STUDY TITLE

Determination of Residues of Imidacloprid and Relevant Metabolites in Nectar, Pollen and Honey of Winter Rape

Amended by Amendment No. 1 from 2002-03-22

Author

[redacted]

Testing Facility

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Study Completion Date

2001-04-02

Study Number

E 370 1887-4

MR-147/01 / MO-02-004997

CERTIFICATION OF AUTHENTICITY

[redacted]

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2001-03-30
Date

Study Director

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STATEMENT OF COMPLIANCE

This study was conducted in compliance with the Principles of Good Laboratory Practice (Chemikaliengesetz, dated 25th July 1994, current version of Anhang 1 and the current OECD Principles of Good Laboratory Practice (GLP)).

The Test facility has been inspected and certified as working in compliance with the Principles of Good Laboratory Practice by the competent authorities (Aktenzeichen IV C 4-31.11.62.03, 4th March 1999).

Signature:

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Author and
Responsible Analyst

2001-03-30
Date

Study Director

2001-04-02
Date

Referat GLP
Quality Assurance Statement

[redacted]

TABLE OF CONTENT

1 INTRODUCTION

Rape flowers, pollen, nectar and honey samples obtained from a German trial station were analysed for residues of Imidacloprid and its olefin- and Hydroxy metabolites. The results are summarized 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 analytical study protocol:	2000-07-13
Start of analytical phase:	2000-08-01
End of analytical phase:	2000-08-09

3 SAMPLE LIST

3.1 Untreated Plot Samples

The following samples were collected from the honeycombs of the untreated plot:

Sample Material	Sample Origin	Date of Sampling
Pollen (from the Comb)	4 (Colony 114)	2000-05-02
Nectar (from the Comb)	5 (Colony 114)	2000-05-02
Nectar (from the Comb)	7 (Colony 86)	2000-05-02
Nectar (from the Comb)	9 (Colony 72)	2000-05-02
Pollen (from the Comb)	19 (Colony 114)	2000-05-08
Pollen (from the Comb)	20 (Colony 86)	2000-05-08
Pollen (from the Comb)	21 (Colony 72)	2000-05-08
Nectar (from the Comb)	22 (Colony 114)	2000-05-08
Nectar (from the Comb)	23 (Colony 86)	2000-05-08
Nectar (from the Comb)	24 (Colony 72)	2000-05-08
Pollen (from the Comb)	28 (Colony 114)	2000-05-15
Pollen (from the Comb)	30 (Colony 86)	2000-05-15
Pollen (from the Comb)	32 (Colony 72)	2000-05-15
Nectar (from the Comb)	29 (Colony 114)	2000-05-15
Nectar (from the Comb)	31 (Colony 86)	2000-05-15
Nectar (from the Comb)	33 (Colony 72)	2000-05-15
Honey (from the Comb)	34 (Colony 15,90,76)	2000-05-22

3.2 Treated Plot Samples (Poncho 80 & 420 FS)

The following samples were collected from the honeycombs of the treated plot:

Sample Material	Sample Origin	Date of Sampling
Nectar (from the Comb)	6 (Colony 126)	2000-05-02
Nectar (from the Comb)	8 (Colony 69)	2000-05-02
Nectar (from the Comb)	10 (Colony 108)	2000-05-02
Pollen (from the Comb)	19 (Colony 126)	2000-05-08
Pollen (from the Comb)	20 (Colony 69)	2000-05-08
Pollen (from the Comb)	21 (Colony 108)	2000-05-08
Nectar (from the Comb)	22 (Colony 126)	2000-05-08
Nectar (from the Comb)	23 (Colony 69)	2000-05-08
Nectar (from the Comb)	24 (Colony 108)	2000-05-08
Pollen (from the Comb)	28 (Colony 126)	2000-05-15
Pollen (from the Comb)	30 (Colony 69)	2000-05-15
Pollen (from the Comb)	32 (Colony 108)	2000-05-15
Nectar (from the Comb)	29 (Colony 126)	2000-05-15
Nectar (from the Comb)	31 (Colony 69)	2000-05-15
Nectar (from the Comb)	33 (Colony 108)	2000-05-15
Honey (from the Comb)	34 (Colony 19,124,35)	2000-05-22

3.3 Samples from the Honeycombs and Flowers from the Untreated and Treated Plot

The following samples were collected from the honeycombs and from the flowers of the untreated and treated plot:

Treatement	Sample Material and Origin	Date of Sampling
Untreated		
	Nectar (from the Flowers)	2000-05-04
	Nectar (from the Flowers)	2000-05-05
	Pollen, Swarm 72 (from the Comb)	2000-05-05
	Pollen, Swarm 86 (from the Comb)	2000-05-05
	Pollen, Swarm 114 (from the Comb)	2000-05-05
	Pollen, Swarm 86 (from the Comb)	2000-05-02
	Pollen, Swarm 72 (from the Comb)	2000-05-02
Treated		
	Nectar (from the Flowers)	2000-05-04
	Pollen, Swarm 69 (from the Comb)	2000-05-05
	Pollen, Swarm 126 (from the Comb)	2000-05-05
	Pollen, Swarm 108 (from the Comb)	2000-05-05
	Pollen, Swarm 69 (from the Comb)	2000-05-02
	Pollen, Swarm 108 (from the Comb)	2000-05-02
	Pollen, Swarm 126 (from the Comb)	2000-05-02

3.4 Pooled Samples (Untreated)

The following samples from the honeycombs of the untreated plot were pooled before the analytical determination:

Sample Material	Sample Origin	Date of Sampling	Sample Number
Nectar (from the Comb)	5 (Colony 114)	2000-05-02	7
	7 (Colony 86)	2000-05-02	
	9 (Colony 72)	2000-05-02	
Pollen (from the Comb)	19 (Colony 114)	2000-05-08	8
	20 (Colony 86)	2000-05-08	
	21 (Colony 72)	2000-05-08	
Nectar (from the Comb)	22 (Colony 114)	2000-05-08	9
	23 (Colony 86)	2000-05-08	
	24 (Colony 72)	2000-05-08	
Pollen (from the Comb)	28 (Colony 114)	2000-05-15	10
	30 (Colony 86)	2000-05-15	
	32 (Colony 72)	2000-05-15	
Nectar (from the Comb)	29 (Colony 114)	2000-05-15	11
	31 (Colony 86)	2000-05-15	
	33 (Colony 72)	2000-05-15	
Honey (from the Comb)	34 (Colony 15,90,76)	2000-05-22	12

3.5 Pooled Samples (Poncho 80 & 420 FS)

The following samples from the honeycombs of the treated plot were pooled before the analytical determination:

Sample Material	Sample Origin	Date of Sampling	Sample Number
Nectar (from the Comb)	6 (Colony 126)	2000-05-02	1
	8 (Colony 69)	2000-05-02	
	10 (Colony 108)	2000-05-02	
Pollen (from the Comb)	19 (Colony 126)	2000-05-08	2
	20 (Colony 69)	2000-05-08	
	21 (Colony 108)	2000-05-08	
Nectar (from the Comb)	22 (Colony 126)	2000-05-08	3
	23 (Colony 69)	2000-05-08	
	24 (Colony 108)	2000-05-08	
Pollen (from the Comb)	28 (Colony 126)	2000-05-15	4
	30 (Colony 69)	2000-05-15	
	32 (Colony 108)	2000-05-15	
Nectar (from the Comb)	29 (Colony 126)	2000-05-15	5
	31 (Colony 69)	2000-05-15	
	33 (Colony 108)	2000-05-15	
Honey (from the Comb)	34 (Colony 19,124,35)	2000-05-22	6

3.6 Pooled Samples (Untreated and Treated Samples)

The following samples from the untreated and treated plot were pooled before the analytical determination:

Treatement	Sample Material	Date of Sampling	Sample Number
Untreated			
	Nectar (from the Flowers)	2000-05-04	17
	Nectar (from the Flowers)	2000-05-05	
	Pollen, Swarm 72 (from the Comb)	2000-05-05	13
	Pollen, Swarm 86 (from the Comb)	2000-05-05	
	Pollen, Swarm 114 (from the Comb)	2000-05-05	
	Pollen, Swarm 86 (from the Comb)	2000-05-02	14
	Pollen, Swarm 72 (from the Comb)	2000-05-02	
Treated			
	Nectar (from the Flowers)	2000-05-04	18
	Pollen, Swarm 69 (from the Comb)	2000-05-05	15
	Pollen, Swarm 126 (from the Comb)	2000-05-05	
	Pollen, Swarm 108 (from the Comb)	2000-05-05	
	Pollen, Swarm 69 (from the Comb)	2000-05-02	16
	Pollen, Swarm 108 (from the Comb)	2000-05-02	
	Pollen, Swarm 126 (from the Comb)	2000-05-02	

3.7 Flower Samples

The following flower samples were analysed:

Sample Material	Sample	Date of Sampling	Sample Number
Flowers	F2233	2000-05-11	1
Flowers	F2234	2000-05-11	2
Flowers	F2235	2000-05-11	3
Flowers	F2236	2000-05-11	4

4 RESULTS

4.1 Results from the Pooled Nectar, Pollen, and Honey Samples from the Untreated and Treated Study Plot

	Sample Material	Number of Pooled Samples	Hydroxy-Imidacloprid [mg/kg]	Olefin-Imidacloprid [mg/kg]	Imidacloprid [mg/kg]
Untreated	Nectar (from the Comb)	7	n.d.	n.d.	n.d.
	Pollen (from the Comb)	8	n.d.	n.d.	n.d.
	Nectar (from the Comb)	9	n.d.	n.d.	n.d.
	Pollen (from the Comb)	10	n.d.	n.d.	n.d.
	Nectar (from the Comb)	11	n.d.	n.d.	n.d.
	Honey (from the Comb)	12	n.d.	n.d.	n.d.
Treated	Nectar (from the Comb)	1	n.d.	n.d.	< LOQ
	Pollen (from the Comb)	2	n.d.	n.d.	n.d.
	Nectar (from the Comb)	3	n.d.	n.d.	< LOQ
	Pollen (from the Comb)	4	n.d.	n.d.	n.d.
	Nectar (from the Comb)	5	n.d.	n.d.	n.d.
	Honey (from the Comb)	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 (< LOQ)
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

4.2 Results from Single Analysis of Nectar Samples from the Treated Study Plot

In two of the analysed pooled samples from the treatment plot, residues below the LOQ were detected. To confirm the residue analytical results the single samples were analysed again.

	Sample Material	**Number of Pooled Samples**	**Hydroxy-Imidacloprid [mg/kg]**	**Olefin-Imidacloprid [mg/kg]**	**Imidacloprid [mg/kg]**
Treated	Nectar (from the Comb)	1/6	n.d.	n.d.	n.d.
	Nectar (from the Comb)	1/8	n.d.	n.d.	< LOQ
	Nectar (from the Comb)	1/10	n.d.	n.d.	< LOQ
	Nectar (from the Comb)	3/22	n.d.	n.d.	< LOQ
	Nectar (from the Comb)	3/23	n.d.	n.d.	< LOQ
	Nectar (from the Comb)	3/24	n.d.	n.d.	< LOQ

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 (< LOQ)
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

4.3 Results from Nectar and Pollen Samples of the Untreated and Treated Study Plot

	Sample Material	**Number of Pooled Samples**	**Hydroxy-Imidacloprid [mg/kg]**	**Olefin-Imidacloprid [mg/kg]**	**Imidacloprid [mg/kg]**
Control	Nectar (from the Flowers)	17	n.d.	n.d.	n.d.
	Pollen (from the Comb)	13	n.d.	n.d.	n.d.
	Pollen (from the Comb)	14	n.d.	n.d.	< LOQ*
Treated	Nectar (from the Flowers)	18	n.d.	n.d.	n.d.
	Pollen (from the Comb)	15	n.d.	n.d.	n.d.
	Pollen (from the Comb)	16	n.d.	n.d.	n.d.

*: There was not enough sample material to repeat the analysis
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 (< LOQ)
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

4.4 Results from Flower Samples:

Sample Number	Hydroxy-Imidacloprid [mg/kg]	Olefin-Imidacloprid [mg/kg]	Imidacloprid [mg/kg]
1	n.d	n.d	n.d
2	n.d	n.d	n.d
3	n.d	n.d	n.d
4	n.d	n.d	n.d

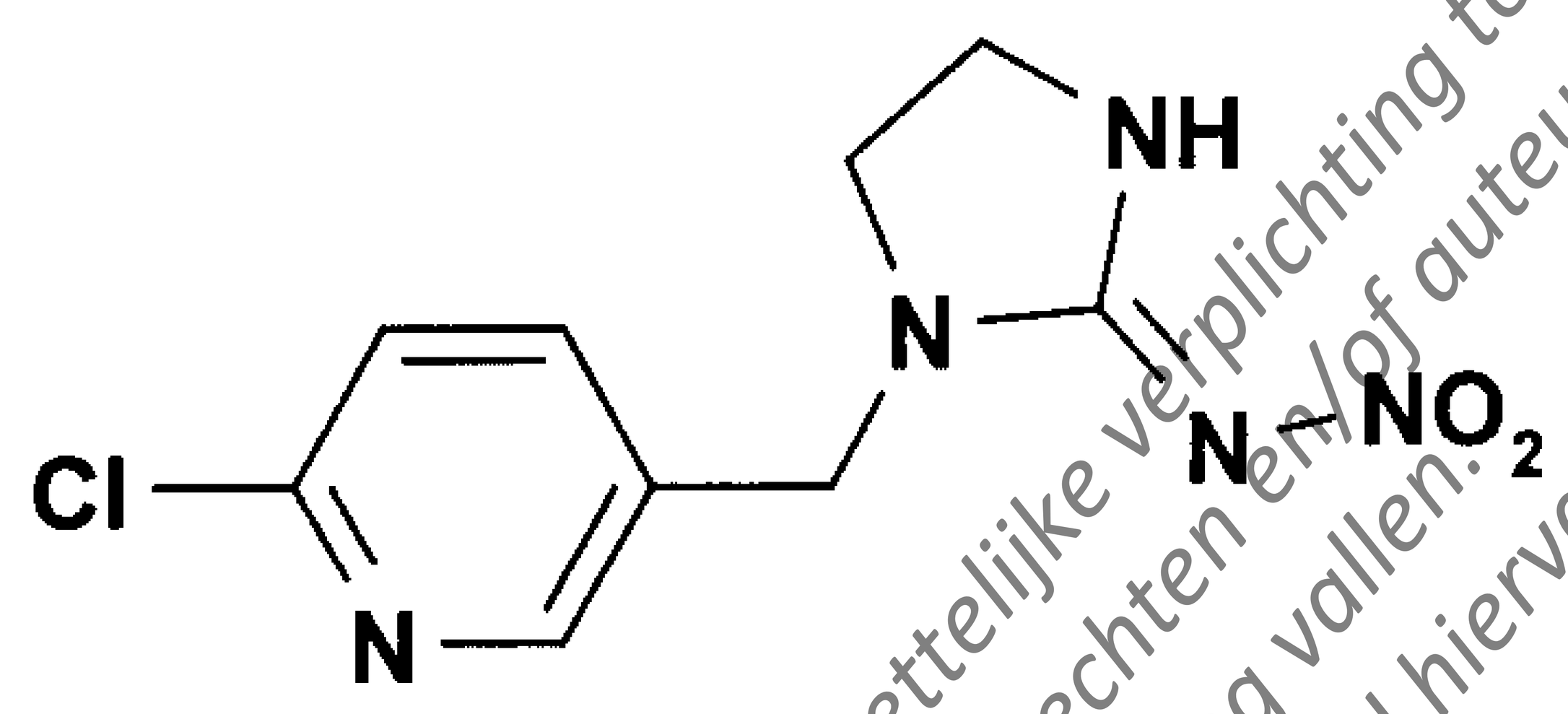
Limit of quantitation: 0.005 mg/kg for Imidacloprid and Hydroxy-metabolite, 0.01 mg/kg for the Olefin-metabolite
< 0.005 and < 0.010 = Residues below the limit of quantitation (< LOQ)
Limit of detection: 0.0015 mg/kg for Imidacloprid and Hydroxy-metabolite, 0.003 mg/kg for the Olefin-metabolite,
n.d.: Residues below the limit of detection

5. EXPERIMENTAL

5.1 Reference Substances

Imidacloprid

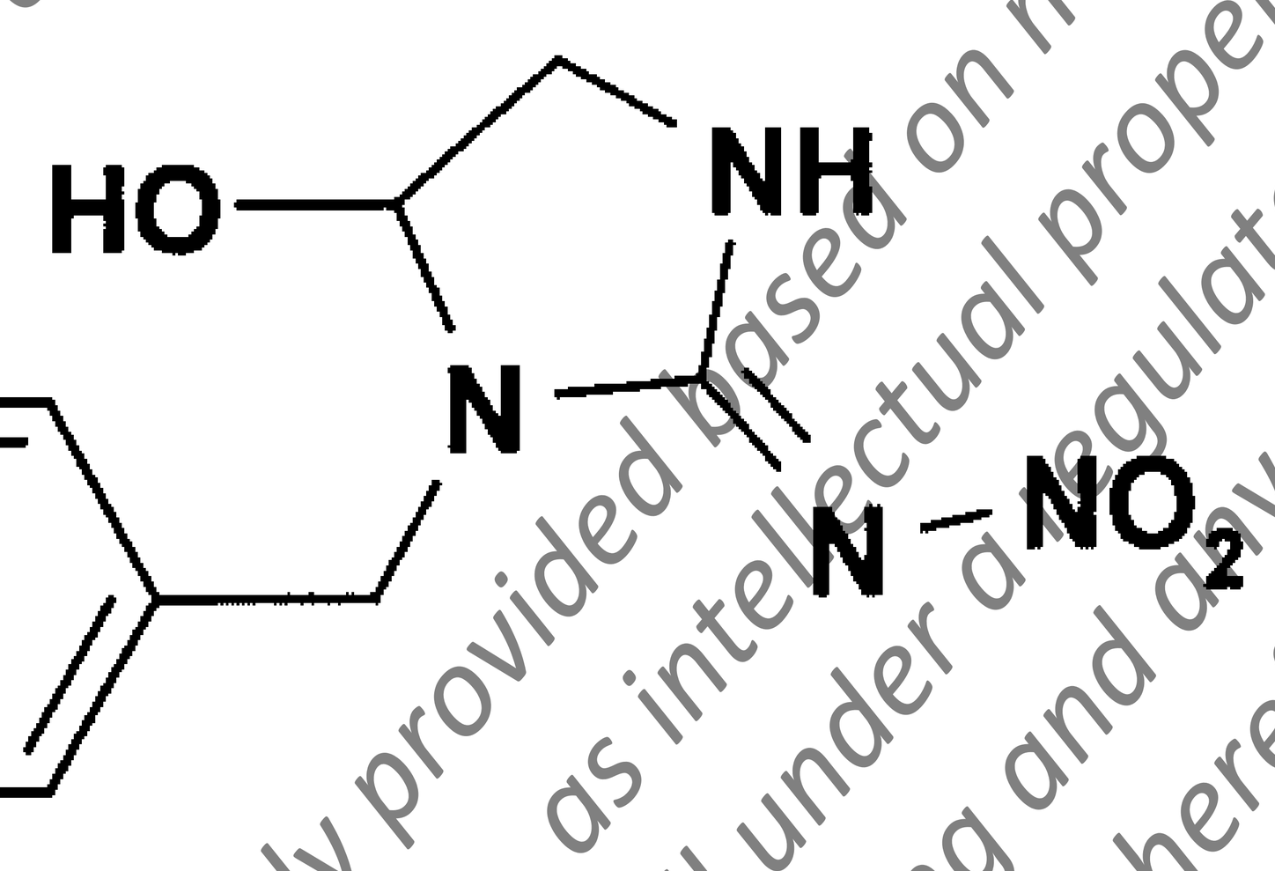
Structural formula:



Empirical formula: $C_9H_{10}ClN_5O_2$
Molecular weight: 255.7 g/mole
Certificate of analysis: M06693, 2000-01-11
Certified assay: 99.8 %
Expiry date: November 2001

Hydroxy-Imidacloprid (WAK 4103)

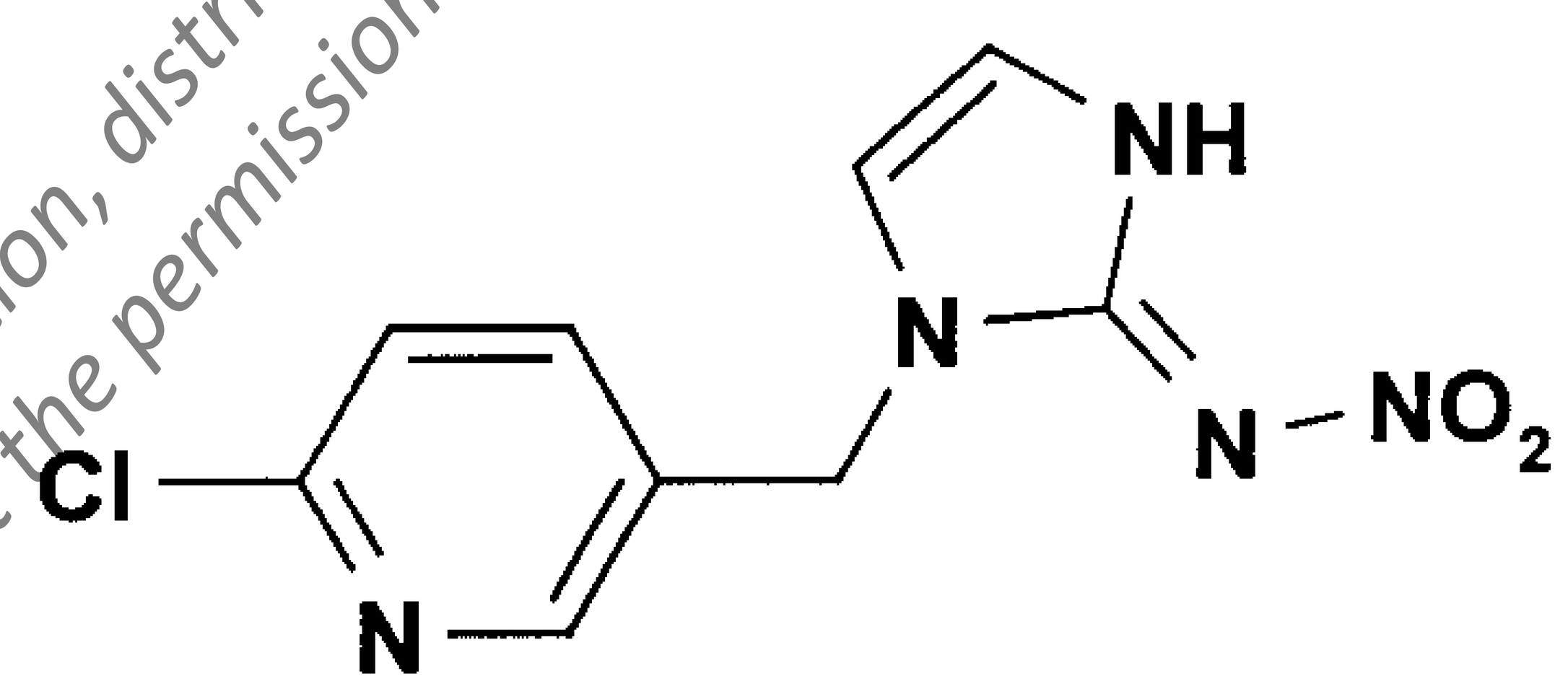
Structural formula:



Empirical formula: $C_9H_{10}ClN_5O_4$
Molecular weight: 271.7 g/mole
Certificate of analysis: 930323ELB03, 2000-05-11
Certified assay: 99.4 %
Expiry date: May 2005

Olefin-Imidacloprid (NTN 35884)

Structural formula:



Empirical formula: $C_9H_8ClN_5O_2$
Molecular weight: 253.6 g/mole
Certificate of analysis: M11453, 2000-07-28
Certified assay: 98.6 %
Expiry date: July 2002

5.2 Residue Analytical Methodology

5.2.1 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.

5.2.2 ChemElut® Column Clean-up

1. Add 5 to 10 mL water to the aqueous solution from 5.2.1 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.

5.2.3 Silica Gel Column Clean-up

1. Dissolve the residues from 5.2.2 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.**

5.3 HPLC-MS/MS Determination of Imidacloprid and Metabolites

5.3.1 Measuring Equipment and HPLC Conditions:

Instrument:	Hewlett Packard 1100
Column:	e.g.: Phenomenex, Luna C18 (2), 5 µm, 15 x 0.46 cm i.D. or Merck, Superspher, RP select-B, 4 µm, 12.5 x 0.4 cm i.D.
Solvent A:	Water/ACN (9/1, v/v) + 0.1 mL Acetic acid/L
Solvent B:	ACN + 0.1 mL Acetic acid/L
Oventemperature:	40 °C
Inject.volume:	50 µL
Flow:	1.0 mL/min
Split:	150 µL into MS from 1000 µL

Time Table	0 min	11.1 % B TO MS
	10 min.	11.1 % B
	10.1 min	90 % B
	11 min	90 % To Waste
	15 min	90 % B
	15.1 min	11.1 % B
	16 min	11.1 % B TO MS
	19 min	11.1 % B

Retention Times:	Olefin-Imidacloprid	approx. 4.8 min
	Hydroxy-Imidacloprid	approx. 5.6 min
	Imidacloprid	approx. 8.5min

5.3.2 MS/MS-Detection

The experiments were performed on a triple-quadrupole mass spectrometer 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, Hydroxy-Metabolite and Olefin-Metabolite (dissolved in acetonitrile / water (2/8, v/v) + 0.1 mL acetic acid per litre) at a flow rate of 5-10 µL/min. Mass axis calibration was done by infusing a polypropylene glycol solution. Unit mass resolution was established and maintained in each mass resolving quadrupole by maintaining a full widht 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, Hydroxy-Metabolite and Olefin-Metabolite were determined. These experiments were performed with nitrogen as collision gas with a collision offset of -19 and -23 eV for Imidacloprid, -23 eV for Hydroxy-Metabolite and –12 and -13 eV for Olefin-Metabolite at an approximate collision gas thickness of 1.56 x 10^{15} atoms/cm^2. Nebulization gas is set at 1.48 L/min, curtain gas is set at 1.44 L/min, CAD gas is set at 0.87 L/min and turbo gas is set at 7 L/min.

Detector:	Triple Quadrupol LC-MS/MS Mass Spectrometer PE Biosystems (Perkin-Elmer Sciex Instruments) API 365, Windows NT 4.0 System	
Interface:	Electrospray, Turbo Ion Spray Potential: + 4400 V Temperature: 400° C (Source)	
Gas:	Nebulization gas:	1.48 L/min (liquid nitogen 5.0)
	Curtain gas:	1.44 L/min (liquid nitogen 5.0)
	Collision gas	0.87 L/min (liquid nitogen 5.0)
	Turbo gas:	7 L/min (liquid nitogen 5.0)
Scan Type:	MRM (Multiple Reaction Monitoring Mode)	
Polarity:	Positive	
Aquisition mode:	Profile	

Mass spectrometer operating parameters

Compound	Precursor Ion Q1 Mass (amu)	Product Ion Q3 Mass (amu)	Dwell Time (msec)	Collision Energy (eV)
Imidacloprid (Cl 37)#	258	211	250	-19
Imidacloprid (Cl 35)	256	209	250	-19
Imidacloprid (Cl 37)#	258	175	250	-23
Imidacloprid (Cl 35)	256	175	250	-23
Hydroxy-Metabolite (Cl 37)#	274	191	250	-23
Hydroxy-Metabolite (Cl 35)	272	191	250	-23
Olefin-Metabolite (Cl 35)#	254	236	250	-12
Olefin-Metabolite (Cl 35)	254	207	250	-13

#= ^{37}Cl isotope of all substances were detected to use as qualifiers

STUDY TITLE

Determination of Residues of Imidacloprid and Relevant Metabolites in Nectar, Pollen and Honey of Winter Rape

Amendment

Amendment No. 1 from 2002-03-22

Author of the Amendment

[redacted]

Testing Facility

Bayer AG
PF-E/OE, Building 6620
51368 Leverkusen, Germany

Study Number

E 370 1887-4

Reason for Amendment

The rape variety used in this study was erroneously described as Summer Rape while it was Winter Rape.

Remark:

The corresponding pages 1 and 4 have been exchanged.

[redacted]

Study Director

2002-03-22
Date

Manager of Test Facility
PF-E/OE) and Representative
of the Sponsor

22.3.02
Date

Quality assurance statement to Amendment 1

Referat GLP
Quality Assurance Statement
[redacted]