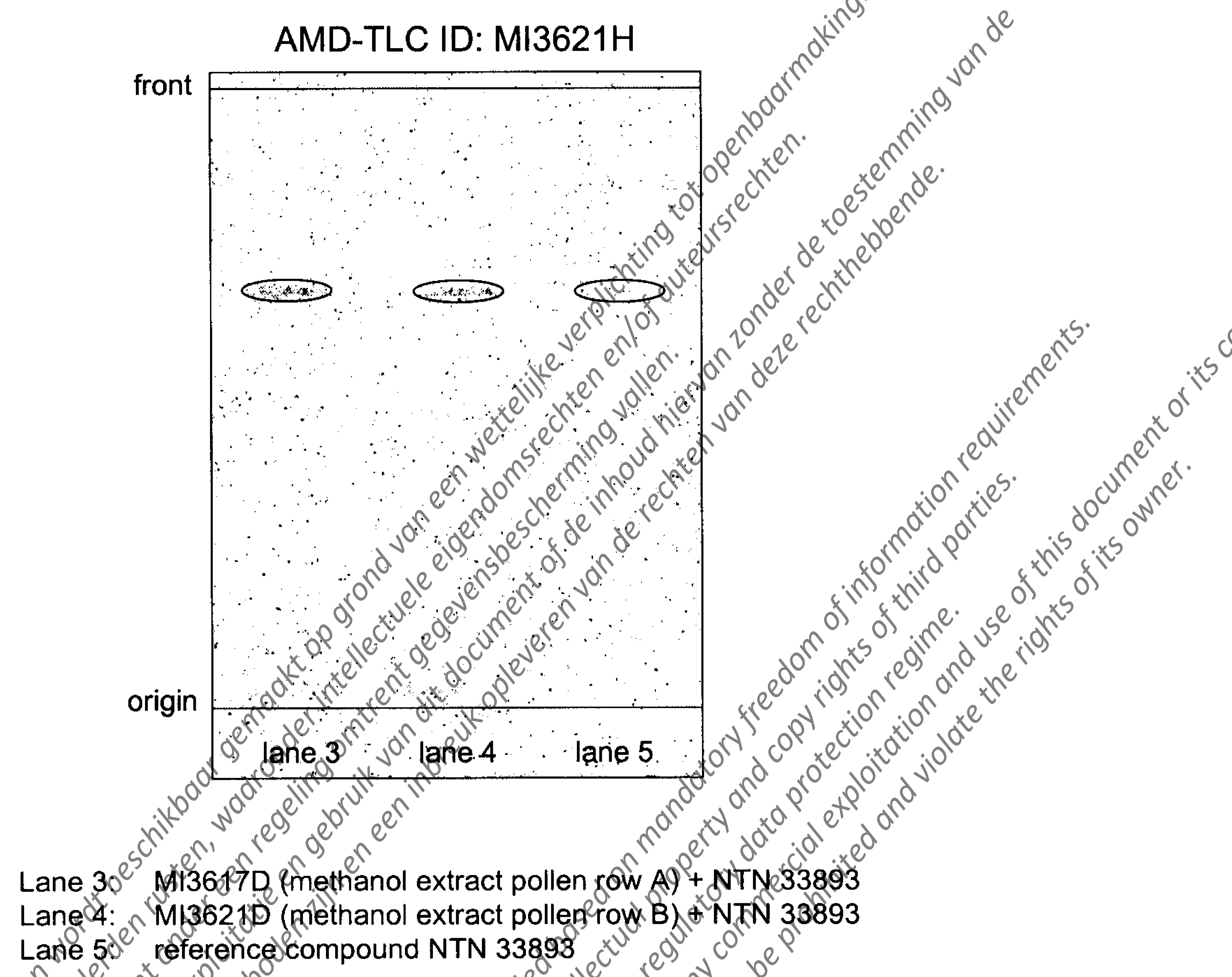
Figure 9: AMD analysis of the methanol extracts of pollen.



Lane 3: MI3617D (methanol extract pollen row A) + NTN 33893
Lane 4: MI3621D (methanol extract pollen row B) + NTN 33893
Lane 5: reference compound NTN 33893

The non-labelled reference compounds were visualized under UV light. They were labelled with circles on the TLC plate and in this figure.

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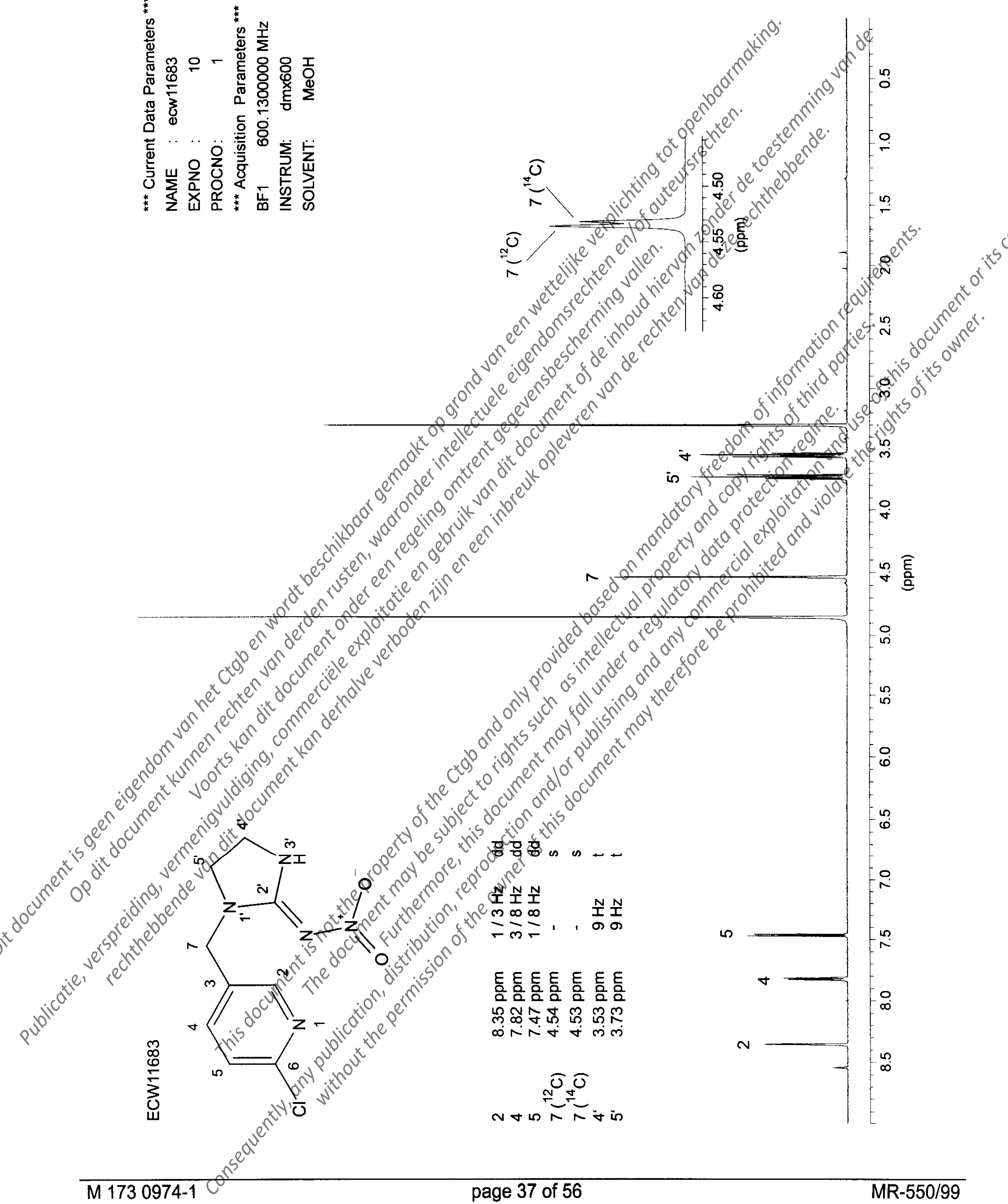
Appendix I: ^{1}H-NMR spectrum of [methylene-^{14}C]imidacloprid.



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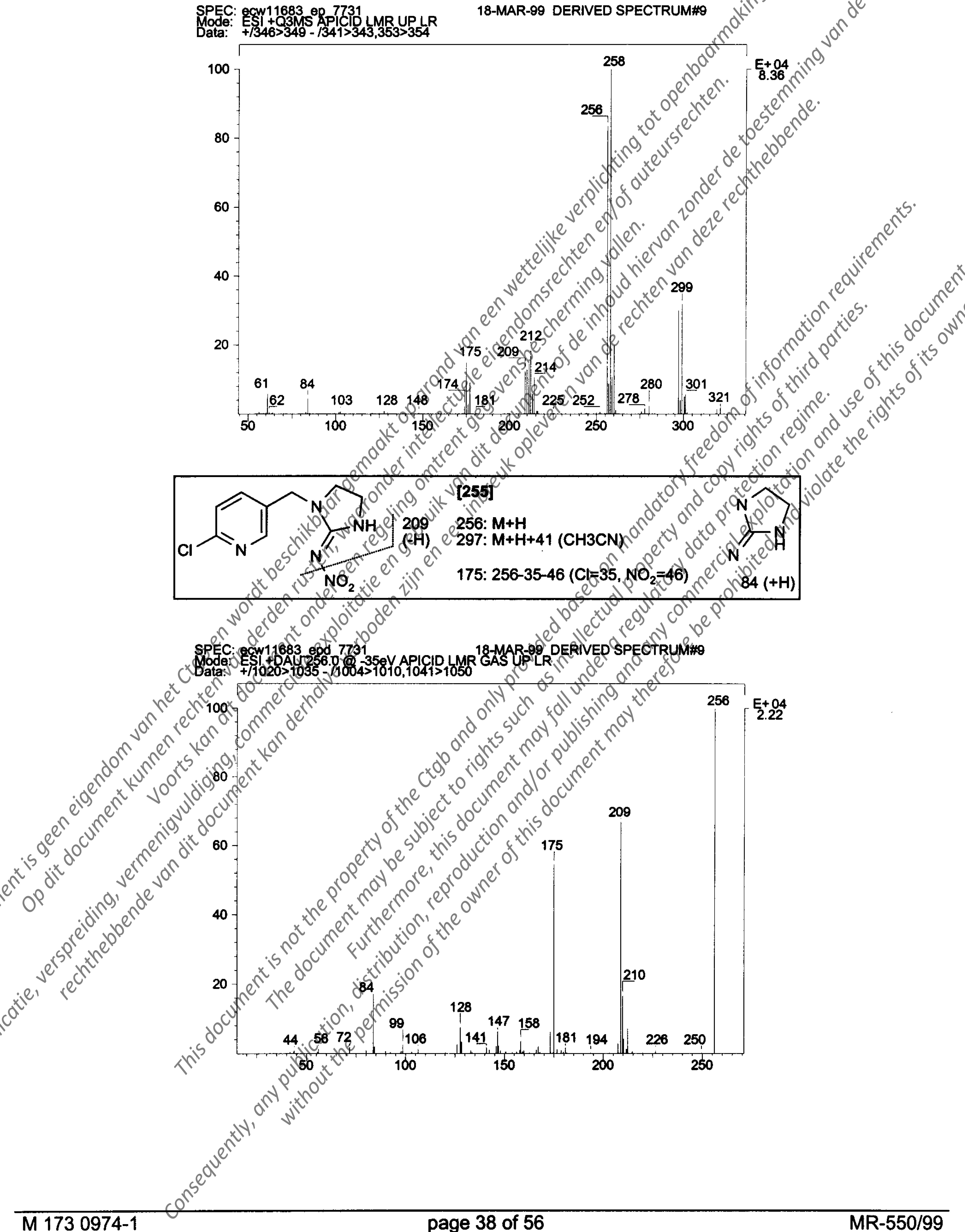
Appendix II: LC/MS-ESI spectrum and daughter ion spectrum of [methylene-^{14}C]imidacloprid (positive ionisation).



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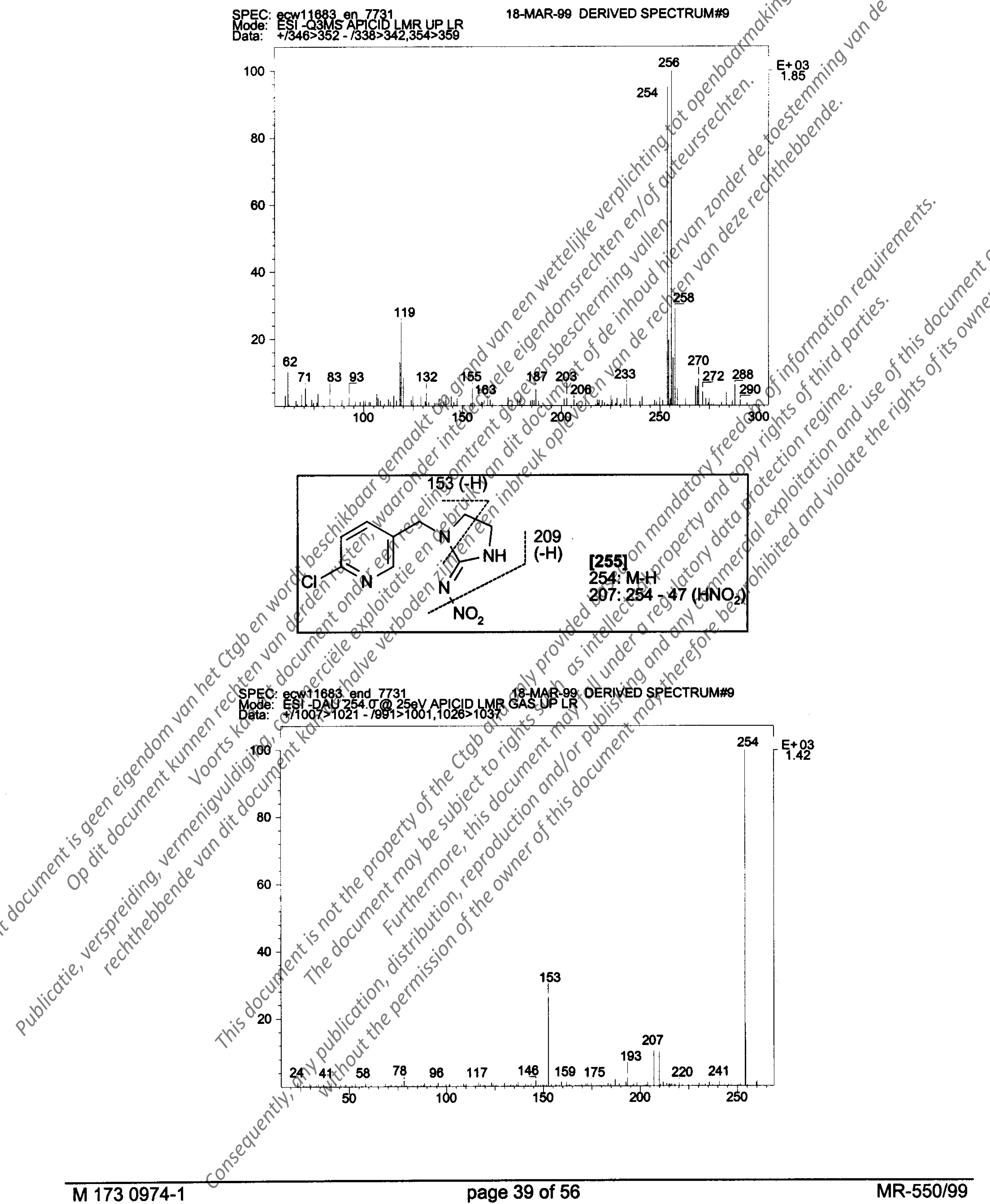
Appendix III: LC/MS-ESI spectrum and daughter ion spectrum of [methylene-^{14}C]imidacloprid (negative ionisation).



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Appendix IV: Radiochemical details of the formulation.

Sample identification	ECW 11691
Type of formulation:	WS 70
Amount of formulation delivered:	40.02 mg
Concentration of a.i. in the formulation:	70.0 %
Amount of active ingredient delivered:	28.01 mg
Specific radioactivity:	3.77 MBq/mg
Total radioactivity delivered:	105.61 MBq
Radiochemical purity:	> 99 %
Code for NMR, LC/MS-/MS:	ECW 11683

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Appendix V: Parameters of the soil "Langenfeld".

Soil analysis conducted by LUFA (Agricultural Experimental and Research Station, Speyer, May 1994).

Physico-chemical data:

Organic carbon [%]:	1.38
pH (in 0.01M $CaCl_2$ sol.):	5.9
Cation exchange capacity [meq/100 g]:	5

Texture analysis:

	according to DIN 19682 diagram	according to USDA soil diagram
Sand [%]:	75.8	77.3
Silt [%]:	19.0	17.5
Clay [%]:	5.2	5.2
Soil type	"schwach lehmiger Sand"	loamy sand

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Appendix VI: Environmental and growth conditions of the sunflowers.

Crop: Sunflowers

Variety: Fleury

Soil type: "Langenfeld" (see **Appendix V**)

Fertilization measures:

Date	Fertilizer	Amount
February 04, 1999	Phosphate-potassium-fertilizer ("Thomaskali")	900 kg/ha
February 04, 1999	Calcium ammonium nitrate	300 kg/ha
From March 15, 1999 weekly	Trace element fertilizer ("Wuchsal super")	1 g/pot

Crop Protection Measures:

none

Climatic data in the greenhouse chamber

Parameter	Value	Comments
Light ①	min. 35 kLux	6 a.m. until 8 p.m.
Air temperature (day)	20/19°C	6 a.m. until 8 p.m.
Air temperature (night)	14/13°C	8 p.m. until 6 a.m.
Air humidity	60 % rel.	

① Osram Power Star. HQI-T 400 W/D.

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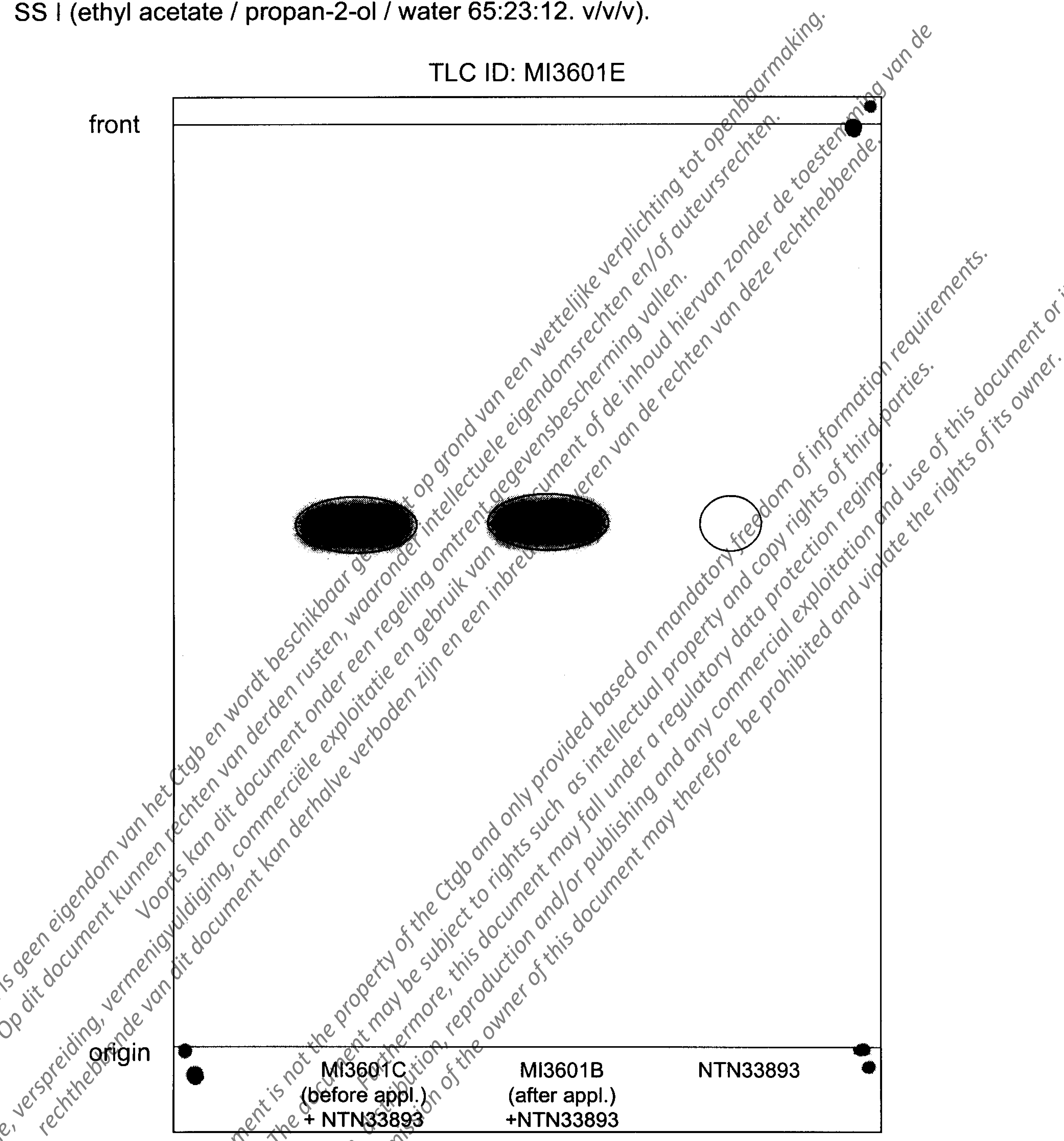
Appendix VII: Radiochemical purity of [methylene-^{14}C]imidacloprid (TLC investigation).

SS I (ethyl acetate / propan-2-ol / water 65:23:12. v/v/v).

TLC ID: MI3601E



The non-labelled reference compounds were visualized under UV light. They were labelled with circles on the TLC plate and in this figure.

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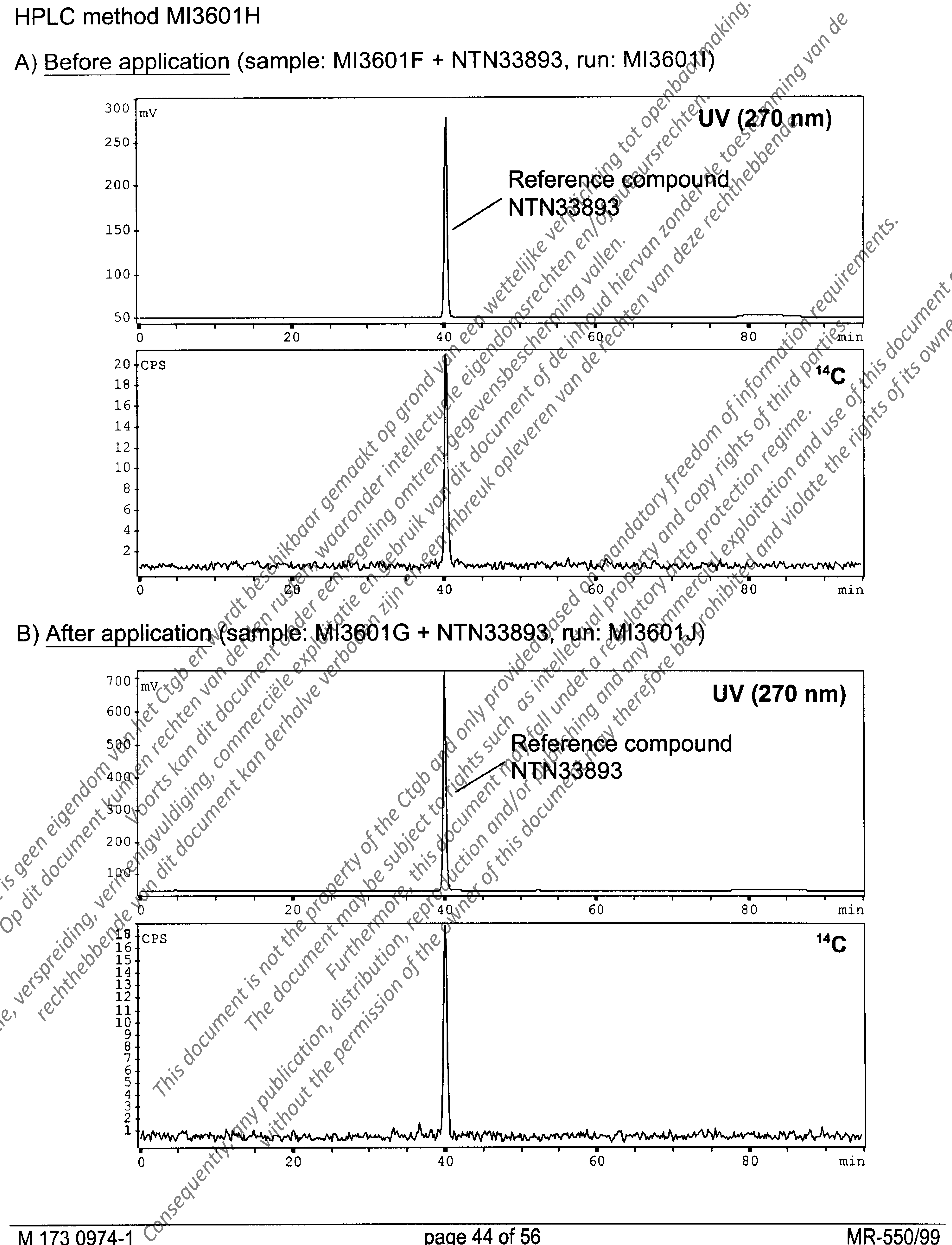
Appendix VIII: Radiochemical purity of [methylene-^{14}C]imidacloprid (HPLC investigation).



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Appendix IX: Time table of the sunflower experiment.

Date		
February 23, 1999	Application (seed dressing) and sowing	
March 01, 1999	Beginning of emergence	
March 04, 1999	Replanting in final pots	
April 26, 1999	Start of flowering row B	
April 30, 1999	Start of flowering row A	**Sampling of nectar and pollen**
May 09, 1999	End of flowering (rows A and B)	
May 10, 1999	Harvest of plants	

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Appendix X: Dilution of nectar.

Sample	Row A		Row B	
	Sample ID	Amount	Sample ID	Amount
Nectar (undiluted) [g]:	MI3605B	1.7150	MI3606B	1.7432
+ Water added [g]:		1.7150		1.7432
= Diluted nectar [g]	MI3608A	3.4300	MI3609A	3.4864
[mL] ①		2.9983		3.0476

① Volume calculated from density of diluted nectar (1.144 g/mL, **Appendix XI**)

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Appendix XI: Determination of the density of the diluted nectar.

Sample	Aliquot No.	Weight of 100 µL [g]	Average [g]	Std. dev. ①	RSD ②
Row A	1	0.1153			
	2	0.1148	0.1144	0.0012	1.00 %
	3	0.1131			
Row B	1	0.1159			
	2	0.1128	0.1143	0.0016	1.36 %
	3	0.1143			
Average		**0.1144**			
Std. dev. ①		0.0012			
RSD ②		1.07 %			

① Standard deviation.

② Relative standard deviation.

The determined density for the diluted nectar is 1.144 g/mL.

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Appendix XII: AMD method for imidacloprid and metabolites.

Device: AMD2 (CAMAG)

General parameters

Volume per development step:	6000 µL
Vacuum test approved with final pressure:	<10.0 mbar
Vacuum test approved with pressure drop:	<10.0 mbar/min
No. of flushings before development:	1
Additional volume in tubes:	0 µL
Chamber saturation:	5 % formic acid in water
HPTLC plate:	Merck no. 15552, cleaned with 2-propanol

Solvents

S1: Methanol (+ 0.25 % HCOOH)
S2: Acetonitrile (+ 0.25% HCOOH)
S3: Dichloromethane
S5: Acetonitrile

Gradient

Run no.	Preconditioning	S1 [$\%_{vol}$]	S2 [$\%_{vol}$]	S3 [$\%_{vol}$]	S5 [$\%_{vol}$]	Distance [mm]	Dry [min]
1	No	10	90	0		10.0	2.0
2	No	10	90	0		10.0	2.0
3	No	10	90	0		10.0	2.0
4	No		77	23		13.0	2.0
5	No		63	37		16.0	2.0
6	No		50	50		19.0	2.0
7	No		46	54		22.0	2.0
8	No		43	57		25.0	2.0
9	No		39	61		28.0	2.0
10	No		35	65		31.0	2.0
11	No			66	34	34.0	2.0
12	No			67	33	37.0	2.0
13	No			67	33	40.0	2.0
14	No			68	32	43.0	2.0
15	No			69	31	46.0	2.0
16	No			70	30	49.0	2.0
17	No			71	29	52.0	2.0
18	No			71	29	55.0	2.0
19	No			72	28	58.0	2.0
20	No			73	27	61.0	2.0
21	No			74	26	64.0	2.0
22	No			75	25	67.0	2.0
23	No			76	24	70.0	2.0
24	No			76	24	73.0	2.0
25	No			77	23	76.0	2.0
26	No			78	22	79.0	2.0
27	No			90	10	82.0	2.0
28	No			100	0	85.0	2.0

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Appendix XIII: Measurement of radioactivity.

Liquid samples:

Number of aliquots:	3
Amount per aliquot:	0.1 - 7.0 mL
Instruments:	1. PW 4700 (Philips/Raytest)
	2. LS 6500 (Beckman Instruments)
Quench correction:	External standard

Solid samples:

Number of aliquots:	3-5 (normally 3)
Amount per aliquot:	50-200 mg
Instruments:	Oxidizer 387 (Canberra Packard Instruments)

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. Carbosorb E (8 mL) + Permafluor E+ (10 mL) (Canberra-Packard):	16-18 cpm
2. Quicksafe A (2 mL, Zinsser Analytic GmbH):	15-20 cpm
3. Quickszint 401 (2 mL, Zinsser Analytic GmbH):	14-18 cpm

Measuring time of samples:

Generally between 10 sec. and 100 min. depending on the amount of radioactivity in the sample. The measurements were stopped after reaching a 2-sigma error of 0.7 %. If this error was not reached within 100 min. the measurement was stopped and the 2-sigma error of the cpm-value reached at that time was printed out. The error of the dpm-value was calculated from the 1-sigma error of the cpm-value and the error of the quench correction curve.

Detection limit:

2 x background

Counting efficiencies:

1. PW 4700 (Philips):	48-82 %
2. LS 6500 (Beckman Instruments):	50-92 %

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Appendix XIV: Raw data values and calculation of the TRR in nectar row A.

Calculation of TRR:

$$TRR = \frac{\text{total radioactivity in nectar [MBq]}}{\text{total weight of nectar [kg]} \cdot \text{specific radioactivity [MBq/mg]}} = \frac{0.00001323\ \text{MBq}}{0.0017150\ \text{kg} \cdot 3.77\ \text{MBq/mg}} = 0.0020\ \text{mg/kg}$$

Sample	Sample volume	Aliquot for LSC	Radioactivity in LSC aliquot		Total radioactivity in sample		
(sample ID)	[mL]	[mL]	[Bq]	average	[Bq]	[%]	[mg/kg] ①
Diluted nectar row A (MI3608A)	2.9983	0.100	0.45 0.43 0.44	0.44	13.23	100.0	0.0020

SPE extraction 1 (processed aliquot: 1.715 mL of 2.9983 mL)

Sample	Sample volume	Aliquot for LSC	Radioactivity in LSC aliquot		Ra. in proc. al.	Total ra. in sample		Total radioactivity in sample ④		
(sample ID)	[mL]	[mL]	[Bq]	average	[Bq] ②	[Bq] ③	[%]	[Bq]	[%]	[mg/kg] ①
Methanol extract (MI3613B)	0.4	0.050	1.03 1.10 1.02	1.05	8.40	14.69	111.1	12.77	96.5	0.0020
Wash sol. (H_2O) (MI3613A)	0.6	0.100	0.06 0.05 0.04	0.05	0.31	0.53	4.0	0.46	3.5	0.0001
Total					**8.71**	**15.23**	**115.1**	**13.23**	**100.0**	**0.0020**

The methanol extract MI3613B was investigated as MI3613C by 2-dimensional TLC (MI3613D, **Figure 4**).

(Appendix continued on next page)

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Appendix XIV (continued).

<u>SPE extraction 2</u> (processed aliquot: 0.600 mL of 2.9983 mL)

Sample	Sample volume	Aliquot for LSC	Radioactivity in LSC aliquot		Ra. in proc. al.	Total ra. in sample		Total radioactivity in sample ④		
(sample ID)	[mL]	[mL]	[Bq]	average	[Bq] ②	[Bq] ③	[%]	[Bq]	[%]	[mg/kg] ①
Methanol extract (MI3613G)	0.5	0.050	0.24 0.21 0.24	0.23	2.31	11.55	87.3	13.16	99.5	0.0020
Wash sol. (H_2O) (MI3613F)	0.5	0.100	<0.01 <0.01 <0.01	<0.01	0.01	0.06	0.4	0.07	0.5	<0.0001
Total					**2.32**	**11.60**	**87.7**	**13.23**	**100.0**	**0.0020**

The methanol extract MI3613G was investigated as MI3613H by AMD (MI3621I, **Figure 6**).

① Calculated for 1.7150 g of undiluted nectar (see **Appendix X**).

② Calculated for processed amount of diluted nectar (SPE extraction 1: 1.715 mL, SPE extraction 2: 0.600 mL).

③ Calculated for total amount (2.9983 mL) of diluted nectar.

④ Values normalised to total radioactivity in diluted nectar:

$$\text{normalised radioactivity [Bq]} = \frac{\text{radioactivity in extract [Bq]} \cdot \text{radioactivity in nectar [Bq]}}{(\text{radioactivity in methanol extract [Bq]} + \text{radioactivity in wash solution [Bq]})}$$

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Appendix XV: Raw data values and calculation of the TRR in nectar row B.

Calculation of TRR:

$$TRR = \frac{\text{total radioactivity in nectar [MBq]}}{\text{total weight of nectar [kg]} \cdot \text{specific radioactivity [MBq/mg]}} = \frac{0.00001195\ \text{MBq}}{0.0017432\ \text{kg} \cdot 3.77\ \text{MBq/mg}} = 0.0018\ \text{mg/kg}$$

Sample	Sample volume	Aliquot for LSC	Radioactivity in LSC aliquot		Total radioactivity in sample		
(sample ID)	[mL]	[mL]	[Bq]	average	[Bq]	[%]	[mg/kg] ①
Diluted nectar row B (MI3609A)	3.0476	0.100	0.37 0.39 0.41	0.39	11.95	100.0	0.0018

SPE extraction (processed aliquot: 1.700 mL of 3.0476 mL)

Sample	Sample volume	Aliquot for LSC	Radioactivity in LSC aliquot		Ra. in proc. al.	Total ra. in sample		Total radioactivity in sample ④		
(sample ID)	[mL]	[mL]	[Bq]	average	[Bq] ②	[Bq] ③	[%]	[Bq]	[%]	[mg/kg] ①
Methanol extract (MI3619B)	0.5	0.050	0.62 0.58 0.61	0.60	6.02	10.80	90.4	11.81	98.8	0.0018
Wash sol. (H_2O) (MI3619A)	0.5	0.100	0.03 0.01 0.01	0.01	0.07	0.13	1.1	0.14	1.2	<0.0001
Total					**6.10**	**10.93**	**91.5**	**11.95**	**100.0**	**0.0018**

The methanol extract MI3619B was investigated as MI3619C by 2-dimensional TLC (MI3619D, **Figure 5**) and AMD (MI3619E, **Figure 6**).

① Calculated for 1.7432 g of undiluted nectar (see **Appendix X**)

② Calculated for processed amount (1.700 mL) of diluted nectar.

③ Calculated for total amount (3.0476 mL) of diluted nectar.

④ Values normalised to total radioactivity in diluted nectar:

$$\text{normalised radioactivity [Bq]} = \frac{\text{radioactivity in extract [Bq]} \cdot \text{radioactivity in nectar [Bq]}}{(\text{radioactivity in methanol extract [Bq]} + \text{radioactivity in wash solution [Bq]})}$$

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Appendix XVI: Raw data values for the determination of the total radioactive residue in pollen row A.

Calculation of TRR:

$$TRR = \frac{\text{radioact. in comb. extr. [MBq]} + \text{radioact. in solids [MBq]}}{\text{weight of pollen [kg]} \cdot \text{specific radioactivity [MBq/mg]}} = \frac{0.00002565\ \text{MBq} + 0.00000455\ \text{MBq}}{0.0020\ \text{kg} \cdot 3.77\ \text{MBq/mg}} = 0.0040\ \text{mg/kg}$$

Extracted amount: 2.0 g of pollen row A (MI3607D)

Sample (sample ID)	Volume / weight of sample	Aliquot for LSC	Radioactivity in LSC aliquot [Bq]	Radioactivity in LSC aliquot average	Total radioactivity in sample [Bq]	Total radioactivity in sample [%]	Total radioactivity in sample [mg/kg]
Combined extracts (MI3617A)	5.2 mL	0.200 mL	0.99 1.00 0.97	0.99	25.65	84.9	0.0034
Solids (MI3617B)	0.44 g	1.00 g ①	12.21 8.98 10.24 9.96 10.35	10.35	4.55	15.1	0.0006
Sum (TRR)					30.20	100.0	**0.0040**

① Aliquots of ca. 50 mg were measured, but raw data are given in Bq/g.

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Appendix XVI (continued).

SPE extraction of combined extracts (5.2 mL of 5.2 mL used)

Sample	Sample volume	Aliquot for LSC	Radioactivity in LSC aliquot		Total radioact. in sample		Total radioactivity in sample ③		
(sample ID)	[mL]	[mL]	[Bq]	average	[Bq]	[%] ①	[Bq]	[%] ②	[mg/kg]
Methanol extract (MI3617D)	2.3	0.050	0.57 0.51 0.52	0.53	24.49	95.5	25.64	84.9	0.0034
Wash sol. (H_2O) (MI3617C)	0.5	0.100	<0.01 <0.01 <0.01	<0.01	0.01	<0.1	0.01	<0.1	<0.0001
Total					**24.49**	**95.5**	**25.65**	**84.9**	**0.0034**

The methanol extract MI3617D was investigated by 2-dimensional TLC (MI3617E, **Figure 7**) and AMD (MI3621H, **Figure 9**).

① Radioactivity in combined extracts (25.65 Bq) = 100 %.

② Radioactivity in extracted amount of pollen (2 g, 30.20 Bq) = 100 %.

③ Values normalised to total radioactivity in combined extracts:

$$\text{normalised radioactivity [Bq]} = \frac{\text{radioactivity in extract [Bq]} \cdot \text{radioactivity in combined extracts [Bq]}}{(\text{radioactivity in methanol extract [Bq]} + \text{radioactivity in wash solution [Bq]})}$$

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Appendix XVII: Raw data values for the determination of the total radioactive residue in pollen row B.

Calculation of TRR:

$$TRR = \frac{\text{radioact. in comb. extr. [MBq]} + \text{radioact. in solids [MBq]}}{\text{weight of pollen [kg]} \cdot \text{specific radioactivity [MBq/mg]}} = \frac{0.00002466\ \text{MBq} + 0.00000380\ \text{MBq}}{0.0020\ \text{kg} \cdot 3.77\ \text{MBq/mg}} = 0.0038\ \text{mg/kg}$$

Extracted amount: 2.0 g of pollen row B (MI3607I)

Sample (sample ID)	Volume / weight of sample	Aliquot for LSC	Radioactivity in LSC aliquot [Bq]	Radioactivity in LSC aliquot average	Total radioactivity in sample [Bq]	Total radioactivity in sample [%]	Total radioactivity in sample [mg/kg]
Combined extracts (MI3621A)	8.0 mL	0.200 mL	0.59 0.62 0.64	0.62	24.66	86.7	0.0033
Solids (MI3621B)	0.42 g	1.00 g ①	8.14 7.80 8.11 9.83 11.32	9.04	3.80	13.3	0.0005
Sum (TRR)					28.46	100.0	**0.0038**

① Aliquots of ca. 50 mg were measured, but raw data are given in Bq/g.

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Appendix XVII (continued).

SPE extraction of combined extracts (8.0 mL of 8.0 mL used)

Sample	Sample volume	Aliquot for LSC	Radioactivity in LSC aliquot		Total radioact. in sample		Total radioactivity in sample ③		
(sample ID)	[mL]	[mL]	[Bq]	average	[Bq]	[%] ①	[Bq]	[%] ②	[mg/kg]
Methanol extract (MI3621D)	3.0	0.050	0.40 0.42 0.41	0.41	24.35	98.7	24.59	86.4	0.0033
Wash sol. (H_2O) (MI3621C)	0.5	0.100	0.01 0.02 0.01	0.01	0.07	0.3	0.07	0.3	<0.0001
Total					**24.42**	**99.0**	**24.66**	**86.7**	**0.0033**

The methanol extract MI3621D was investigated by 2-dimensional TLC (MI3621E, **Figure 8**) and AMD (MI3621H, **Figure 9**).

① Radioactivity in combined extracts (24.66 Bq) = 100 %.

② Radioactivity in extracted amount of pollen (2 g, 28.64 Bq) = 100 %.

③ Values normalised to total radioactivity in combined extracts:

$$\text{normalised radioactivity [Bq]} = \frac{\text{radioactivity in extract [Bq]} \cdot \text{radioactivity in combined extracts [Bq]}}{(\text{radioactivity in methanol extract [Bq]} + \text{radioactivity in wash solution [Bq]})}$$

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STOR 2

Study Title

Residues of ^{14}C-NTN 33893 (Imidacloprid) in Blossoms of Sunflower *(Helianthus annuus)* after Seed Dressing

Amendment to Report No. 1

Data Requirement

Supplementary Study

Author

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Laboratory Project ID

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Version 2

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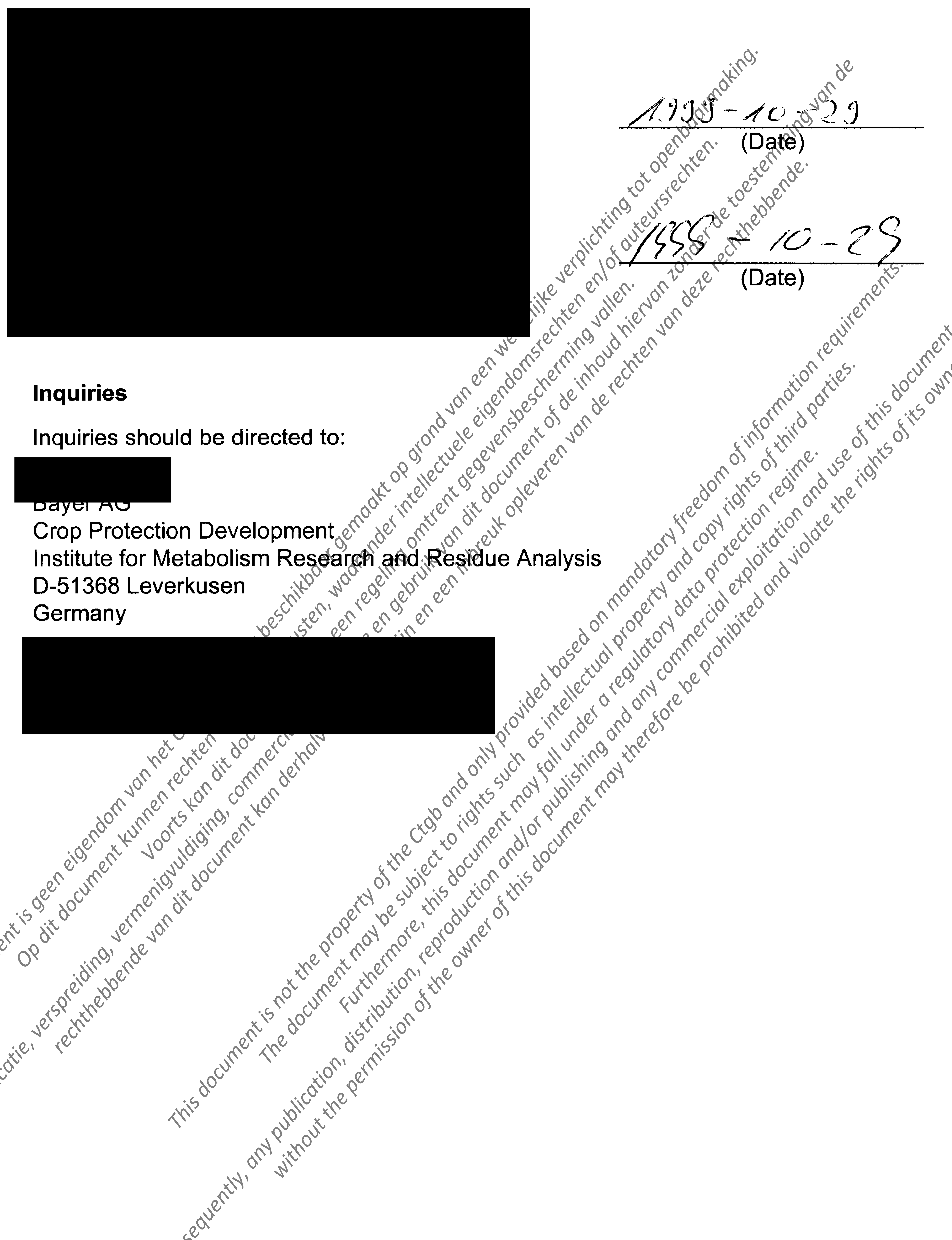

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RATIONALE

The radioactive zones on the TLC plates were detected using a BAS 2000 BioImaging Analyzer (Fuji) (**report p. 16**). This amendment has been prepared in order to calculate the detection limit of compounds with the TLC analysis used.

The chapters of this amendment should be added to the report according to the chapter numeration.

4.4.1 Calculation of the detection limit

The sensitivity of the BAS 2000 BioImaging Analyzer (Fuji) has been tested previously (Klein & Clark, 1993). After 16 h of exposure, zones containing 8 dpm were significantly detected. The authors reported, that even 2 dpm were detected when longer exposure times were used. This is true for this study, since all the TLC plates were exposed 6 days (= 144 h). The detection limit of 2 dpm (0.033 Bq) was confirmed using GLP conform procedures.

Using this assumption, the detection limit expressed as a concentration (e.g. mg/kg) is just dependent on the amount of the sample which has been spotted on the TLC plate.

Sunflower nectar:

The TRR in nectar was 0.0019 mg/kg (average of row A and B) and the total radioactivity in the total nectar samples was 13 Bq (row A) and 12 Bq (row B) (**report Table II**). From each sample, an aliquot of 2.1 Bq was spotted on a TLC plate (as a methanol extract). From the above mentioned assumption it can be stated, that a zone containing an amount of radioactivity equivalent to less than 0.0001 mg/kg was detectable:

$$\frac{0.0019\text{mg/kg} \cdot 0.033\text{Bq}}{2.1\text{Bq}} < 0.0001\text{mg/kg}$$

Sunflower pollen:

The TRR in pollen was 0.0039 mg/kg (average of row A and B) and the total radioactivity in the total nectar samples was 72 Bq (row A) and 69 Bq (row B) (**report Table II**). The smallest aliquot of a methanol extract spotted on a TLC plate was 0.25 Bq (row B). From the above mentioned assumption it can be stated, that a zone containing an amount of radioactivity equivalent to 0.0005 mg/kg was detectable:

$$\frac{0.0039\text{mg/kg} \cdot 0.033\text{Bq}}{0.25\text{Bq}} = 0.0005\text{mg/kg}$$

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10 REFERENCES

Klein, O; Clark, T (1993): "The advantages of a new bio-imaging analyzer for investigation of the metabolism of ^{14}C-radiolabeled pesticides", Journal of Planar Chromatography **6**, 368-371.

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