TITLE PAGE

Residue Levels of Imidacloprid and Imidacloprid Metabolites in Nectar, Blossoms and Pollen of Sunflowers Cultivated on Soils with Different Imidacloprid Residue Levels and Effects of These Residues on Foraging Honeybees

Test Location: farmland "Höfchen" - 1999

AUTHOR

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TESTING FACILITY

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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:

[redacted]

Study Director — 27.9.99

Title — Date

Responsible Analyst
Biological Samples — 27.9.99

Title — Date

Responsible Analyst
Soil Samples — 28.9.99

Title — Date

CERTIFICATION OF AUTHENTICITY

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TABLE OF CONTENTS

1.0 SUMMARY

Report: [redacted] (1999). Residue Levels of Imidacloprid and Imidacloprid Metabolites in Nectar, Blossoms and Pollen of Sunflowers Cultivated on Soils with Different Imidacloprid Residue Levels and Effects of These Residues on Foraging Honeybees. Test Location: farmland "Höfchen" - 1999
Bayer AG, unpublished report No: SXR/Am 006; 1999/08/27.
(appendix I and III report data from study MR471-99 and MR-516/99, respectively).)

Guidelines: Internal Testing Method
Deviations: not applicable

GLP: yes (certified laboratory)

Material and methods: sunflower seed (variety "Fleury") either dressed (variant "1999")with 150 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 (control and variants "1997 and 1998" were drilled on 10 May 1999 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 0.5 U/ha. During peak flowering of the sunflowers (end of July) small bee colonies (2,000 to 3,000 honeybees) were caged on these plots (appr. 50 m^2) as a sampling device for sunflower nectar and pollen. In addition, some pollen and flowers were sampled by hand. The honeybees used as samplers were observed for signs of behavioral impacts. All samples and a small sample of honeybees were subjected to a residue analysis for imidacloprid and its relevant metabolites.

Dates of biological work: July 25 – September 3, 1999.

Dates of soil analysis: August 9 – 11, 1999.

Dates of analysis of biological samples: August 25 - September 21, 1999.

Findings: Residues in soil and in sunflower plant matrices 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 number 502) – imidacloprid-free seed in imidacloprid-free soil			
Soil sample (0-30 cm)	n.d.	--	--
Leaves (produced latest)	n.d.	n.d.	n.d.
Flowers (male / female flowers)	n.d.	n.d.	n.d.
Nectar sampled from the hive combs	n.d.	n.d.	n.d.
Pollen sampled from the hive combs	n.d.	n.d.	n.d.
Pollen sampled from the plants	n.d.	n.d.	n.d.
Honeybees exposed to the sunflowers	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) = 150,000 seed

Type of Sample	Residue Level [mg/kg] *		
	Imidacloprid	Olefin-NTN	Hydroxy-NTN
Variant „1997“ (field number 502) – imidacloprid-free seed in imidacloprid-contaminated soil			
Soil sample (0-30 cm)	0.018	--	--
Leaves (produced latest)	n.d.	n.d.	n.d.
Flowers (male / female flowers)	n.d.	n.d.	n.d.
Nectar sampled from the hive combs	n.d.	n.d.	n.d.
Pollen sampled from the hive combs	n.d.	n.d.	n.d.
Pollen sampled from the plants	n.d.	n.d.	n.d.
Honeybees exposed to the sunflowers	n.d.	n.d.	n.d.
Variant „1998“ (field number 507) – imidacloprid-free seed in imidacloprid-contaminated soil			
Soil sample (0-30 cm)	< LOQ	--	--
Leaves (produced latest)	n.d.	n.d.	n.d.
Flowers (male / female flowers)	n.d.	n.d.	n.d.
Nectar sampled from the hive combs	n.d.	n.d.	n.d.
Pollen sampled from the hive combs	n.d.	n.d.	n.d.
Pollen sampled from the plants	n.d.	n.d.	n.d.
Honeybees exposed to the sunflowers	n.d.	n.d.	n.d.
Variant „1999“ (south of field number 502) – Gaucho-dressed seed in imidacloprid-free soil			
Soil sample (0-20 cm)	n.d.	--	--
Leaves (produced latest)	0.007	n.d.	< LOQ
Flowers (male / female flowers)	n.d.	n.d.	n.d.
Nectar sampled from the hive combs	n.d.	n.d.	n.d.
Pollen sampled from the hive combs	n.d.	n.d.	n.d.
Pollen sampled from the plants	n.d.	n.d.	n.d.
Honeybees exposed to the sunflowers	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 behavioral impacts (e.g. apathy, exaggerated motility, discoordinated movements) or suspicious mortality was observed on the honeybees used for collecting sunflower nectar and pollen.

2.0 INTRODUCTION

According to EU directive 91/414/EWG 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 sunflower 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 Scenario 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
Indication:	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, 2000
Physical appearance:	white powder
Specific density:	not applicable
Storage conditions:	room temperature
Seed dressing rate(s) tested in the study:	150 g/U (1 U = 150,000 seed) (= nominal content: 105 g/U Imidacloprid; analytical findings, FAR 671-00: 89.3 g/U Imidacloprid).
Seed drilling rate tested in the study:	0.5 U/ha (= 1,800 seed per four 240 m² study plots) (sunflower variety: „Fleury“; standard fungicidal treatment: Carbendazim, Metalaxyl and Cu-Oxyquinolat)
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 May 10, 1999. Sampling of nectar, pollen, flowers and honeybees and the behavioral observations were performed between July 25 – September 3, 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 006

3.4 *Origin of Honeybees and Preparation of Hive Nuclei*

Honeybee colonies used for pollen and nectar sampling were supplied by a German beekeeper [redacted] Preparation of the hive colonies used for the test started on 22 May 1999. From large commercially managed beehives, 20 combs were removed and combined. After the brood of these combs had ecdysed (5 June 1999), the combs were removed and allocated to 10 three-comb-colonies (two combs from the combined hive and a new comb matrix) with about 2,000 – 3,000 workerbees. Two days before colony installment on the test plots, the two original combs were replaced by new comb matrices and the colonies received 0.5 litre ready-to-use syrup (1:1). Hive installment was on 26 July 1999.

3.5 *Procedure of Seed Dressing*

The sunflower seeds (variety: „Fleury“) 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 combined fungicide (Carbendazim, Metalaxyl and Cu-Oxyquinolat). This fungicidal treatment was also applied to all imidacloprid-free seeds which were drilled on study plots of test variants 97, 98 and the control.

3.6 *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 percise 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.

3.7 Treatment Design

After the previous crop had been destroyed (4 l/ha Glyfos and subsequent ploughing), all study plots were drilled with 0.5 U/ha sunflower seed (1 U = 150,000 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 sunflower seed (variety: Fleury). Drilling distance was 50 cm between rows and 22.8 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 maize or rape plants.

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

- Control plot: untreated grass area since 1996. Drilled with imidacloprid-free sunflower 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 sunflower seed on 10 May 1999.
- Variant „1998“: cropped in fall 1998 by Gaucho treated winter barley (52 g ai/ha). Drilled with imidacloprid -free sunflower seed on 10 May 1999.
- Variant „1999“: untreated grass area since 1996. Drilled with Gaucho WS 70 treated sunflower seed on 10 May 1999 (45 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).

Shortly before full sunflower blossom, tunnel cages of 10 x 5 m and 3 m height were installed on each study plot. The tunnel cages consisted of an aluminium frame covered by gauze material (2 x 2 mm mesh size). For operational purposes, a walkway was created by removing all plants along a 50 cm transect between the tunnel entrance and the opposite end.

3.8 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.

3.9 Sampling Procedure

Installment of bee hives

One bee colony with 3 combs (about 2,000 – 3,000 honeybees) was placed left from the entrance on each study plot on 26 July 1999 hive nuclei no. 51, 46, 60, 57). Bee colonies remained in the tunnels till 5 August 1999.

Sampling of Foraging Honeybees

On days 2 and 3 following hive installment (= 28 and 29 July 1999) approximately 100 honeybees were sampled with glass tubes while foraging on the sunflowers within the tunnel. Sampled honeybees were killed by placing the sampling glass tubes into a container with dry ice. At the end of each sampling day at the latest, collected honeybees were transferred into a refrigerator (-20°C) where they were retained until residue analysis (see 3.10). This sampling was primerily done to get sunflower nectar from the honeybulbs and sunflower pollen from the pollen pockets. In terms of time and sample quantity, however, other sampling methods (see below) proved to be more efficient. For this reason, no further processing of honeybees was done as initially intended but the honeybees kept in reserve in case that other questions may arise during or after the analytical work. A small number of honeybees (approximately 1 g) were analyzed for residues to examine whether or not any residues in nectar and/or pollen would also be detected in this matrix.

Sampling of Sunflower Nectar

On days 2, 4 and 8 following colony installment (= 28 July, 30 July and 3 August 1999) combs were removed from the beehives to take pollen samples (only on 30 July 1999, sufficient pollen stores were present to do this sampling). During these sampling events, some freshly collected sunflower nectar was detected in the combs and 1 ml samples were taken from each colony for residue analysis. After sampling, the nectar samples were stored on dry ice in the field. At the end of each sampling day at the latest, the nectar samples were transferred into a refrigerator (-20°C) where they were retained until residue analysis (see 3.10).

Sampling of Pollen from Sunflowers and the Beehives

On days 2, 4 and 8 following hive installment (= 28 July, 30 July and 3 August 1999) combs were removed from the beehives to cut out pollen stores. Only on 30 July 1999, sufficient pollen stores were present to do this sampling. Additional pollen sampling campaigns were made between 25 July and 30 July by shaking pollen out of the sunflower heads directly. After sampling (either from combs or from sunflower heads), the pollen samples were stored on dry ice in the field. At the end of each sampling day at the latest, they were transferred into a refrigerator (-20°C) where they were retained until residue analysis (see 3.10).

Sampling of Sunflower Flowers

About 20 g of each, male and female sunflower flowers were sampled from the sunflower plants during the peak flowering period. After sampling, the flowers were stored on dry ice in the field. At the end of each sampling day at the latest, they were transferred into a refrigerator (-20°C) where they were retained until residue analysis (see 3.10).

Sampling of Sunflower Leaves

Sunflower leaves were collected on 23 July 1999. After sampling, the leaves were stored on dry ice in the field. At the end of the sampling day, the leaf samples were transferred into a refrigerator (-20°C) where they were retained until residue analysis (see 3.10).

3.10 Sample Processing and Residue Analysis

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

3.11 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

While hive nuclei were confined within the tunnels, climatic records were made during each evaluation. The following conditions were recorded (high noon till 3 p.m):

Day after first exposure	Precipitation [mm]	Air temperature [°C]	Soil temperature [°C]	Remarks
0	0	24	32	Free sky, slightly windy
1	0	23	32	Free sky, slightly windy
2	0	26	28	Free sky, slightly windy
3	0	27	39	Free sky, slightly windy
7	0	29	37	40% cloudy, calm
8	0	30	34	40% cloudy, calm

3.12 Observations on Honeybees

All behavioral anomalies of the honeybees were recorded together with the date of observations. In particular, the following behavioural components were noted:

Foraging intensity:	Six times (days 0, 1, 2, 3, 7 and 8 after 1st exposure) the number of bees foraging on randomly selected 100 sunflower heads was recorded.

Behavioral Anomalies:	Whenever observed, the following behavioral anomalies were recorded with the date and daytime of observation: - exaggerated motility - discoordinated movements (trembling, shaking, apathy)
Mortality:	In front of the hive nuclei, linen sheets (60x50 cm) were placed on the ground to trap the dead bees which were removed from the beehives during the time while nuclei were confined within the tunnel cages. In addition, the number of dead honeybees around the cage margin were counted as an indicator whether a higher number of bees tried to leave the tunnel or failed to return to the hive. Also, any conspicous numbers of dead bees in the study plots was recorded but no formal counts were made.

4.0 FILING

All raw data, the study protocol and the original of the report are filed in the Central GLP archive of PF/F, 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

Soil Samples

Analytical findings 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 determined 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.

Biological Samples

Residue levels of imidacloprid and of its toxicologically relevant metabolites (olefin- and hydroxy-imidacloprid) were below the limit of detection (= 0.0015 mg/kg and 0.03 mg/kg, respectively) in all bee-relevant sunflower parts (nectar, pollen), in the florets and in the exposed honeybees. Even in leaves of that sunflowers which were planted as succeeding crop, no residues were detected. Only in the leaves of plants which were raised from Gaucho-dressed seed, traces of imidacloprid (0.007 mg/kg) and of the hydroxy-metabolite (< LOQ) were detected.

5.2 Biological Observations on Foraging Honeybees

All hive nuclei decreased in weight during their confinement on the study plots (Tab. 2). The weight decrease was 4.8, 5.9, 3.0 and 5.3% of the initial weight for the control, test variants „1997", „1998", and „1999", respectively. These data show that the weight decrease was comparable troughout study plots and no treatment-related effect was indicated.

Foraging activity (recorded during 6 occasions between 26 July and 3 August 1999) averaged 100, 130, 82, and 94 honeybees per 50 sunflower heads in the control, test variants „1997", „1998", and „1999", respectively (Tab. 2). No treatment-related effect is evident from these data.

No treatment-related effect is evident for mortality as well. The treatment-specific mortality figures are 17, 17, 18, and 18 honeybees for the control, test variants „1997", „1998", and „1999", respectively (Tab. 2).

During the foraging period, no behavioral impacts (e.g. apathy, exaggerated motility, discoordinated movements) were observed. Flight and foraging intensity was not different between bees foraging on control and on treatment plots. Likewise, returning frequency of honeybees was not affected by the treatment (Tab. 2).

A month later, all nucleus hives were evaluated for their further development. There were no differences in population density, food stores or brood status between the control and the treatment hives.

In summary, it can be concluded that honeybees were not adversely affected by any of the examined exposure scenario.

FIGURES

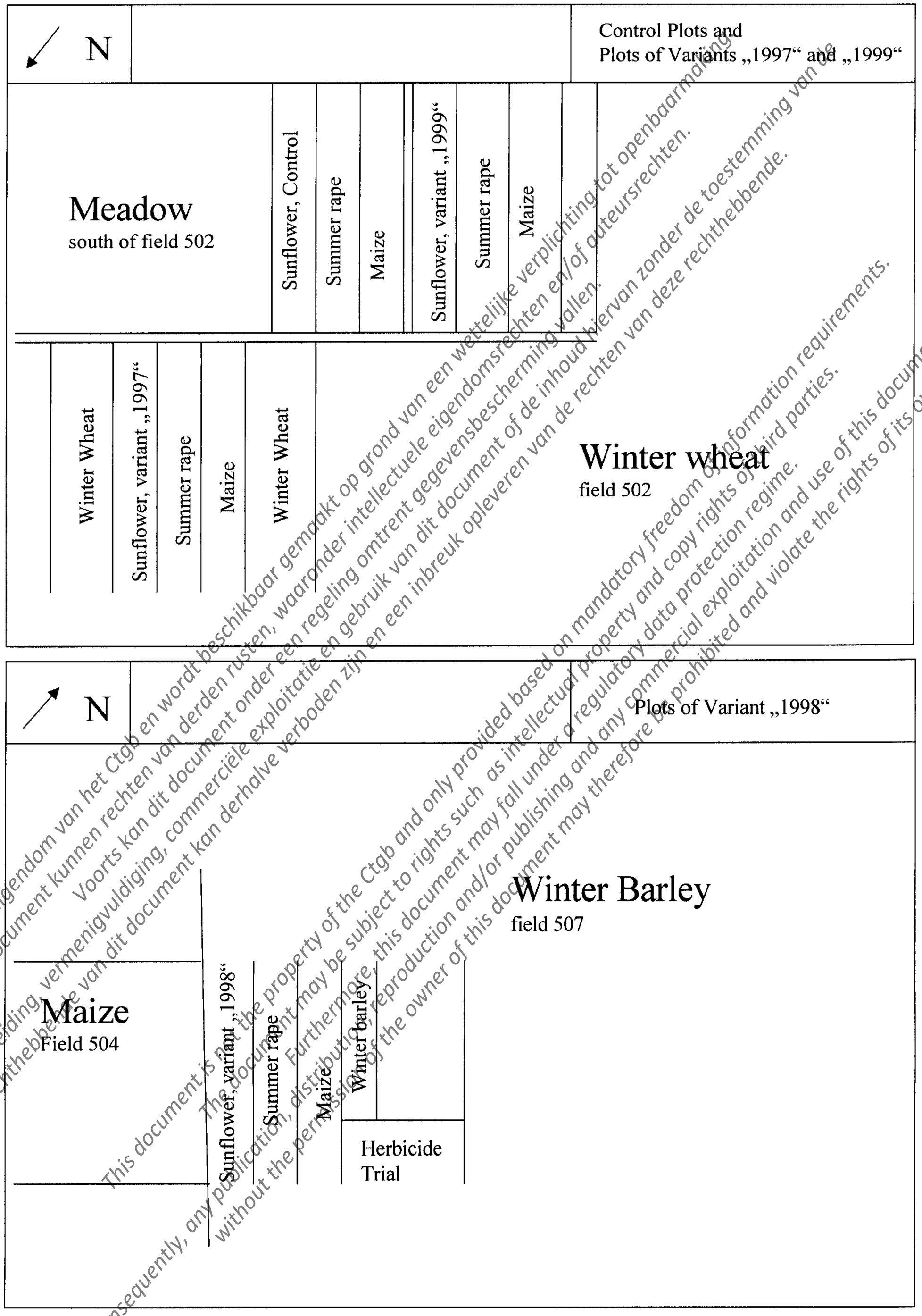


Figure 1: Arrangement of the study plots on study fields 502 and 507 (Farm „Höfchen“, D).
Each study plot had a size of 8 x 30 m with distances of 50 cm between rows and 22.8 cm in-row.

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: 24 April 1999 (50 g ai/ha)
- Variant „1998“: last imidacloprid treatment: 24 Sept. 1998 (52 g ai/ha)
- Variant „1999“: last imidacloprid treatment: before 1996; drilled on 12 May 1999 with Gaucho-treated sunflower seed (45 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“ (same study field as for control = 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: Biological observations on the hive nuclei used for sampling sunflower nectar and pollen.

Hive installment was on 26 July 1999. Plot history is as follows:

- Control plot: last imidacloprid treatment: before 1996
- Variant „1997“: last imidacloprid treatment: 24 April 1999 (50 g ai/ha)
- Variant „1998“: last imidacloprid treatment: 24 Sept. 1998 (52 g ai/ha)
- Variant „1999“: last imidacloprid treatment: before 1996; drilled on 12.May 1999 with Gaucho-treated sunflower seed (45 g ai/ha).

Weight Development

Treatment	Hive nucleus no.	Hive weight at study initiation [g]	Hive weight at study termination [g]	Weight changes
Control	51	7140	6795	- 4.8
Variant 97	46	7140	6720	- 5.9
Variant 98	60	6935	6730	- 3.0
Variant 99	57	6810	6450	- 5.3

Foraging Activity

Treatment	Days after first exposure	No. foraging honeybees per 50 sunflower Left of walkway	No. foraging honeybees per 50 sunflower Right of walkway	No. foraging honeybees per 50 sunflower Average
Control	0	10	12	
	1	33	35	
(= hive	2	48	53	
nucleus	3	49	53	
no. 51)	7	83	87	
	8	76	63	100.3 ± 47.5
Variant 97	0	25	29	
	1	49	63	
(= hive	2	58	65	
nucleus	3	71	97	
no. 46)	7	86	90	
	8	69	76	129.7 ± 40.7
Variant 98	0	10	9	
	1	48	54	
(= hive	2	45	60	
nucleus	3	50	66	
no. 60)	7	38	48	
	8	29	37	82.3 ± 32.5
Variant 99	0	10	14	
	1	30	33	
(= hive	2	51	57	
nucleus	3	50	56	
no. 57)	7	59	74	
	8	57	70	93.5 ± 38.3

Table 2: cont`d.

- Control plot: last imidacloprid treatment: before 1996
- Variant „1997“: last imidacloprid treatment: 24 April 1999 (50 g ai/ha)
- Variant „1998“: last imidacloprid treatment: 24 Sept. 1998 (52 g ai/ha)
- Variant „1999“: last imidacloprid treatment: before 1996; drilled on 12 May 1999 with Gaucho-treated sunflower seed (45 g ai/ha).

Mortality

Treatment	Days after first exposure	No. of dead honeybees In front of beehive	No. of dead honeybees At the tent margin	No. of dead honeybees Total
Control	1	3	2	
(= hive	2	1	6	
nucleus	3	2	1	
no. 51)	7	1	0	
	8	0	1	17
Variant 97	1	0	2	
(= hive	2	0	3	
nucleus	3	0	3	
no. 46)	7	1	4	
	8	0	4	17
Variant 98	1	0	4	
(= hive	2	0	3	
nucleus	3	0	3	
no. 60)	7	0	5	
	8	0	3	18
Variant 99	1	0	2	
(= hive	2	1	3	
nucleus	3	0	4	
no. 57)	7	0	3	
	8	2	3	18

Long-term Development (evaluation 3 September 1999)

Treatment	Occupation degree of central comb by honeybees	Brood development	Occupation of side combs by honeybees, food stores or brood
Control(= hive nucleus no. 51)	+++	+++	+
Variant 97 (= hive nucleus no. 46)	+++	+++	+
Variant 98 (= hive nucleus no. 60)	+++	+++	-
Variant 99 (= hive nucleus no. 57)	+++	+++	++

- = no honeybees/food stores/brood cells on combs
+ = singular honeybees/food stores/brood cells on comb
++ = partly occupied combs (honeybees/food stores/brood cells)
+++ = well occupied combs (honeybees), good brood amount, high food stores.

Table 3: Plant Residue Level of Imidacloprid and Toxicologically Relevant Metabolites at the Different Study Sites.

The details of the analytical work are given in appendix III. Plot history is as follows:

- Control plot: last imidacloprid treatment: before 1996
- Variant „1997“: last imidacloprid treatment: 24 April 1999 (50 g ai/ha)
- Variant „1998“: last imidacloprid treatment: 24 Sept. 1998 (52 g ai/ha)
- Variant „1999“: last imidacloprid treatment: before 1996; drilled on 12 May 1999 with Gaucho-treated sunflower seed (45 g ai/ha).

Type of Sample	Residue Level [mg/kg] *		
	Imidacloprid	Olefin-NTN	Hydroxy-NTN
Control Plot (south of field number 502)			
Leaves (produced latest)	n.d.	n.d.	n.d.
Flowers (male / female flowers)	n.d.	n.d.	n.d.
Nectar sampled from the hive combs	n.d.	n.d.	n.d.
Pollen sampled from the hive combs	n.d.	n.d.	n.d.
Pollen sampled from the plants	n.d.	n.d.	n.d.
Honeybees exposed to the sunflowers	n.d.	n.d.	n.d.
Variant „1997“ (field number 502)			
Leaves (produced latest)	n.d.	n.d.	n.d.
Flowers (male / female flowers)	n.d.	n.d.	n.d.
Nectar sampled from the hive combs	n.d.	n.d.	n.d.
Pollen sampled from the hive combs	n.d.	n.d.	n.d.
Pollen sampled from the plants	n.d.	n.d.	n.d.
Honeybees exposed to the sunflowers	n.d.	n.d.	n.d.
Variant „1998“ (field number 507)			
Leaves (produced latest)	n.d.	n.d.	n.d.
Flowers (male / female flowers)	n.d.	n.d.	n.d.
Nectar sampled from the hive combs	n.d.	n.d.	n.d.
Pollen sampled from the hive combs	n.d.	n.d.	n.d.
Pollen sampled from the plants	n.d.	n.d.	n.d.
Honeybees exposed to the sunflowers	n.d.	n.d.	n.d.
Variant „1999“ (south of field number 502)			
Leaves (produced latest)	0.007	n.d.	< LOQ
Flowers (male / female flowers)	n.d.	n.d.	n.d.
Nectar sampled from the hive combs	n.d.	n.d.	n.d.
Pollen sampled from the hive combs	n.d.	n.d.	n.d.
Pollen sampled from the plants	n.d.	n.d.	n.d.
Honeybees exposed to the sunflowers	n.d.	n.d.	n.d.

* Limit of quantitation: 0.005 mg/kg (imidacloprid & hydroxy-metabolite), 0.01 mg/kg (olefin-metabolite); n.d. = below limit of detection (0.0015 mg/kg and 0.003 mg/kg, respectively)

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

MR-471/99

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 **PAGE**

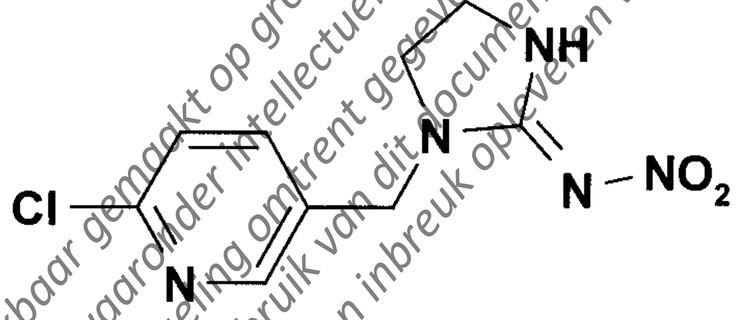
1 INTRODUCTION

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.

Crop management before 24 March 1999 was not conducted and recorded under GLP regulations.

- 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.

Crop management before 24 March 1999 was not conducted and recorded under GLP regulations.

- 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	sunflower	Carbendazim [F] Cu-Oxyquinolat [F] Metalaxyl [F]	
12 May	sunflower	3 L/ha Racer [H]	
8 June	sunflower	3.0 kg/ha Mesurol RB 4 [I]	
18 June	sunflower	1.2 L/ha Unden [I]	
Variant 1999			
24 March	Grass (*Lolium perenne*)	4 L/ha Glyfos [H]	
5 May	uncropped		60 kg/ha KAS
10 May	Sunflower [45 g imidacloprid/ha]	Carbendazim [F] Cu-Oxyquinolat [F] Metalaxyl [F] 150 g/U Gaucho WS 70 [I]	
12 May	sunflower	3 L/ha Racer [H]	
8 June	sunflower	3.0 kg/ha Mesurol RB 4 [I]	
18 June	sunflower	1.2 L/ha Unden [I]	
Variant 1997			
15 March	winter wheat		60 kg/ha KAS
24 March	winter wheat	4 L/ha Glyfos [H]	
23 April	uncropped	71.5 g Gaucho WS 70 spray	
5 May	uncropped		60 kg/ha KAS
10 May	sunflower	Carbendazim [F] Cu-Oxyquinolat [F] Metalaxyl [F]	
12 May	sunflower	3 L/ha Racer [H]	
8 June	sunflower	3.0 kg/ha Mesurol RB 4 [I]	
18 June	sunflower	1.2 L/ha Unden [I]	

APPENDIX II: cont´d.

Crop management before 1999 was not conducted and recorded under GLP regulations.

- 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

Study Plot / Year	Cropping	Pesticidal Treatments	Fertilizer Treatments
Variant 1998			
12 March	winter barley		60 kg/ha KAS
24 March	winter barley	4 L/ha Glyfos [H]	
5 Mayh	uncropped		60 kg/ha KAS
10 May	sunflower	Carbendazim [F] Cu-Oxyquinolat [F] Metalaxyl [F]	
12 May	sunflower	3 L/ha Racer [H]	
8 June	sunflower	3.0 kg/ha Mesurol RB 4 [I]	
18 June	sunflower	1.2 L/ha Unden [I]	

[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

STUDY TITLE

Residue Levels of Imidacloprid and Imidacloprid Metabolites in Nectar, Blossoms and Pollen of Sunflowers 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 27, 1999

Study Number

E 370 1552-3

TABLE OF CONTENT

1 INTRODUCTION

Sunflower 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 25, 1999
End of Experimental Phase:	September 21, 1999
Completion of Report:	September 27, 1999

3 RESULTS OF NECTAR, POLLEN, FLOWER, BEE AND GREEN MATERIAL SAMPLES :

3.1 Nectar Samples.

Sample Name	Sample description	Sample weight [g]	Hydroxy-NTN [mg/kg]	Olefin-NTN [mg/kg]	Imidacloprid [mg/kg]
E15523K001	Nectar Honeycomb Gilli	4	n.d.	n.d.	n.d.
E15523E97001	Nectar Honeycomb Gilli	3.6	n.d.	n.d.	n.d.
E15523E98001	Nectar Honeycomb Gilli	4.8	n.d.	n.d.	n.d.
E15523E99001	Nectar Honeycomb Gilli	4.3	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 Pollen Samples:

Sample Name	Sample description	Sample weight [g]	Hydroxy-NTN [mg/kg]	Olefin-NTN [mg/kg]	Imidacloprid [mg/kg]
E15523K002	Pollen	6.1	n.d.	n.d.	n.d.
E15523K003	Pollen Honeycomb Gilli	2	n.d.	n.d.	n.d.
E15523E97002	Pollen	4.3	n.d.	n.d.	n.d.
E15523E97003	Pollen Honeycomb Gilli	1.7	n.d.	n.d.	n.d.
E15523E98002	Pollen	1.9	n.d.	n.d.	n.d.
E15523E98003	Pollen Honeycomb Gilli	2.3	n.d.	n.d.	n.d.
E15523E99002	Pollen	2.1	n.d.	n.d.	n.d.
E15523E99003	Pollen Honeycomb Gilli	1.8	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.

3.3 Flower Samples:

Sample Name	Sample description	Sample weight [g]	Hydroxy-NTN [mg/kg]	Olefin-NTN [mg/kg]	Imidacloprid [mg/kg]
E15523K004	Sunflower Flower Male	23.2	n.d.	n.d.	n.d.
E15523K005	Sunflower Flower Female	12.2	n.d.	n.d.	n.d.
E15523E97004	Sunflower Flower Male	11.5	n.d.	n.d.	n.d.
E15523E97005	Sunflower Flower Female	21.5	n.d.	n.d.	n.d.
E15523E98004	Sunflower Flower Male	14.6	n.d.	n.d.	n.d.
E15523E98005	Sunflower Flower Female	20.7	n.d.	n.d.	n.d.
E15523E99004	Sunflower Flower Male	14.5	n.d.	n.d.	n.d.
E15523E99005	Sunflower Flower Female	12.5	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.

3.4 Bee Samples:

Sample Name	Sample description	Sample weight [g]	Hydroxy-NTN [mg/kg]	Olefin-NTN [mg/kg]	Imidacloprid [mg/kg]
E15523K006	Bees of Flowers	19.1	n.d.	n.d.	n.d.
E15523E97006	Bees of Flowers	16.8	n.d.	n.d.	n.d.
E15523E98006	Bees of Flowers	22	n.d.	n.d.	n.d.
E15523E99006	Bees of Flowers	18.8	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.

3.5 Green Material Samples:

Sample Name	Sample description	Sample weight [g]	Hydroxy-NTN [mg/kg]	Olefin-NTN [mg/kg]	Imidacloprid [mg/kg]
E15523K007	Green Material	383	n.d.	n.d.	n.d.
E15523E97007	Green Material	358	n.d.	n.d.	n.d.
E15523E98007	Green Material	334	n.d.	n.d.	n.d.
E15523E99007	Green Material	339	< LOQ	n.d.	0.0074

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.

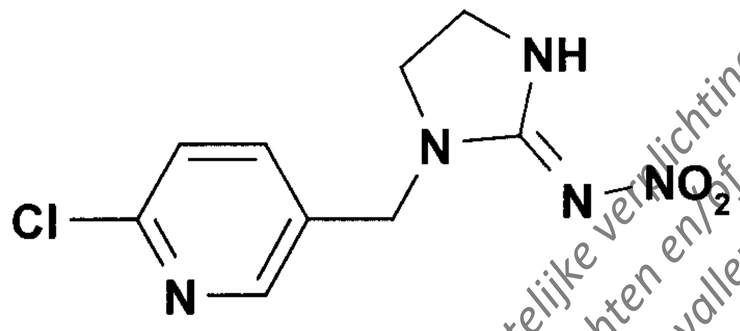
Limit of detection: 0.0015 mg/kg for Imidacloprid and Hydroxy-Metabolite, 0.003 mg/kg for the Olefin-Metabolite; n.d.: Residues below the limit of detection.

4 EXPERIMENTAL

4.1 Reference Substances

Imidacloprid

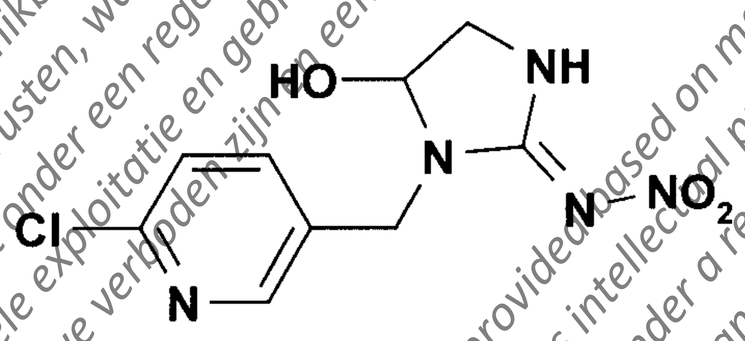
Structural formula:



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)

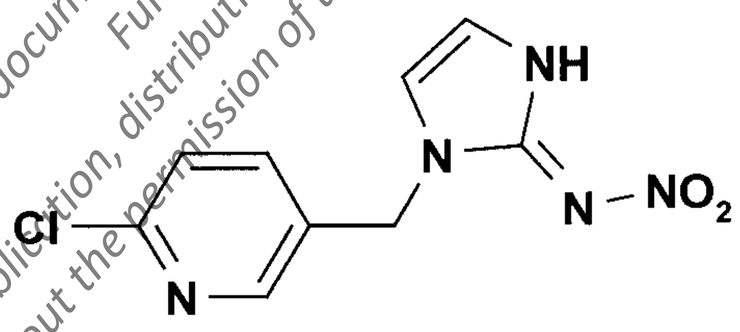
Structural formula:



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 CH_2Cl_2. 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.

4.3 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

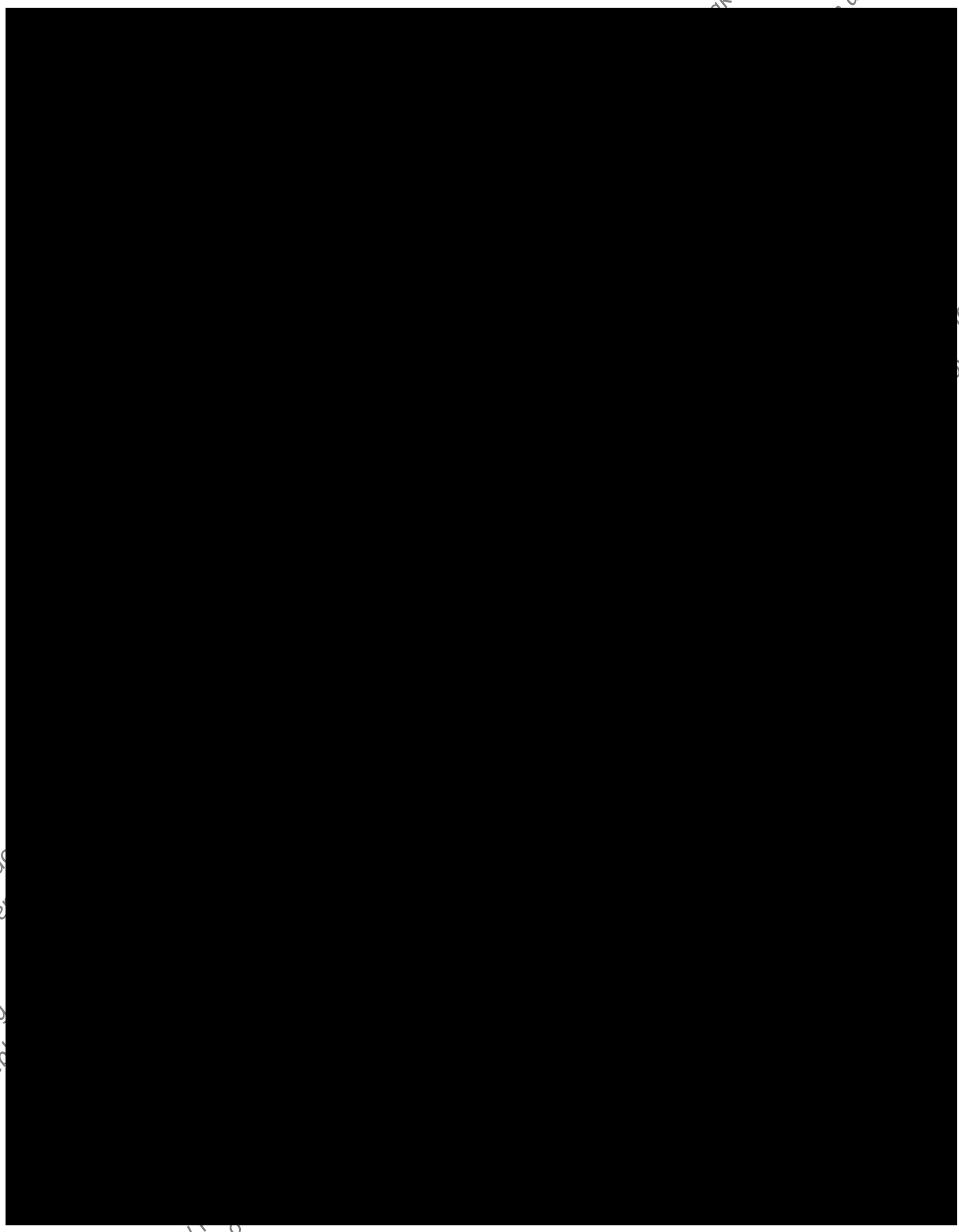
Mass spectrometer operating parameters:

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: (continued):

[redacted]

Appendix V: Quality Assurance Statement

Referat GLP
Quality Assurance Statement

[redacted]