TITLE PAGE

Residue Levels of Imidacloprid and Imidacloprid Metabolites in Honeybees Orally Dosed with Imidacloprid in Standardized Toxicity Tests (EPPO 170)

AUTHOR

[redacted]

TESTING FACILITY

BAYER AG
Crop Protection-Development
Institute For Environmental Biology

D-51368 Leverkusen-Bayerwerk

GLP STUDY NUMBER

E 370 1725 - 5

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SXR/Am 013

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STUDY COMPLETION DATE:

30 September 1999

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]).

[redacted]

CERTIFICATION OF AUTHENTICITY

[redacted]

Study Director

Title

30/9/99

Date

Responsible Analyst
Biological Samples

Title

30.9.99

Date

Head of Institute for
Environmental Biology

Title

30.9.99

Date

INQUIRIES

Inquiries should be directed to:

[redacted]
BAYER AG
Crop Protection - Development
Institute for Environmental Biology
D-51368 Leverkusen - Bayerwerk, FRG

[redacted]

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1.0 SUMMARY

Report: [redacted] 1999): Residue Levels of Imidacloprid and Imidacloprid Metabolites in Honeybees Orally Dosed with Imidacloprid in Standardized Toxicity Tests (EPPO 170)
Bayer AG, unpublished report No: SXR/Am 013; 1999/09/30.
(tab 1 report data from IBACON study 6400036; app I contains data from MR study –575/99).

Guidelines: Internal Testing Method (deviations: not applicable)

GLP: yes (certified laboratory)

Material and methods: test substance specification: imidacloprid techn., batch no. M00680, purity 99.4%. Adult honeybees were orally dosed with either 0.0001, 0.0008, 0.0015, 0.0031, 0.006, 0.012, 0.023 or 0.041 µg/honeybee imidacloprid techn.. Honeybees which died during the study were removed from the test boxes at each evaluation and stored at –20°C. At study termination, alive honeybees were killed by CO_2 asphyxiation and retained also at –20°C till residue analysis. After shipping the honeybee samples to Bayer AG, they were analysed for residues of imidacloprid and toxicologically relevant metabolites , i.e. olefin- and hydroxy-imidacloprid.

Dates of biological work: July 6 – 10, 1999 (IBACON study 6400036).
Dates of analysis of biological samples: September 15 – 17, 1999.

Findings: Residues in honeybees orally dosed with imidacloprid techn.:

Dose Applied [ng/bee]	Time to Death [h]	Sample weight [g]	Hydroxy-NTN [mg/kg]	Olefin-NTN [mg/kg]	Imidacloprid [mg/kg]
0.1	-- **	3.8	n.d.	n.d.	n.d.
0.8	-- **	4.1	n.d.	n.d.	n.d.
1.5	-- **	3.7	n.d.	n.d.	n.d.
3.1	4	0.3	n.d.	n.d.	< LOQ
	24	0.6	< LOQ	n.d.	n.d.
	-- **	2.7	< LOQ	n.d.	n.d.
6.0	24	0.7	< LOQ	n.d.	n.d.
	-- **	2.7	< LOQ	n.d.	n.d.
12.2	24	0.5	< LOQ	n.d.	n.d.
	-- **	2.8	0.006	n.d.	n.d.
22.9	24/48	0.4	0.010	< LOQ	< LOQ
	-- **	3.3	0.010	< LOQ	n.d.
40.9	1/1.15	0.2	n.d.	n.d.	< LOQ
	24	0.6	0.006	n.d.	0.017
	48/72	0.7	0.040	0.010	< LOQ
	-- **	1.8	0.006	< LOQ	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)
** Honeybees were asphyxiated by CO_2 at study termination.

Observations: Oral doses of 1.5 ng/bee or less had no observable adverse effects on honeybees and no residues of imidacloprid or the olefin- and hydroxy-metabolite could be detected in those bees. All other doses caused adverse effects and residues of imidacloprid or the olefin- and hydroxymetabolite could be detected in the respective honeybee samples. In most cases, the highest residue level was found for the hydroxy-metabolite which may be a suitable indicator for a significant exposure of honeybees to imidacloprid.

2.0 INTRODUCTION

The objective of this study was to consider whether or not imidacloprid-related bee incidents could be identified by a characteristic residue pattern in honeybees which had been exposed to harmful imidacloprid doses.

Honeybees were orally dosed with imidacloprid techn. in an acute toxicity test according to EPPO guideline no. 170 performed by a contract research facility (IBACON, Roßdorf). The honeybees from this test were asphyxiated by CO_2 at test termination at the latest and retained at –20°C till residue analysis. The residue findings on these honeybees are reported here.

3.0 EXPERIMENTAL

3.1 Test Substance

Test substance:	imidacloprid techn.
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:	insecticidal seed dressing
Formulation/batch number:	M00680
Date of certificate:	March 30, 1998
Purity (acc. to analysis):	99.4%
Analytical method:	HPLC
Date of analysis:	March 13, 1998
Expiry date:	March 2000
Physical appearance:	colourless crystals
Specific density:	not applicable
Storage conditions:	room temperature
Doses applied in the LD50 study:	0.1, 0.8, 1.5, 3.1, 6, 12.2, 22.9 and 40.9 ng/honeybee.
Safety precaution:	Routine hygienic precautions

3.2 Reference Substance

For this type of study, a reference compound is not applicable.

3.3 Execution of the Test

The acute LD50 study was performed between 6 July and 10 July 1999 by the contract research institute IBACON GmbH, Roßdorf, Germany (IBACON report no. 6400036). The honeybees from this study were shipped to the Bayer AG on 20. August 1999.

Sponsor:	BAYER AG GB Plant Protetion Marketing - Seed Treatment D-40789 Monheim
Study Director:	[redacted]
Responsible Analyst:	
Study Technicians:	
Quality Assurance:	
Laboratory Study Number:	SXR/Am 013

3.4 *Origin of Honeybees*

Honeybees (*Apis mellifera mellifera* L.) of different age were sampled from the flight board of disease-free and queen-right bee hives managed by IBACON GmbH, Roßdorf, Germany.

3.5 *Test Cages and Labelling Procedure*

The honeybees were caged in groups of 10 individuals each in stainless steel chambers (10 x 8.5cm and 5.5 cm high) with the front side consisting of a removable glass plate. The test chambers were perforated with 98 ventilation holes (d = 1 mm) and were lined with filter paper. For each dosage 3 replicates with 10 honeybees each were prepared. The test chambers were uniquely identified with the study number, application date, treatment, test concentration and the replicate number.

3.6 *Food Provided to the Honeybees During the Test*

After oral dosing, honeybees received a commercial ready-to-use syrup (Apiinvert: 30% saccharose, 31% glucose, 39% fructose) *ad libitum* till test termination.

3.7 *Study Procedure*

After collecting honeybees were allocated impartially to the test chambers of the different dose groups and starved for 70 minutes. Some 20 mg of a commercial ready-to-use syrup per honeybee was mixed with 8 different amounts of imidacloprid and offered in syringes which were weight before and after the dosing phase (dosing phase was restricted to 3 hours). During the dosing and the subsequent 96 hr observation phase honeybees were maintained in incubators at 29°C and 50-70% RH in darkness (except during evaluations).

3.7 *Evaluation*

Two parameters were assessed during the study: mortality and behavioural abnormalities such as vomiting, apathy, intensive cleaning, exaggerated and/or discoordinated movements. The number of dead and behaviorally impacted bees were recorded on the exposure day 1, 2 and 4 hr after honeybees had ingested the syrup. Further evaluations were made 24, 48, 72 and 96 hr later. After the final evaluation, all survivors were asphyxiated by CO_2 and stored at –20°C. All bees which died before study termination were removed from the cages and also stored in a freezer.

3.8 *Sample Processing and Residue Analysis*

Honeybee samples were shipped to the Agricultural Centre of the Bayer AG in Monheim on 20 August 1999. During shipment they were retained on dry ice at –20°C. After arrival, they were transferred into a freezer and retained at –20°C till residue analysis. Sample processing and analytical methods are described in detail in appendix I.

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 Biological Observations on Foraging Honeybees

Oral doses up to 1.5 ng/bee had no observable adverse effect on the honeybees (table 1). At doses between 3.1 and 12.2 ng/bee the number of affected bees and effect duration increased in a dose-dependent manner. Higher doses (22.9 and 40.9 ng/bee) did not further increase the number of affected bees. This may be attributed by an impact on the trophallactic behavior (= social feeding) of the honeybees.

5.2 Analytical Findings

No residues of imidacloprid or the olefin- and hydroxy-metabolite could be detected in honeybees fed with imidacloprid doses which had no observable effects on the bees. At all doses which caused effects residues above the limit of detection were found for either the parent compound or the olefin- and hydroxy-metabolite. The residue levels found corresponded well with the applied imidacloprid doses. From the time to appearance of the respective metabolite it appears that imidacloprid is readily degraded in honeybees. In most cases, th highest residue level was found for the hydroxy-metabolite which may be a suitable indicator for a significant exposure of honeybees to imidacloprid.

TABLES

Table 1: Time to Death and Symptoms Observed During the LD50 Study.
These data originate from an oral LD50 study (IBACON 6400036).

Dose Applied	Time to Death [time to death in h / no. of dead bess]	Symptoms Observed [type of symptoms / no of affected bees]	
Control	2 h / 1	apathy (2-4 h)	1
	24 h / 1		
	48 h / 1		
0.1	-- / 0	--	--
0.8	-- / 0	--	--
1.5	4 h / 1	--	--
3.1	4 h / 3	apathy (2-4h)	4
	24 h / 7	exaggerated (4 h)	4
		discoordination(2-4 h)	2
6.0	4 h / 2	apathy (2-4h)	12
	24 h / 8	discoordination(2-4 h)	9
12.2	1 h / 1	apathy (2-24h)	29
	24 h / 6	exaggerated (4 h)	19
	48 h / 2	discoordination(2-4 h)	19
22.9	1 h / 1	apathy (1-24h)	28
	24 h / 3	exaggerated (1-4 h)	13
	48 h / 1	discoordination(1-24 h)	14
40.9	1 h / 2		
	24 h / 6	apathy (1-24h)	28
	48 h / 7	exaggerated (1 -48h)	8
	72 h / 1	discoordination(1-24 h)	18

Table 2: Residue Levels of Imidacloprid and Toxicologically Relevant Metabolites in Honeybees Orally Dosed with Imidacloprid.
The honeybee samples originate from an acute LD50 study (IBACON 6400036).

Dose Applied [ng/bee]	Time to Death [h]	Sample weight [g]	Hydroxy-NTN [mg/kg]	Olefin-NTN [mg/kg]	Imidacloprid [mg/kg]
0.1	96	3.8	n.d.	n.d.	n.d.
0.8	96	4.1	n.d.	n.d.	n.d.
1.5	96	3.7	n.d.	n.d.	n.d.
3.1	4	0.3	n.d.	n.d.	< LOQ
	24	0.6	< LOQ	n.d.	n.d.
	96	2.7	< LOQ	n.d.	n.d.
6.0	24	0.7	< LOQ	n.d.	n.d.
	96	2.7	< LOQ	n.d.	n.d.
12.2	24	0.5	< LOQ	n.d.	n.d.
	96	2.8	0.006	n.d.	n.d.
22.9	24/48	0.4	0.010	< LOQ	< LOQ
	96	3.3	0.010	< LOQ	n.d.
40.9	1/1.15	0.2	n.d.	n.d.	< LOQ
	24	0.6	0.006	n.d.	0.017
	48/72	0.7	0.040	0.010	< LOQ
	96	1.8	0.006	< LOQ	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 Bee Samples.

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

D-51368 Leverkusen

September 29, 1999
Report No.: MR-575/99

STUDY TITLE

Residue Levels of Imidacloprid and Imidacloprid Metabolites in Honeybees Orally Dosed with Imidacloprid in Standardized Toxicity Tests (EPPO 170)

Author

[redacted]

Testing Facility

Bayer AG
PF-E/MR, Building 6610
51368 Leverkusen, Germany

Study Completion Date

September 29, 1999

Study Number

E 370 1725 - 5

TABLE OF CONTENT

1 INTRODUCTION

Honeybee samples from an acute LD50 study (IBACON 6400036) 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:	September 15, 1999
Start of Experimental Phase:	September 15, 1999
End of Experimental Phase:	September 17, 1999
Completion of Report:	September 29, 1999

3 RESULTS FOR BEE MATERIAL SAMPLES :

3.1 Bee Samples:

Dose Applied [ng/bee]	Time to Death [h]	Sample weight [g]	Hydroxy-NTN [mg/kg]	Olefin-NTN [mg/kg]	Imidacloprid [mg/kg]
0.1	96	3.8	n.d.	n.d.	n.d.
0.8	96	4.1	n.d.	n.d.	n.d.
1.5	96	3.7	n.d.	n.d.	n.d.
3.1	4	0.3	n.d.	n.d.	< LOQ
	24	0.6	< LOQ	n.d.	n.d.
	96	2.7	< LOQ	n.d.	n.d.
6.0	24	0.7	< LOQ	n.d.	n.d.
	96	2.7	< LOQ	n.d.	n.d.
12.2	24	0.5	< LOQ	n.d.	n.d.
	96	2.8	0.006	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.

Dose Applied [ng/bee]	Time to Death [h]	Sample weight [g]	Hydroxy-NTN [mg/kg]	Olefin-NTN [mg/kg]	Imidacloprid [mg/kg]
22.9	24/48	0.4	0.010	< LOQ	< LOQ
	96	3.3	0.010	< LOQ	n.d.
40.9	1/1.15	0.2	n.d.	n.d.	< LOQ
	24	0.6	0.006	n.d.	0.017
	48/72	0.7	0.040	0.010	< LOQ
	96	1.8	0.006	< LOQ	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.

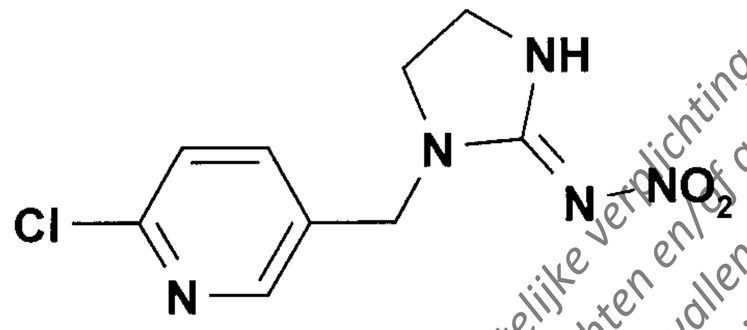
Limit of detection: 0.0015 mg/kg for Imidacloprid and Hydroxy-Metabolite, 0.003 mg/kg for the Olefin-Metabolite; n.d.: Residues below the limit of detection.

4 EXPERIMENTAL

4.1 Reference Substances

Imidacloprid

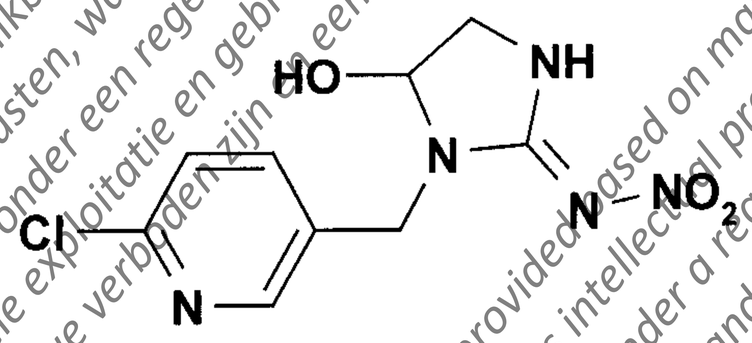
Structural formula:



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)

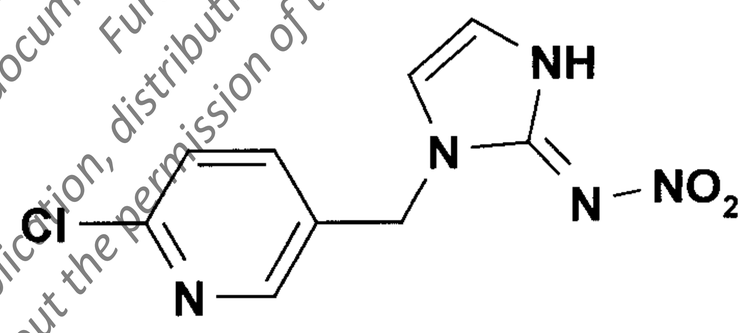
Structural formula:



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 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 one 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 silica gel.

2. The flow rate during loading and elution of the column should not be too high, since otherwise losses may occur leading to recoveries below 70 % and less effective clean-up.

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: Conditions 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 its 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 x 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

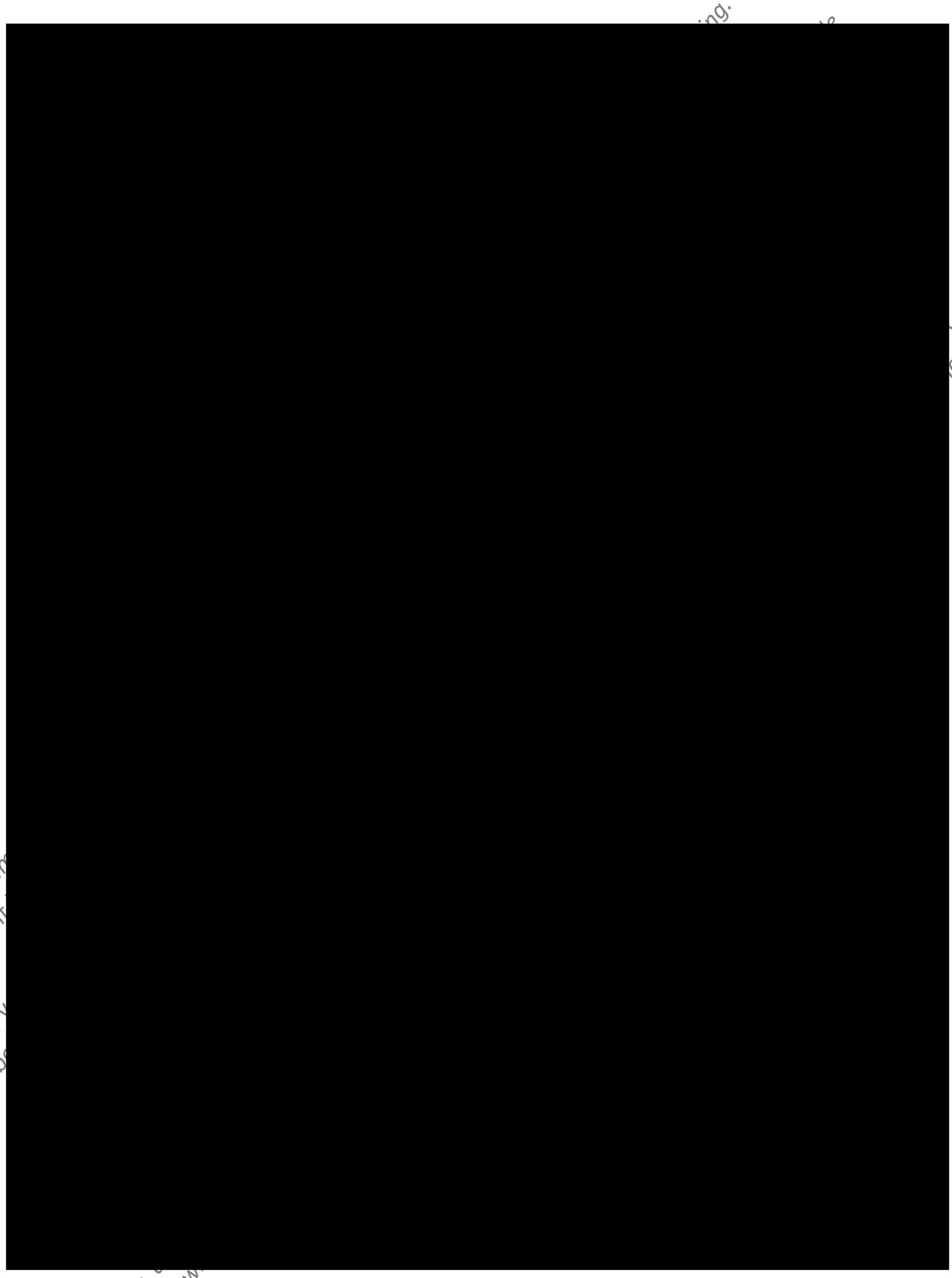
Mass spectrometer operating parameters:

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 II: Copy of the GLP Certificate



Appendix II: cont`d.

[redacted]

Appendix III: Quality Assurance Statement

Referat GLP
Quality Assurance Statement

[redacted]