TITLE PAGE

Residue Levels of Imidacloprid and Imidacloprid Metabolites in Pollen of Maize Plants Cultivated on Soils with Different Imidacloprid Residue Levels

Test Location: Farmland "Höfchen" -1999

AUTHOR

[redacted]

TESTING FACILITY

BAYER AG
Crop Protection-Development
Institute For Environmental Biology
D-51368 Leverkusen-Bayerwerk

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E 370 1551 - 2

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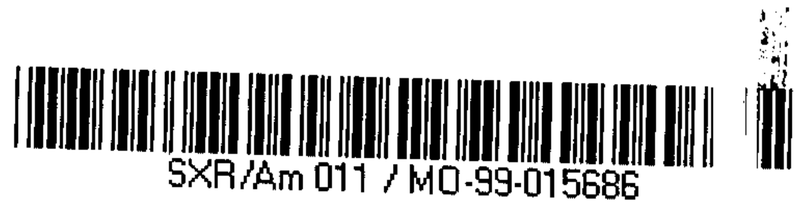

STATEMENT OF COMPLIANCE

This study was conducted in compliance with the Principles of Good Laboratory Practice (Chemicals Law (ChemG) of July 25, 1994, Annex 1 and OECD Principles of Good Laboratory Practice (GLP) of November 26, 1997 [C(97) 186/Final]).

Signature:

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CERTIFICATION OF AUTHENTICITY

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Date

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30.9.99
Date

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Date

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1.0 SUMMARY

Report: [redacted] (1999): Residue Levels of Imidacloprid and Imidacloprid Metabolites in Pollen of Maize Plants Cultivated on Soils with Different Imidacloprid Residue Levels. Test Location: Farmland "Höfchen" - 1999
Bayer AG, unpublished report No: SXR/Am 011; 1999/09/28.
(appendix I and III report data from study MR471-99 and MR-514/99, respectively).

Guidelines: Internal Testing Method
Deviations: not applicable

GLP: yes (certified laboratory)

Material and methods: maize seed (variety "Ilias") either dressed with 70 g/U[i] Gaucho WS 70 (a.i. content: 72.5% imidacloprid; batch no. 233 614 749, developmental no. 04 175 778) or imidacloprid-free were drilled on 10 May 99 in soils with different imidacloprid residue levels. Soil samples for an analytical determination of the imidacloprid residue level were taken immediately before drilling. Drilling rate was 2 U/ha. During peak flowering of the maize plants (end of July) pollen was harvested from the male flowers. These pollen samples were subjected to a residue analysis for imidacloprid and its relevant metabolites.

Dates of biological work: July 22 – 29, 1999.
Dates of soil analysis: August 9 – 11, 1999.
Dates of analysis of biological samples: August 31 - September 22, 1999.

Findings: Residues in soil, and in pollen of maize planted as succeeding crop. (detects above the LOQ are highlighted):

Type of Sample	Residue Level [mg/kg] *		
	Imidacloprid	Olefin-NTN	Hydroxy-NTN
Control Plot (south of field field number 502)			
Soil sample (0-30 cm)	n.d.	--	--
Leaves (produced latest)	n.d.	n.d.	n.d.
Pollen sampled from the plants	n.d.	n.d.	n.d.
Variant „1997" (field number 502)			
Soil sample (0-30 cm)	0.018	--	--
Leaves (produced latest)	n.d.	n.d.	n.d.
Pollen sampled from the plants	n.d.	n.d.	n.d.

* Limit of quantitation for soil samples: 0.006 mg/kg for imidacloprid; n.d. = below limit of detection (0.002 mg/kg)
Limit of quantitation for biological samples: 0.005 mg/kg for imidacloprid and hydroxy-imidacloprid, 0.01 mg/kg for olefin-imidacloprid. n.d. = below limit of detection (0.0015 and 0.003 mg/kg).

[i] 1 U (Unit) = 50,000 seed

Type of Sample	Residue Level [mg/kg] *		
	Imidacloprid	Olefin-NTN	Hydroxy-NTN
Variant „1998“ (field number 507)			
Soil sample (0-30 cm)	< LOQ	--	--
Leaves (produced latest)	n.d.	n.d.	n.d.
Pollen sampled from the plants	n.d.	n.d.	n.d.
Variant „1999“ (south of field number 502)			
Soil sample (0-30 cm)	n.d.	--	--
Leaves (produced latest)	0.011	n.d.	< LOQ
Pollen sampled from the plants	n.d.	n.d.	n.d.

* Limit of quantitation for soil samples: 0.006 mg/kg for imidacloprid; n.d. = below limit of detection (0.002 mg/kg)
Limit of quantitation for biological samples: 0.005 mg/kg for imidacloprid and hydroxy-imidacloprid, 0.01 mg/kg for olefin-imidacloprid. n.d. = below limit of detection (0.0015 and 0.003 mg/kg).

Observations: No residue levels at or above the limit of detection could be detected in pollen of maize planted as succeeding crop in soil previously cropped with Gaucho-dressed plants. Even in pollen of seed-dreesed maize plants, no residues of imidacloprid above the limit of detection were found. In the latest leaf stages, a residue level of 11 µg/kg imidacloprid and traces of the hydroxy-metabolite (< LOQ) were detected.

2.0 INTRODUCTION

According to EU directive 91/414/EEC the impacts of pesticides on honeybees have to be examined. Besides the intrinsic toxicity of a pesticide the concentration to which a honeybee may be exposed under field conditions is an integral component for the hazard assessment. The present study aims to examine the exposure in greater detail for a refined risk assessment.
The maize pollen samples were analysed for residues of imidacloprid and its olefin- and hydroxy-metabolites. These metabolites were considered as relevant, since they have a chemical structure closely related to the parent molecule and were observed in plant metabolism studies in significant proportions (up to approx. 10 %).

3.0 EXPERIMENTAL

3.1 Test Substance Used for Test Variant „1999“

Test substance:	Gaucho WS 70
Active ingredient(s):	Imidacloprid (NTN 33893)
Chemical name(s) of ai(s):	2-Imidazolidinimine, 1-[(6-chloro-3-pyridinyl)methyl]-N-nitro-
CAS number of ai(s):	138 261-41-3
Indikation:	seed dressing
Developmental/article number	04 175 778
Formulation/batch number:	233 614 749
No. of certificate:	FAR-No. 559-01
AI content (acc. to analysis):	72.5%
Analytical method:	HPLC, ext. std.
Date of analysis:	February 1, 1999
Expiry date:	August 1, 1999
Physical appearance:	white powder
Specific density:	not applicable
Storage conditions:	room temperature
Seed dressing rate(s) tested in the study:	70 g/U (1 U = 50,000 seed) (= nominal content: 49 g/U imidacloprid; analytical findings, FAR 668-00: 44.6 g/U imidacloprid).
Seed drilling rate tested in the study:	2 U/ha (= 2,400 seed per four 240 m² study plots) (maize variety: „Ilias“; standard fungicidal treatment: TMTD)
Safety precaution:	Routine hygienic precautions

3.2 Reference Substance

For this type of material and use pattern, a reference compound is not specified.

3.3 Execution of the Test

The sampled study plots were drilled on 10 May 1999. Pollen was sampled between 26 and 29 July 1999.

Sponsor: BAYER AG
GB Plant Protection
Marketing - Seed Treatment
D-40789 Monheim

Study Director: [redacted]

Cultivar Manager:

Trials Officer:

Responsible Analyst (soil)

Responsible Analyst (biological samples):

Study Technicians:

Quality Assurance:

Laboratory Study Number: SXR/Am 011

3.4 *Procedure of Seed Dressing*

The maize seeds (variety: „Ilias“) used for test "variant 99" were dressed by a commercial seed dressing company (SUET Saat- und Erntetechnik GmbH, D-37257 Eschwege) and delivered to Bayer on 1 April 1999. Besides the insecticidal treatment, the seed were treated with a standard fungicide (TMTD). This fungicidal treatment was also applied to all imidacloprid-free seeds which were drilled on study plots of test "variants "1997", "1998" and the control.

3.5 *Location of the Trial Site and Description of the Study Plots*

The trial site was located within the Bayer AG´s experimental farmland "Höfchen", approximately 1 km from Burscheid (Germany, 205 m above sea level). The precise field location was as follows:

- Control plot: field area „Auf dem Brachfeld“, south of field number 502
- Variant „1997“: field area „Auf dem Brachfeld“, field number 502
- Variant „1998“: field area „Auf dem Brachfeld“, field number 507
- Variant „1999“: field area „Auf dem Brachfeld“, south of field number 502

The soil characteristics of the study plots were determined for another study at a site close to the study fields (OE No. 2566, sampling date: 8 December 1998). The soil at this site was classified as a "loamy silt" with particle size fractions of 7.1 % sand, 83.9 % silt and 9.1 % clay. The pH value (KCL) at the study site was determined to be 6.72. Soil organic carbon was 1.95% by weight. The water holding capacity was 64.47 g water per 100 g dry soil.

3.6 *Treatment Design*

After the previous crop had been destroyed (4 l/ha Glyfos and subsequent ploughing), all study plots were drilled with 2 U/ha maize seed (1 U = 75,000 seed) on 10 May 1999. For each test variant and for the control, plots of 8 x 30 m were drilled with either imidacloprid-free or Gaucho WS 70 dressed maize seed (variety: Ilias). Drilling distance was 80 cm between rows and 12.5 cm in-row. Prior to sowing the proper functioning of the equipment was tested. The equipment was adjusted according to the preconditions (e.g. seed density). The test plots were adjacent to similar test plots which were cultivated with either sunflower or rape plants.

With regard to imidacloprid, study plots received the following treatments:

- Control plot: untreated grass area since 1996. Drilled with imidacloprid-free maize seed on 10 May1999
- Variant „1997“: cropped in fall 1997 with Gaucho treated winter wheat (59 g ai/ha), sprayed on 30 April 1999 with 71.5 g/ha Gaucho WS 70 (= 50 g ai/ha imidacloprid; batch no. 233 614 749, 72.5% imidacloprid according to FAR no. 559-01). Drilled with imidacloprid-free maize seed on 10 May 1999.
- Variant „1998“: cropped in fall 1998 by Gaucho treated winter barley (52 g ai/ha). Drilled with imidacloprid -free maize seed on 10 May 1999.
- Variant „1999“: untreated grass area since 1996. Drilled with Gaucho WS 70 treated maize seed on 10 May 1999 (89 g ai/ha).

On the day of drilling, soil samples were taken to analytically verify the residue level of the study plots. From each study field 20 soil cores of 5 cm diameter and a depth of 30 cm were sampled. Sampling points were distributed along the two diagonals of each study field with equal distances between the points, i.e. 10 samples per diagonal. Depending on the plot arrangement, the total size of the sampled area was:

- Control plot/Variant „1999“: 30 x 50 m.
- Variant „1997“: 24 x 30 m.
- Variant „1998“: 24 x 30 m.

Immediately after sampling, soil samples were divided into two subsamples, one subsample contained the 0-20 cm top soil layer and the other subsample the 20-30 cm soil fraction. After dividing, all subsamples were stored at –20°C until residue analysis. Residue levels of the different subsamples are reported in the pertinent analytical report (appendix I).

3.7 Plot History and Cultivation of the Plots during the Study

Plot history and 1999 treatments of the study plots are reported in detail in appendix II.

3.8 Sampling Procedure

Sampling of Pollen from Maize Plants

Pollen was sampled between 26 and 29 July 1999 by shaking pollen out of the maize flowers directly. After sampling, the pollen samples were stored on dry ice in the field. At the end of each sampling day at the latest, the pollen samples were transferred into a refrigerator (-20°C) where they were retained until residue analysis (see 3.10).

3.9 Sample Processing and Residue Analysis

Sample processing and analytical methods are described in detail in appendix I (soil samples) and appendix III (biological samples).

3.10 Climatic Conditions During the Study

During cultivation of the study plots, temperature and precipitation events were continously recorded by weather stations located adjacent to the study sites (within a 3 km distance). The following records were made during this time period:

Month	Precipitation [mm]	Min. air temperature 2m [°C]	Max. air temperature 2m [°C]	Soil temperature 0 cm [°C]	Energy input [kJ/cm²]
April	70.6	0.1 – 10.9	4.9 – 20.9	0.1 – 12.7	38.7
May	39.5	3.7 – 15.3	12.5 – 27.6	9.5 – 21.6	56.7
June	80.3	6.8 – 15.0	13.3 – 28.1	11.8 – 19.3	54.5
July	29.7	11.0 – 18.4	17.0 – 30.4	13.8 – 28.7	60.7
August	86.6	7.8 – 18.6	15.9 – 30.1	12.2 – 29.9	46.4

4.0 FILING

All raw data, the study protocol and the original of the report are filed in the Central GLP archive of PF/E, Crop Protection Center 40789 Monheim, FRG. Reserve samples of the test substance are stored in the pertinent archive of that test facility which provided or certified the test substance.

5.0 RESULTS AND DISCUSSION

5.1 Analytical Findings on Soil Samples

Analytical findings on soil samples are summarized in table 1 and given in detail in the analytical report (appendix I). In the control plots, no residues at or above the limit of quantitation was detected. In the treated plots, residue levels were well in the range as expected from plot and/or treatment history. Within the 0-30 cm soil layer, imidacloprid concentrations of 17.8 µg/kg and < LOQ were detected for the test variants „1997“ and „1998“, respectively (Tab. 1). No residues were detected in the 0-30 cm soil samples of the „1999/control“ field.

5.2 Analytical Findings on Maize Pollen Samples

No residue levels at or above the limit of detection could be detected in pollen of maize planted as succeeding crop in soil previously cropped with Gaucho-dressed plants. Even in pollen of seed-dreesed maize plants, no residues of imidacloprid above the limit of detection were found. In the latest leaf stages, a residue level of 11 µg/kg imidacloprid and traces of the hydroxy-metabolite (< LOQ) were detected.

FIGURES

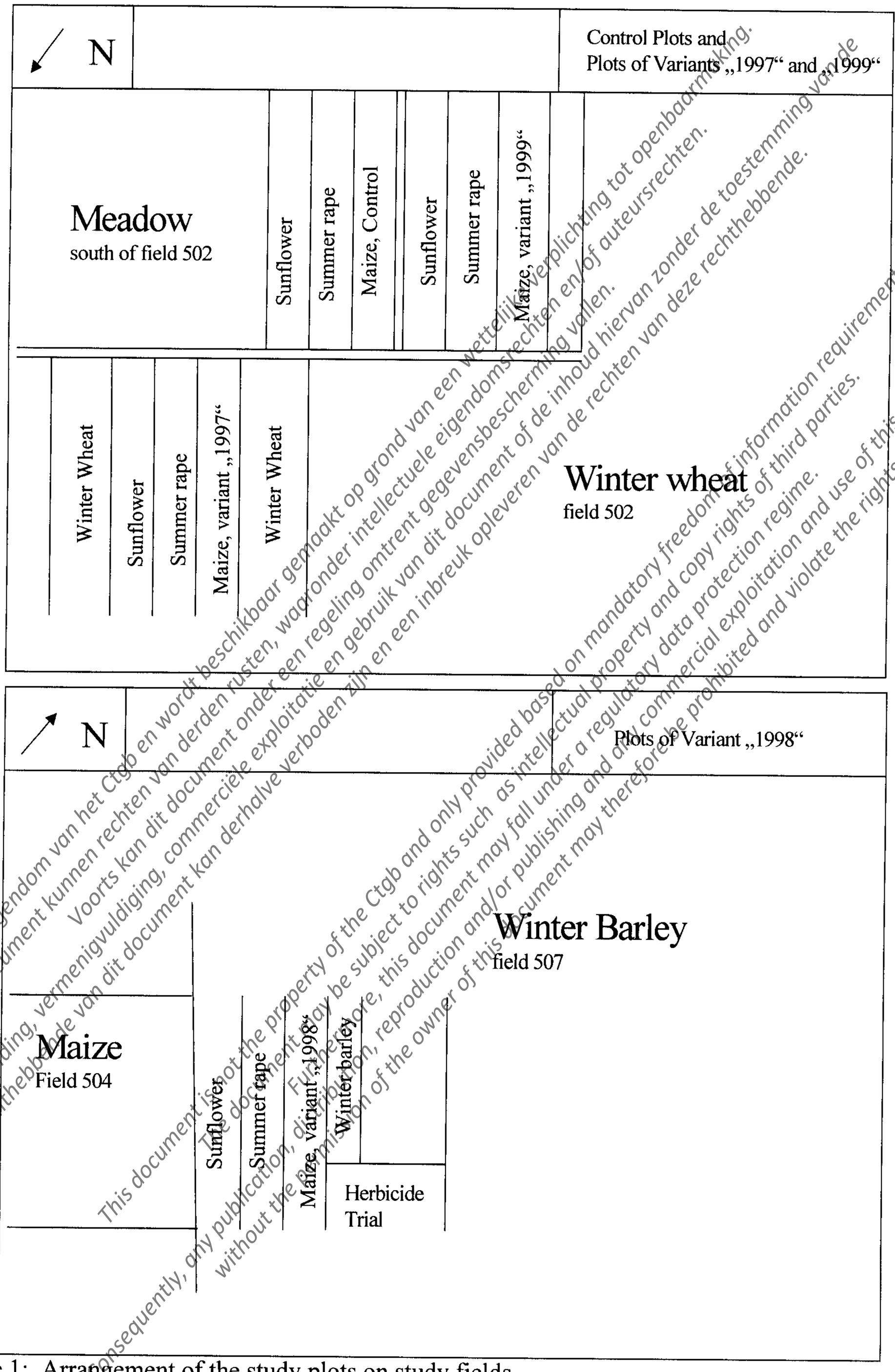


Figure 1: Arrangement of the study plots on study fields.
Each study plot had a size of 8 x 30 m with distances of 80 cm between rows and 12.5 cm in-row.

TABLES

Table 1: Soil Residue Level of Imidacloprid at the Different Study Sites.

The details of the analytical work are given in appendix I. Residue data refer to the level immediately before seed drilling on 10 May 1999. Plot history was as follows:

- Control plot: last imidacloprid treatment: before 1996
- Variant „1997“: last imidacloprid treatment: 30 April 1999 (50 g ai/ha)
- Variant „1998“: last imidacloprid treatment: 26 Sept. 1998 (52 g ai/ha)
- Variant „1999“: last imidacloprid treatment: before 1996; drilled on 10 May 1999 with Gaucho-treated maize seed (89 g ai/ha).

Sample No.	Sample description	Soil Layer	Imidacloprid Residue Level [µg/kg]
1	Control Plot (south of field number 502)	0-20 cm	< LOQ
		0-30 cm	n.d.
2	Variant „1997“ (field number 502)	0-20 cm	24.5
		0-30 cm	17.8
3	Variant „1998“ (field number 507)	0-20 cm	8.7
		0-30 cm	< LOQ
4	Variant „1999“ (south of field number 502)		< LOQ
			n.d.

LOQ (Limit of quantification): 0.006 mg/kg.

n.d.: Residue levels below the limit of detection: 0.002 mg/kg.

Table 2: Plant Residue Level of Imidacloprid and Toxicologically Relevant Metabolites at the Different Study Sites.

The details of the analytical work are given in appendix I. Residue data refer to the level immediately before seed drilling on 10 May 1999. Plot history was as follows:

- Control plot: last imidacloprid treatment: before 1996
- Variant „1997“: last imidacloprid treatment: 30 April 1999 (50 g ai/ha)
- Variant „1998“: last imidacloprid treatment: 26 Sept. 1998 (52 g ai/ha)
- Variant „1999“: last imidacloprid treatment: before 1996; drilled on 10 May 1999 with Gaucho-treated maize seed (89 g ai/ha).

Type of Sample	Residue Level [mg/kg] *		
	Imidacloprid	Olefin-NTN	Hydroxy-NTN
Control Plot (south field number 502)			
Leaves (produced latest)	n.d.	n.d.	n.d.
Pollen sampled from the plants	n.d.	n.d.	n.d.
Variant „1997“ (field number 502)			
Leaves (produced latest)	n.d.	n.d.	n.d.
Pollen sampled from the plants	n.d.	n.d.	n.d.
Variant „1998“ (field number 507)			
Leaves (produced latest)	n.d.	n.d.	n.d.
Pollen sampled from the plants	n.d.	n.d.	n.d.
Variant „1999“ (south of field number 502)			
Leaves (produced latest)	0.011	n.d.	< LOQ
Pollen sampled from the plants	< LOQ	n.d.	n.d.

* LOQ (Limit of quantitation): 5 µg/kg for imidacloprid & hydroxy-imidacloprid; 10 µg/kg for olefine-imidacloprid

n.d. (below limit of detection): 1.5 µg/kg for imidacloprid & hydroxy-imidacloprid; 3.0 µg/kg for olefine-imidacloprid

APPENDICES

APPENDIX I: Analytical Report for Soil Samples.

Bayer AG, Crop Protection Business Group
Crop Protection - Development
Institute for Metabolism Research and Residue Analysis
51368 Leverkusen, Germany

August 31, 1999

Title

Analysis of Soil Samples from

E 370 1548 – 8
E 370 1549 – 9
E 370 1550 – 0
E 370 1551 – 2
E 370 1552 – 3
E 370 1553 – 4

for Residues of Imidacloprid

Responsible Scientist

[redacted]

Bayer AG, Crop Protection Business Group
Crop Protection - Development
Institute for Metabolism Research and Residue Analysis (PF-E/MR)
51368 Leverkusen, Germany

Experimental Starting Date

August 09, 1999

Experimental Completion Date

August 11, 1999

Study Numbers

E 370 1548 – 8
E 370 1549 – 9
E 370 1550 – 0
E 370 1551 – 2
E 370 1552 – 3
E 370 1553 – 4

TABLE OF CONTENTS

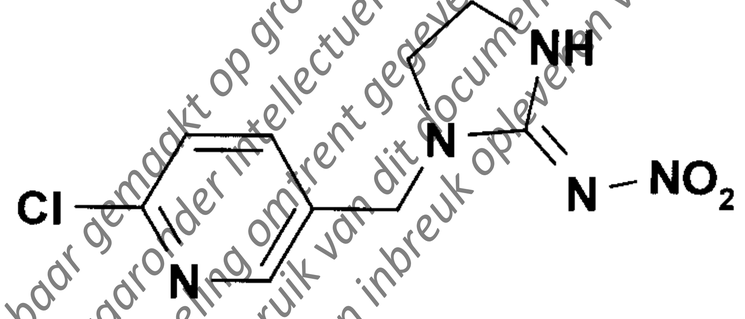
1 INTRODUCTION

Soil samples of the German trial stations "Höfchen" and "Laacher Hof" were analyzed for residues of Imidacloprid. The results are tabulated in Table 2 and 3. Extraction of soil samples and determination of Imidacloprid by HPLC-UV were performed according to method 00267 (MR-53/92) [3]. The limit of quantification (LOQ) was 6 µg/kg. The limit of detection (LOD) was 2 µg/kg.

2 REFERENCE SUBSTANCE

The following substance will be used as reference substance in recovery experiments and for preparation of external standard solutions.

Imidacloprid



Empirical formula:	$C_9H_{10}ClN_5O_2$
Molecular weight:	255.7 g/mol
Reference Substance No:	M00680
Purity:	99.4 % (HPLC), identity ensured by MS
Expiry date:	March 2000

3 PERFORMANCE

3.1 Extraction

Soil samples are extracted in a Soxtec extraction device with boiling methanol. The oil-bath temperature is set at 200 °C.

Soil samples of 25 g are weighed into an extraction thimble and covered with a defatted cotton wool plug. 40 mL of methanol and some boiling chips are placed into aluminum cups. Thimbles and cups are inserted in the Soxtec extraction device.

The extraction time takes one hour. Afterwards the thimbles are placed in rinse position for 30 minutes until the extraction is terminated.

The residue is flushed quantitatively into a 50 mL centrifuge tube by two times rinsing the aluminium cups with about 5 mL of ethanol. The extract is evaporated to dryness in a Turbo-Vap evaporator at 50 °C and reconstituted in 2 mL of acetonitrile/water 50/50 (v/v).

3.2 High Performance Liquid Chromatographic Measurement

Liquid chromatograph: Hewlett Packard 1090

Column:	LiChrospher 60 RP-Select B (5 µm) 125 x 4 mm
Solvent A:	Water + 1g Sodium-dihydrogenphosphate-2-hydrate per L
Solvent B:	Acetonitrile
Oven temperature:	40 °C
Inject. volume:	25 µL
Flow rate:	1.5 mL/min
Detector wavelength:	270 nm

Table 1: Gradient for the HPLC-UV measurement

Time Table	
0 min	10 % B
10 min.	25 % B
13 min	90 % B
18 min	90 % B
20 min	10 % B
30 min	10 % B

Retention time of Imidacloprid: approx. 6.4 min

3.3 Method of Confirmation

Within each series of analyses the identity of Imidacloprid was determined by LC/MS/MS according to method 00537 (MR-551/98) [4]. Therefore, one standard sample (recovery experiment), one control sample and one sample from the trials were analysed for the characteristic mass-to-charge ratio of Imidacloprid.

4 Results

Table 2: Concentrations of Imidacloprid for trial station "Höfchen" (E3701551-2, E3701552-3 and E3701553-4)

Sample No.	Sample description	Soil layer	Imidacloprid [µg/kg]
No.1	Control sample (identical with test sample 1999)	0-20 cm	< LOQ
No.2	Control sample (identical with test sample 1999)	0-30 cm	n.d.
No.3	Test sample 1998	0-20 cm	8.7
No.4	Test sample 1998	0-30 cm	< LOQ
No.5	Test sample 1997	0-20 cm	24.5
No.6	Test sample 1997	0-30 cm	17.8

< LOQ: Concentrations of Imidacloprid below the limit of quantification of the analytical method of 6 µg/kg.

n.d.: Concentrations of Imidacloprid below the limit of detection of the analytical method of 2 µg/kg.

Table 3: Concentrations of Imidacloprid for trial station "Laacher Hof" (E3701548-8, E3701549-9 and E3701550-1)

Sample No.	Sample description	Soil layer	Imidacloprid [µg/kg]
No.1	Control sample (identical with test sample 1999)	0-20 cm	< LOQ
No.2	Control sample (identical with test sample 1999)	0-30 cm	n.d.
No.3	Test sample 1998	0-20 cm	15.3
No.4	Test sample 1998	0-30 cm	12.7
No.5	Test sample 1998 (replicate)	0-20 cm	16.1
No.6	Test sample 1998 (replicate)	0-30 cm	14.3
No.7	Test sample 1997	0-20 cm	17.3
No.8	Test sample 1997	0-30 cm	15.7

< LOQ: Concentrations of Imidacloprid below the limit of quantification of the analytical method of 6 µg/kg.

n.d.: Concentrations of Imidacloprid below the limit of detection of the analytical method of 2 µg/kg.

Table 4: Recovery Rates of Imidacloprid

Fortification [µg/kg]	Soil	Soil layer	Imidacloprid [%]
6.02	Höfchen	0-20 cm	94.0
6.02	Laacher Hof	0-20 cm	95.7
60.2	Höfchen	0-20cm	92.2
60.2	Laacher Hof	0-20 cm	92.3

5 References

1. Chemikaliengesetz, attachment 1, dated July 25, 1994

2. OECD Principles of Good Laboratory Practice (GLP), dated November 26, 1997 [C(97) 186/Final]

3. [redacted] Method for high-performance liquid chromatographic determi-nation of residues of the insecticide Imidacloprid in soil. Reference: MR-53/92, Method 00267 dated January 23, 1992

4. [redacted] Residue Analytical Method for the Determination of Residues of Imidacloprid, Hydroxy-Metabolite and Olefin-Metabolite in Nectar, Honey, Rape Flower, Rape Pollen and Bee Samples by HPLC with Electrospray MS/MS detection. Reference: MR-551/98, Method 00537 dated January 15, 1999

APPENDIX II: Plot History and Cultivation of the Plots during the Study.

- Control plot: field area „Auf dem Brachfeld", south of field number 502
- Variant „1997": field area „Auf dem Brachfeld", field number 502
- Variant „1998": field area „Auf dem Brachfeld", field number 507
- Variant „1999": field area „Auf dem Brachfeld", south of field number 502

Plot History

Study Plot / Year	Cropping	Pesticidal Treatments	
Control			
1996 - 1998	grassland	none	
Variant 1999			
1996 - 1998	grassland	none	
Variant 1997			
1996	winter barley	3.0	L/ha Econal [H]
		0.3	L/ha Bulldock [I]
		0.75	L/ha Starane [H]
		0.8	L/ha Camposan [H]
	winter rape		
1997	winter rape	1.5	L/ha Folicur [F]
		1.0	L/ha CCC 720 [H]
		2.0	L/ha Butisan Star [H]
		12	kg/ha Mesurol slug pellet 2% [I]
	winter wheat	0.3	L/dt Arena/Gaucho 350 FS [F/I]
	(= 58.5 g imidacl./ha)	0.5	kg/ha Herold [H]
		2.0	L/ha Duplosan KV [H]
		1.2	L/ha Cycocel 720 [H]
1998	winter wheat	0.6	L/ha Metasystox R [I]
		0.2	L/ha Bulldock [I]
		5.0	L/ha Glyfos [H]
	winter wheat	0.2	L/dt Arena [F]
Variant 1998			
1996	winter barley	0.5	kg/ha Herold [H]
		3.0	L/ha Fenikan [H]
		1.5	L/ha Pronto Plus [F]
		1.0	L/ha Folicur [F]
		0.8	L/ha Camposan [H]
	winter barley		
1997	winter barley		
		various developmental herbicides	
	grass		
1998	grass		
	winter barley	0.5	L/dt Manta plus [F/I]
	(= 51.8 g imidacl./ha)	3.0	kg/ha Mesurol RB 2

[H] = herbicide/plant growth regulator, [F] = fungicide, [I] = insecticide

APPENDIX II: cont´d.

- Control plot: field area „Auf dem Brachfeld“, south of field number 502
- Variant „1997“: field area „Auf dem Brachfeld“, field number 502
- Variant „1998“: field area „Auf dem Brachfeld“, field number 507
- Variant „1999“: field area „Auf dem Brachfeld“, south of field number 502

1999 Treatments

Study Plot / Year	Cropping	Pesticidal Treatments	Fertilizer Treatments
Control			
24 March	Grass (*Lolium perenne*)	4 L/ha Glyfos [H]	
5 May	uncropped		60 kg/ha KAS
10 May	maize	TMTD [F]	
20 May	maize	1.0 kg/ha Terano [H]	
11 June	Maize	1.0 L/ha Metivell [H]	
Variant 1999			
24 March	Grass (*Lolium perenne*)	4 L/ha Glyfos [H]	
5 May	uncropped		60 kg/ha KAS
10 May	maize [89 g imidacloprid/ha]	TMTD [F] 150 g/U Gaucho WS 70 [I]	
20 May	maize	1.0 kg/ha Terano [H]	
11 June	Maize	1.0 L/ha Metivell [H]	
Variant 1997			
15 March	winter wheat		60 kg/ha KAS
24 March	winter wheat	4 L/ha Glyfos [H]	
23 April	uncropped [50 g imidacloprid/ha]	71.5 g Gaucho WS 70 spray	
5 May	uncropped		60 kg/ha KAS
10 May	maize	TMTD [F]	
20 May	maize	1.0 kg/ha Terano [H]	
Variant 1998			
12 March	winter barley		60 kg/ha KAS
24 March	winter barley	4 L/ha Glyfos [H]	
5 May	uncropped		60 kg/ha KAS
10 May	maize	TMTD [F]	
20 May	maize	1.0 kg/ha Terano [H]	

[H] = herbicide, [F] = fungicide, [I] = insecticide

APPENDIX III: Analytical Report for Biological Samples.

Bayer AG
Crop Protection Development
Institute for Metabolism Research
and Residue Analysis

D-51368 Leverkusen

September 28, 1999
Report No.: MR-514/99

STUDY TITLE

Residue Levels of Imidacloprid and Imidacloprid Metabolites in Pollen of Maize Cultivated on Soils with Different Imidacloprid Residue Levels and Effects of These Residues on Foraging Honeybees

Test Location: farmland "Höfchen"

Author

[redacted]

Testing Facility

Bayer AG

PF-E/MR, Building 6610

51368 Leverkusen, Germany

Study Completion Date

September 28, 1999

Study Number

E 370 1551-2

TABLE OF CONTENT

1 INTRODUCTION

Maize samples of the Germany trial station "Höfchen" were analysed for residues of Imidacloprid and its Olefin- and Hydroxy Metabolites. The results are tabulated in the table below. Extraction, sample clean up and determination of Imidacloprid, Hydroxy- and Olefin-Metabolite by HPLC-MS/MS were performed according to method 00537/E001 (MR-568/99) The limit of quantitation was 0.005 mg/kg for Imidacloprid and the Hydroxy-Metabolite and 0.01 mg/kg for the Olefin-Metabolite. The limit of detection was 0.0015 mg/kg for Imidacloprid and the Hydroxy-Metabolite and 0.003 mg/kg for the Olefin-Metabolite.

2 TIME SCHEDULE

The experimental work was performed during the following time period:

Signature of Study Protocol:	March 22, 1999
Start of Experimental Phase:	August 31, 1999
End of Experimental Phase:	September 22, 1999
Completion of Report:	September 28, 1999

3 RESULTS OF POLLEN AND GREEN MATERIAL SAMPLES :

3.1 Pollen Samples:

Sample Name	Sample description	Sample weight [g]	Hydroxy-NTN [mg/kg]	Olefin-NTN [mg/kg]	Imidacloprid [mg/kg]
E15512K001	Pollen of Mais-Rispen	25	n.d.	n.d.	n.d.
E15512E97001	Pollen of Mais-Rispen	14.5	n.d.	n.d.	n.d.
E15512E98001	Pollen of Mais-Rispen	16.6	n.d.	n.d.	n.d.
E15512E99001	Pollen of Mais-Rispen	15.6	n.d.	n.d.	n.d.

Limit of quantitation: 0.005 mg/kg for Imidacloprid and Hydroxy-Metabolite, 0.01 mg/kg for the Olefin-Metabolite, < 0.005 and <0.010 = Residues below the limit of quantitation

Limit of detection: 0.0015 mg/kg for Imidacloprid and Hydroxy-Metabolite, 0.003 mg/kg for the Olefin-Metabolite, n.d.: Residues below the limit of detection

3.2 Green Material Samples:

Sample Name	Sample description	Sample weight [g]	Hydroxy-NTN [mg/kg]	Olefin-NTN [mg/kg]	Imidacloprid [mg/kg]
E15512K002	Green Material	147	n.d.	n.d.	n.d.
E15512E97002	Green Material	327	n.d.	n.d.	n.d.
E15512E98002	Green Material	145	N.d.	n.d.	n.d.
E15512E99002	Green Material	227	< LOQ	n.d.	0.011

Limit of quantitation: 0.005 mg/kg for Imidacloprid and Hydroxy-Metabolite, 0.01 mg/kg for the Olefin-Metabolite, < 0.005 and <0.010 = Residues below the limit of quantitation

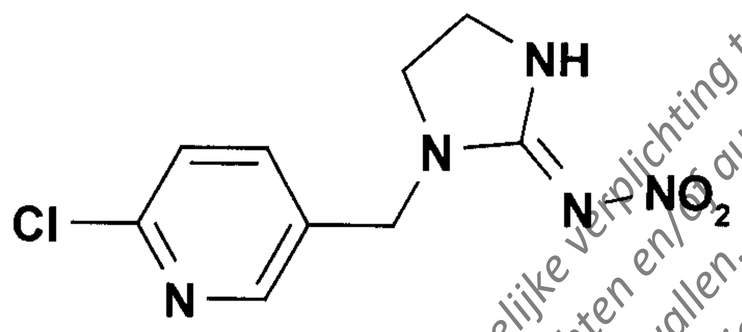
Limit of detection: 0.0015 mg/kg for Imidacloprid and Hydroxy-Metabolite, 0.003 mg/kg for the Olefin-Metabolite, n.d.: Residues below the limit of detection

4 EXPERIMENTAL

4.1 Reference Substances

Imidacloprid

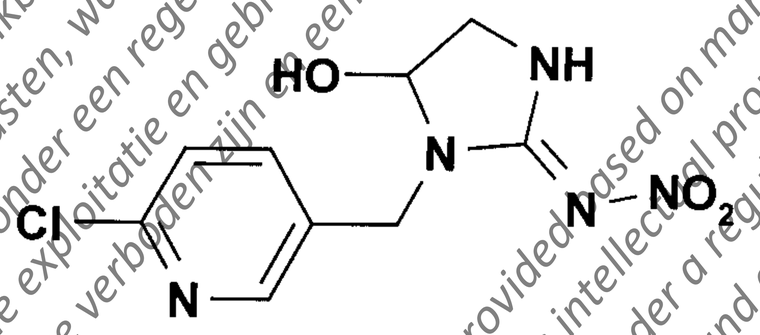
Structural formula:



Empirical formula:	$C_9H_{10}ClN_5O_2$
Molecular weight:	255.7 g/mole
Certificate of Analysis:	M00680, 03/13/98
Certified Assay:	99.4 %
Expiry Date:	March 2000

Hydroxy-Imidacloprid (WAK 4103)

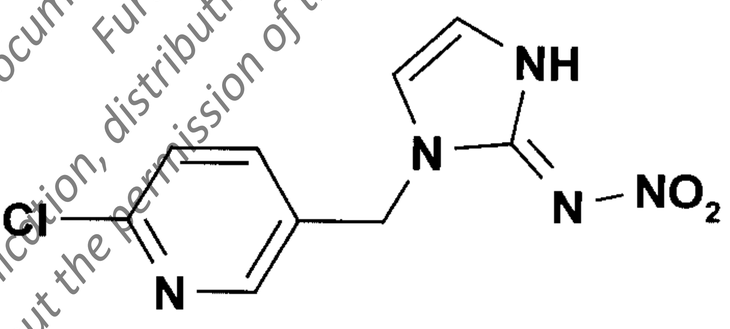
Structural formula:



Empirical formula:	$C_9H_{10}ClN_5O_4$
Molecular weight:	271.7 g/mole
Certificate of Analysis:	930323ELB03, 06/07/95
Certified Assay:	99.4 %
Expiry Date:	June 2000

Olefin-Imidacloprid (NTN 35884)

Structural formula:



Empirical formula:	$C_9H_8ClN_5O_2$
Molecular weight:	253.6 g/mole
Certificate of Analysis:	M00804, 07/22/98
Certified Assay:	98 %
Expiry Date:	June 2000

4.2 Residue Analytical Methodology

4.2.1 Extraction and Sample Clean-up

1. Place for e.g. 2.0 g of the sample material in a 150-ml beaker.
   Add 30 ml of methanol/water (3/1,v/v) and allow the sample to soak for 30 min.

2. Blend the sample using an ultra-turrax blender (or equivalent) for approximately 1 min.

3. Vacuum filter the suspension through 2.5 g of Celite filter aid using Schwarzband filter paper supported on a Büchner funnel into a 250-ml vacuum filter flask.

4. Wash the filtered solids with a total of 30 ml of methanol/water (3/1, v/v). Press residual solvent from the solids using rubber damming. Discard the filtered solids.

5. Transfer the filtrate to a 100-ml graduated cylinder. Determine the total volume of the extracts. Mix the solution well, and transfer the half (e.g. 1.0 g sample equivalent) to a 250-ml brown glass round-bottomed flask.

6. Concentrate the aliquot to an aqueous remainder of 5 to 10 ml using a rotary evaporator with a max. bath temperature of 50 °C.

4.2.2 ChemElut® Column Clean-up

1. Add 5 to 10 ml water to the aqueous solution from 4.2.1 step 6 to bring the total volume of the extracts to approx. 20 ml.

2. Place the aqueous solution on the top of the ChemElut® CE 1020 (20 ml volume) column fitted with a disposable stainless steel needle and wait for approx. 15 minutes to achieve an uniform distribution of the liquid on the column.

3. Elute the residues from the column with 140 ml of CH2Cl2. Collect the eluate in a 250-ml brown glass round-bottomed flask.

4. Evaporate the eluate from step 3 to dryness using a vacuum rotary evaporator and a max. bath temperature of 40 °C.

4.2.3 Silica Gel Column Clean-up

1. Dissolve the residues from 4.2.2 step 4 in 2 ml of toluene/ethyl acetate (85/15, v/v).

2. Apply the organic solution from step 1 onto a 0.5 g (3 ml) silica gel (SiOH) column (e.g. Varian).

3. Allow the solution to pass through the column at a flow rate of 1 ml/min.

4. Rinse the 250-ml brown glass round-bottomed flask with 10 ml of toluene/ethyl acetate (70/30, v/v) and apply the solution onto the column, too.

5. Elute the residues with 5 ml of acetonitrile at a flow rate of 1 ml/min. Collect the eluate in a 25-ml brown glass pear-shaped flask.

6. Evaporate the eluate from step 5 to dryness using a vacuum rotary evaporator and a max. bath temperature of 40 °C. Dissolve the residues in e.g. 1.00 ml of acetonitrile/water (2/8, v/v) and determine the residues with HPLC-MS/MS.

NOTE

1. The volumes to be used for flushing the column with toluene/ethyl acetate and for elution with acetonitrile must be newly determined for each batch of SiOH-column!

2. The flow rate should not be too high, since otherwise losses of the residues in may occur with recoveries below 70 % and the clean-up is less effective.

3. The Hydroxy-Metabolite may be converted to the Olefin-Metabolite (especially under acidic conditions).

4. The Olefin-Metabolite is degraded by light (ca. 50% in one day at natural daylight). Therefore, all solutions containing the Olefin-Metabolite must be protected from light and stored in a cool and dark place.

3.3 HPLC-MS/MS determination of Imidacloprid and Metabolites

4.3.1 Measuring equipment and HPLC conditions:

Instrument:	HP 1100
Injector:	HP 1100
Column:	Phenomenex, Luna C18 (2), 5 µm, 15 cm, 0.46 cm i.d. or equivalent
Injection Volume:	50 µl
Oven temperature:	40 °C
Mobile Phase:	A: Water/ACN (90/10, v/v)+ 0.1 ml acetic acid per litre B: Acetonitrile + 0.1 ml acetic acid per litre

Time Table	0 min	11.1 % B
	10 min	11.1 % B
	10.1 min	90 % B
	15 min	90 % B
	15.1 min	11.1 % B
	19 min	11.1 % B

Stoptime:	19 min	
Flow (Column):	1.0 ml/min	
Flow (into MS):	0.15 ml/min	
Retention Time:	Olefin-Metabolite:	approx. 4.6 min
	Hydroxy-Metabolite:	approx. 5.5 min
	Imidacloprid:	approx. 9.1 min

NOTE: Conditiones may be adapted for other HPLC-MS/MS systems.

4.3.2 MS/MS-Detection

The experiments were performed on a triple-quadrupole mass spectrometer system, fitted with an electrospray interface operated in the positive ion mode under MRM conditions.
The mass spectrometer was tuned by infusing a standard solution of 0.5 mg/l Imidacloprid and ist metabolites (dissolved in water/acetonitrile 8/2 + 0.1 ml acetic acid per l) at a flow rate of 10-20 µl/min. Mass axis calibration was done by infusing a polypropylene glycol 3000 solution. Unit mass resolution was established and maintained in each mass resolving quadrupole by maintaining a full width at half-maximum of between 0.8 and 1.0 DA. After tuning and calibration, optimal collision-activated dissociation (CAD) conditions for fragmentation of Imidacloprid and its metabolites were determined. These experiments were performed with nitrogen as collision gas with a collision offset of -19 eV for Imidacloprid, -21 eV for the Hydroxy-Metabolite and -13 eV for the Olefin-Metabolite and at an approximate collision gas thickness of 1.46×10^{15} atoms/cm^2. Nebulizer gas is set at 1.48 l/min, curtain gas is set at 1.44 l/min collision gas is set at 0.87 l/min and turbo gas is set at 6.0 l/min.

Detector: Triple Quadrupol LC-MS/MS Mass Spectrometer, e.g.,
Perkin-Elmer Sciex Instruments
API 300, Apple™ Macintosh System® 8.1

Interface: Electrospray, Turbo Ion Spray
Potential: +4400 V
Temperature: 400 °C
Nebulizer Gas: Nitrogen 5.0 (99.999 % purity), 1.48 l/min
Curtain Gas: Nitrogen 5.0 (99.999 % purity), 1.44 l/min
Turbo Gas: Nitrogen 5.0 (99.999 % purity), 6.0 l/min

Scan Type: MRM (Multiple Reaction Monitoring Mode)

Polarity: Positive

Collision Gas: Nitrogen 5.0 (99.999 % purity), 0.87 l/min

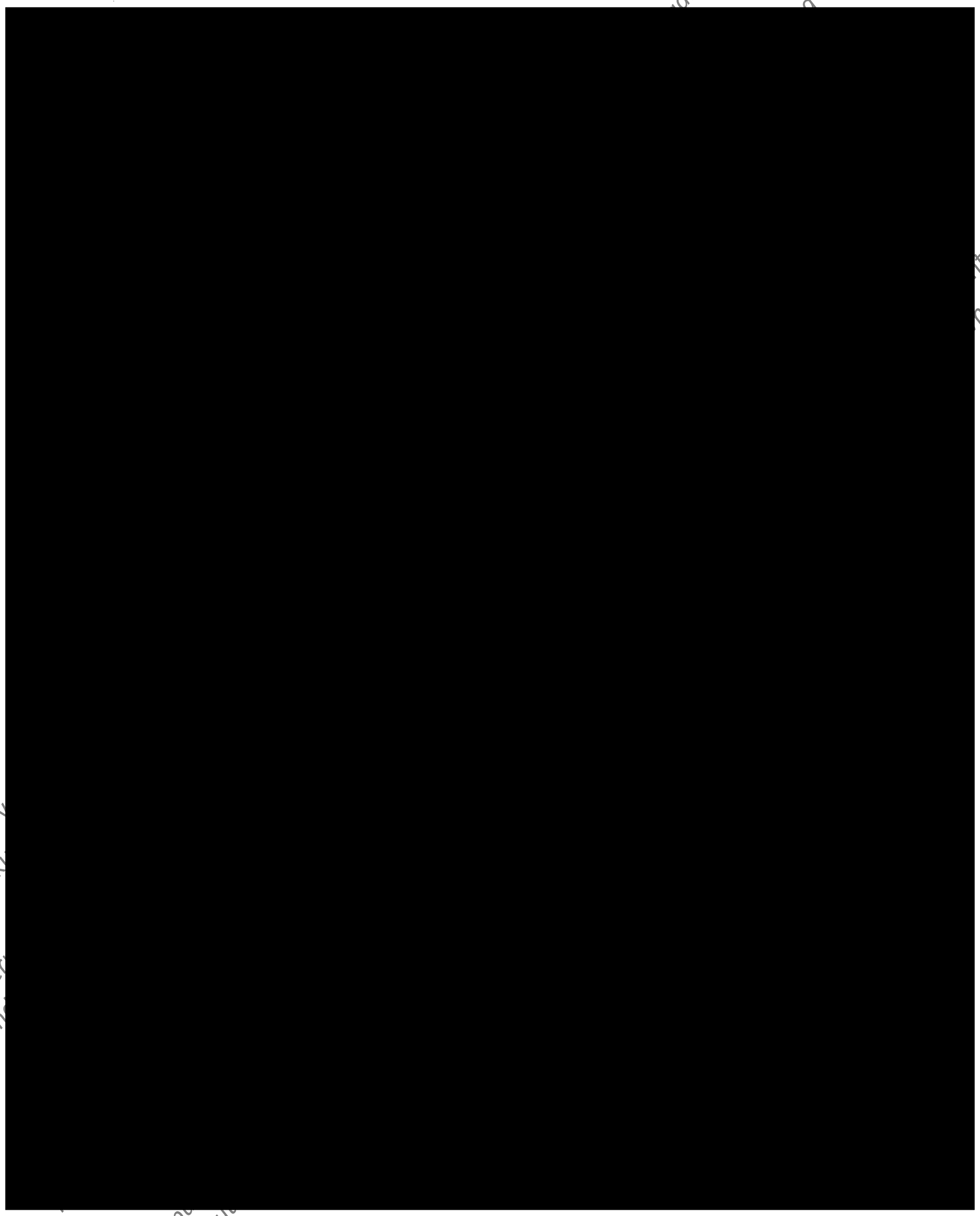
Mass spectrometer operating parameters:

Compound	Precursor Ion Q1 Mass (amu)	Product Ion Q3 Mass (amu)	Dwell Time (msec)	Collision Energy (eV)
Olefin-Metabolite (37)	256#	238	250	-13
Olefin-Metabolite (35)	254	236	250	-13
Hydroxy-Metabolite (37)	274#	191	250	-21
Hydroxy-Metabolite (35)	272	191	250	-21
Imidacloprid (37)	258#	211	500	-19
Imidacloprid (35)	256	209	500	-19

#: The Cl 37 isotope of all substances was detected to build the isotopes ratio

NOTE: Different MS/MS-instruments or instrument parameters may result in different ion transitions and different relative intensities.

Appendix IV: Copy of the GLP Certificate



Appendix IV: cont`d.

Appendix V: Quality Assurance Statement

Referat GLP
Quality Assurance Statement

[redacted]