TITLE PAGE

Residues of Imidacloprid and Imidacloprid Metabolites in Nectar, Blossoms, Pollen and Honey Bees Sampled from a British Summer Rape Field and Effects of These Residues on Foraging Honeybees

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24 June 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

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INQUIRIES

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1.0 SUMMARY

Report: [redacted]: Residues of Imidacloprid and Imidacloprid Metabolites in Nectar, Blossoms, Pollen and Honey Bees Sampled from a British Summer Rape Field and Effects of These Residues on Foraging Honeybees
Bayer AG, unpublished report No: SXR/Am 003; 1999/06/24.
(Appendix I contains data from study RA-2038/98)

Guidelines: Internal Testing Method
Deviations: not applicable

GLP: yes (certified laboratory)

Material and methods: Poncho FS 500, a.i. content: 78.3 g/L Beta-Cyfluthrin & 428.2 g/l Imidacloprid; specification (formulation No.: 030 based on 06200/0029, developmental No.: 00195939); test product: rape seed dressed with 2.5 l/dt Poncho FS 500; drilling rate: 5 kg/ha. Under field conditions small beehives (appr. 5,000 honeybees) were caged on flowering summer rape plots (60 m ², drilled on 20 March 98) as a sampling device for rape nectar and rape pollen. Nectar was also directly sampled from flowers via micropipettes. In addition, flowers were sampled by hand. The honeybees used as samplers were observed for signs of behavioral impacts. All samples including the honeybees were subjected to a residue analysis for imidacloprid and its relevant metabolites.

Dates of biological work: June 22 - 24, 1998 (soil analysis: September 25 – 29, 1998).
Dates of analytical work: June 30 – July 28, 1998.

Findings: Residues in rape plant matrices and in the foraging honeybees

Type of Sample	Residue Level [mg/kg] *		
	Imidacloprid	Olefin-NTN	Hydroxy-NTN
Control Samples			
Honeybees before exposure	< 0.01	< 0.01	< 0.01
Honeybees after exposure	< 0.01	< 0.01	< 0.01
Rape nectar sampled by bees	< 0.01	< 0.01	< 0.01
Rape nectar sampled with micro-capillaries from the flowers	< 0.01	< 0.01	< 0.01
Rape blossoms	< 0.01	< 0.01	< 0.01
Rape pollen sampled by bees	< 0.01	< 0.01	< 0.01
Treatment Samples			
Honeybees before exposure	< 0.01	< 0.01	< 0.01
Honeybees after exposure	< 0.01	< 0.01	< 0.01
Rape nectar sampled by bees	< 0.01	< 0.01	< 0.01
Rape nectar sampled with micro-capillaries from the flowers	< 0.01	< 0.01	< 0.01
Rape blossoms	< 0.01	< 0.01	< 0.01
Rape pollen sampled by bees	< 0.01	< 0.01	< 0.01

* Limit of quantitation: 0.01 mg/kg.

Observations: No behavioral impacts (e.g. apathy, exaggerated motility, discoordinated movements) or suspicious mortality was observed on the honeybees used for collecting rape nectar and rape 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 rape 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

Test Substance:	Poncho FS 500
Active Ingredient(s):	(a) Beta-Cyfluthrin (FCR 4545) (b) Imidacloprid (NTN 33893)
Chemical Name(s) of AI(s):	(a) CYCLOPROPANECARBOLXYLIC ACID,= 3-(2,2-DICHLOROETHENYL)-2,2-= DIMETHYL-,CYANO(4-FLUORO-3-= PHENOXYPHENYL)METHYL ESTER (b) 1-<(6-CHLORO-3-PYRIDINYL)=METHYL>-= N-NITRO-2-=IMIDAZOLIDINIMINE
CAS Number of AI(s):	(a) 68359 - 37 - 5 (b) 138261 -41 - 3
Indikation:	Seed dressing
Product Number	0195939
Formulation/Batch Number:	FL 0030 based on form. no. 06200/0029
No. of Certificate:	FAR-No. 446-01
AI Content (acc. to Analysis):	(a) 78.3 g/l (b) 428.2 g/l
Analytical Method:	(a) GLC, int. Std. (b) HPLC, ext. Std.
Date of Analysis:	February 4, 1998
Expiry Date:	February 4, 1999
Physical Appearance:	dark blue suspension
Specific Density:	1.151 g/ml
Storage Conditions:	Room temperature
Seed Dressing Rate(s) Tested in the Study:	2.5 l Poncho 500 FS per 100 kg oilseed rape (= 1050 g/dt Imidacloprid & 200 g/dt beta-Cyfluthrin) (analytical findings*: 1019 g/dt Imidacloprid).
Seed Drilling Rate Tested in the Study:	5 kg seed per ha (30 g per 60 m²plot) (seed variety: „Lisonne“; summer rape)
Safety Precaution:	Routine hygienic precautions

3.2 Reference Substance

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

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* Dressed seeds were analysed for imidacloprid only (P 67294708, MR-311/99).

3.3 *Execution of the Test*

The sampled study plots were drilled on March 20, 1998 (reserve plots drilled on April 6, 1998 but these plots were not used in this study). Sampling of nectar, pollen, flowers and honeybees and the behavioral observations were performed between June 23 and 24, 1998.

Sponsor:	BAYER AG GB Plant Protection Marketing - Seed Treatment (Dr. Krohn/Altmann) D-40789 Monheim
Study Director:	[redacted]
Cultivar Manager:	
Trials Officer:	
Responsible Analyst:	
Study Technicians:	
Quality Assurance:	
Laboratory Study Number:	SXR/Am 003

3.4 *Origin of Honeybees*

The honeybees used for pollen and nectar sampling were supplied by a commercial British beekeeper [redacted]
The nucleus beehives (appr. 5,000 bees each) used for the test were transported to the study site in the evening of June 22 and returned to the original place on June 25, 1998.

3.5 *Procedure of Seed Dressing and Dispatch of Test Product*

Rape seeds (variety: „Lisonne"; TGW: 2.8 g) were coated in a Centauer coating machine. Some 562.5 ml Poncho FS 500 were added to 22.5 kg rape seed together with 450 g Talcum blue (Product no. 868426) and mixed over 45 seconds at 300 RPM.

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

The trial site was located in the vicinity of the Bayer UK experimental Elm Farm. Before summer rape, the field was grown with grass in 1997.

There were two rows with three plots of rape plants each separated by a 0.5 m buffer strip. Plots in each row were separated by 1 m buffer strips. Each plot had a size of 4x15 m with a distance of 20 cm between plant rows. The left plot in each row was planted with rape seeds treated with a developmental compound. The right plot in each row was drilled with the control seed. The test substance was drilled in the middle plot of each row. Sampling was done only in the lower row (upper row served only as a reserve plot). Plot management is reported in detail in study RA-2038/98).

3.7 *Drilling of the Rape Seed and Calibration of the Seed Machinery*

The control plot was drilled with 5 kg/ha (= 30 g per 60 m²plot) untreated rape seed whereas the treatment plot received 5 kg/ha rape seed coated with 2.5 l/ha Pocho FS 500.

Prior to sowing the proper functioning of the equipment was tested. The equipment was adjusted according to the preconditions (e.g. seed density).

3.8 *Cultivation of the Plots*

Treated and untreated plots were cultivated in the same way according to the practice of the region. Before initiation of sampling, no protection treatments other than the seed treatment was necessary.

3.9 *Sampling Procedure*

Installment of bee hives

At the time of full rape blossom, tents of 4x4 m and 2 m height were installed on the control and the treatment plot (see Fig. 1). The tents consisted of an aluminium frame covered by gauze material (2x2 mm mesh size). For handling purposes, a walkway was created by removing all plants along a 50 cm transect between the tent entrance and the opposite end. One bee hive was placed at the end of the walkway opposite to the entrance in the treatment plot. The day after installment, hive entrances were disclosed and honeybees were allowed to forage on the study plot within the tent area. Before placing the beehives on the plots, appr. 100-200 honeybees were sampled to get blank samples of honeybees and honeybulbs for the residue work. The sampled honeybees were processed as described in the subsequent chapter.

Sampling of Nectar from the Honeybulbs

On the two days day following hive installment a total of about 200 honeybees were sampled with tweezers directly from rape flowers after watching them for feeding over about 10-30 seconds. All sampled bees were killed by freezing (dry ice). Dead bees were stored on dry ice in the field and, at the end of each sampling day at the latest, transferred to a refrigerator (-19 to -20°C). At the end of the study the samples were shipped to Monheim on dry ice and further retained at - 20°C until preparation of the honeybulbs. Honeybulbs were sampled by fixing the frozen honeybees with tweezers and drawing off the abdomen from the body. The honeybulb was deliberated from attached fluid and tissues and then dissected from the oesophagus. All honeybulbs from one treatment group were pooled witin an Eppendorf cap which was stored on dry ice. After all honeybees of a respective treatment were prepared (at the end of each preparation day at the latest), sampled honeybulbs were stored in a refrigerator at -20°C until residue analysis (see 3.10).

Sampling of Pollen from the Honeybees

From the prepared bees, pollen pockets were removed and stored in a refrigerator at -20°C until residue analysis (see 3.10).

Sampling of Nectar from the Rape Flowers

The day before sampling, between 10 and 20 flowering rape plants outside the caged area were covered by plastic bags to prevent insects from foraging on that flowers. On the next day, nectar was directly drawn from that flowers by 5 µl micropipettes After sampling, the micropipettes were emptied into a 1.5 ml Eppendorf tube which was stored on dry ice in the field. At the end of each sampling day at the latest, these samples were transferred into a refrigerator (-20°C) where they were retained until residue analysis (see 3.10).

As a check of possible contamination of the sampled material from e.g. soil particles, dust, sampling people had to fill a second Eppendorf tube with uncontaminated tap water during sampling activities. Filling of this checking tube was done in several steps (5-10 pipetting events) from a water storage bottle. The analysis of these check samples

revealed no residues indicating that the sampling people did not transfer inadvertently any residues from outside the flower into the nectar samples (see analytical report, page 26).

Sampling of Rape Flowers

About 20 g of rape flowers were sampled from plants outside the tent area. 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).

Soil Samples

Some 200 g soil were sampled from the study field to characterize the soil at the study site. The soil at the study field can be classified as „sandy loam". The organic carbon content was 1.5 % by weight. The water holding capacity was determined to be 55.3 g H_2O/100 g dry soil. The pH - value (1 KCL) was determined to be 5.4. At the time of flowering, the soil contained 17.4 g water per 100 g dry soil (= 31% of the water holding capacity).

3.10 Sample Processing and Residue Analysis

Sample processing and analytical methods are described in detail in appendix III.

3.11 Climatic Conditions During the Study

During the study, temperature and precipitation events were recorded two times per day. The following records were made:

Date	Precipitation [mm]	Min. temperature [°C]	Max. temperature [°C]	Remarks
22 June	0	14	22	partly cloudy, dry
23 June	2.5	11	22	stormy, transitorily cloudy
24 June	0	15	26	partly cloudy, dry

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:

Flight intensity: Three times per day, over a period of 10 minutes, the number of bees leaving the hive and returning to the hive is recorded.

Foraging intensity: Three times per day the number of bees foraging within a haphazardly assigned area of 1 m² of flowering rape within the tent was recorded during a 3 minute period.

Returning frequency: Three times per day, over a period of 10 minutes, the number of bees arriving at the alighting board and returning to the hive is 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: Any conspicous numbers of dead bees in comparison to the controls during and after the test were 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

Analytical findings are summarized in table 1 and given in detail in the analytical report (appendix I). No residues at or above the limit of quantitation were found in any of the examined matrices for either the parent compound or the relevant metabolites (olefin- and hydroxy-imidacloprid).

5.2 Observations on Foraging Honeybees

No behavioral impacts (e.g. apathy, exaggerated motility, discoordinated movements) or suspicious mortality was observed on the honeybees used for collecting rape nectar and rape pollen. 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 (Table 2).

FIGURES

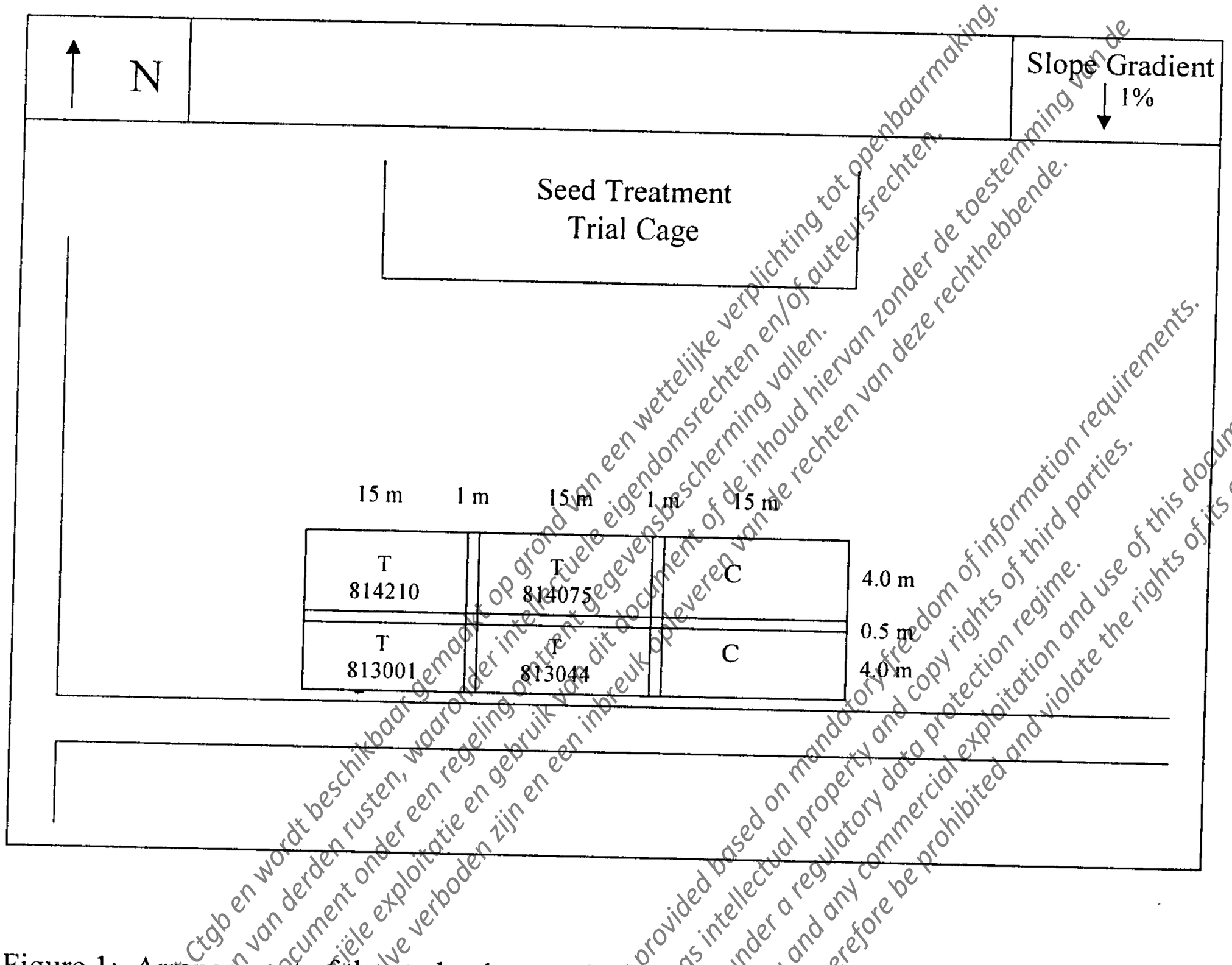


Figure 1: Arrangement of the study plots on the study field (close to Elm Farm, UK).
There were two rows with three plots of rape plants each separated by a 0.5 m buffer strip. Plots in each row were separated by 1 m buffer strips. Each plot had a size of 4x15 m with a distance of 20 cm between plant rows. The left plot in each row was planted with rape seeds treated with a developmental compound. The right plot in each row was drilled with the control seed. The test substance was drilled in the middle plot of each row. Sampling was done only in the lower row (upper row served only as a reserve plot). Plot management is reported in detail in study RA-2038/98).

TABLES

Table 1: Summary of the Analytical Findings.

Type of Sample	Residue Level [mg/kg] *		
	Imidacloprid	Olefin-NTN	Hydroxy-NTN
Control Samples			
Honeybees before exposure	< 0.01	< 0.01	< 0.01
Honeybees after exposure	< 0.01	< 0.01	< 0.01
Rape nectar sampled by bees	< 0.01	< 0.01	< 0.01
Rape nectar sampled with micro-capillaries from the flowers	< 0.01	< 0.01	< 0.01
Rape blossoms	< 0.01	< 0.01	< 0.01
Rape pollen sampled by bees	< 0.01	< 0.01	< 0.01
Treatment Samples			
Honeybees before exposure	< 0.01	< 0.01	< 0.01
Honeybees after exposure	< 0.01	< 0.01	< 0.01
Nectar from honeybulbs of bees before exposure	< 0.01	< 0.01	< 0.01
Rape nectar sampled by bees	< 0.01	< 0.01	< 0.01
Rape nectar sampled with micro-capillaries from the flowers	< 0.01	< 0.01	< 0.01
Rape blossoms	< 0.01	< 0.01	< 0.01
Rape pollen sampled by bees	< 0.01	< 0.01	< 0.01

* Limit of quantitation: 0.01 mg/kg

Table 2: Records on Flight and Foraging Activity of Honeybees During the Sampling Period.

Day after hive installment	No. of bees which left the hive during the 10 min. observation period	No. of bees which returned to the hive during the 10 min. observation period	No. of bees which foraged on the flowering rape during the check
Control Plots			
+ 1, 09:10 – 09:23	11	0	0
+ 1, 12:00 – 12:12	11	7	2
+ 1, 15:10 – 15:23	146	210	22
+ 2, 08:56 – 09:10	58	9	18
+ 2, 11:50 – 12:05	50	22	8
+ 2, 15:35 – 15:43	37	45	7
Treatment Plots			
+ 1, 09:20 – 09:30	6	2	0
+ 1, 12:20 – 12:30	12	1	1
+ 1, 15:30 – 15:50	133	159	17
+ 2, 09:15 – 09:30	88	55	13
+ 2, 12:15 – 12:25	107	83	12
+ 2, 15:10 – 15:25	126	93	10

APPENDICES

APPENDIX I: Analytical Report.

[redacted]

Study No.: E 370 1356-5
(contains data from study no. RA-2038/98)

STUDY TITLE

Analysis of Rape Nectar, Rape Blossoms, and Rape Pollen for Residues of Imidacloprid and Imidacloprid Metabolites and Preliminary Observations of Effects on Domestic Honeybees in Great Britain

Residue Analytical Method for the Determination of Imidacloprid, Hydroxy- and Olefin-Metabolite in Rape Flower, Rape Pollen, Bee and Nectar Samples by HPLC-MS/MS

1) Nectar-Samples:

Extraction and sample clean-up:

1. Place for e.g. 1.0 g of the sample material in a 150-ml beaker.
2. Add 10 ml of water and place the sample for 2 min into a Ultrasonic Bath.
3. Add 20 ml of methanol.
4. Blend the sample using an ultra-turrax blender (or equivalent) for approximately 1 min.
5. 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.
6. Wash the filtered solids with a total of 20 ml of methanol/water (3/1, v/v). Press residual solvent from the solids using rubber damming. Discard the filtered solids.
7. Transfer the filtrate into a 250-ml brown glass round-bottom flask.
8. Concentrate the filtrate to an aqueous remainder of 5 to 10 ml using a rotary evaporator with a max. bath temperature of 50 °C.
9. Add 5 to 10 ml water to the aqueous solution from step 8 to bring the total volume of the extracts to approx. 20 ml.
10. 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.
11. Elute the residues from the column with 140 ml of CH_2Cl_2. Collect the eluate in a 250-ml brown glass round-bottom flask.
12. Evaporate the eluate from step 11 to dryness using a vacuum rotary evaporator and a max. bath temperature of 40 °C.
13. Dissolve the residues in 1.00 ml of acetonitrile/water (2/8, v/v) and determine the residues with HPLC-MS/MS.

2.) Bee-Samples, Rape Flowers, Rape Pollen:

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-bottom 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.

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

8. 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.

9. Elute the residues from the column with 140 ml of CH_2Cl_2. Collect the eluate in a 250-ml brown glass round-bottom flask.

10. Evaporate the eluate from step 9 to dryness using a vacuum rotary evaporator and a max. bath temperature of 40 °C.

11. Dissolve the residues in 2 ml of toluene/ethyl acetate (85/15, v/v).

12. Apply the organic solution from step 11 onto a 0.5 g (3 ml) silica gel (SiOH) column (e.g. Varian).

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

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

15. 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.

16. Evaporate the eluate from step 15 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.

<u>NOTE</u>

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.) HPLC-MS/MS determination of Imidacloprid, Hydroxy and Olefin Metabolite:

A) 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 + 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	20 % B
	10 min.	20 % B
	11 min	90 % B
	15 min	90 % B
	16 min	20 % B
	19 min	20 % B
	Stoptime	19 min

Retention Times:

Olefin-Imidacloprid	approx. 4.5 min
Hydroxy-Imidacloprid	approx. 5.5 min
Imidacloprid	approx. 8.5 min

B) Mass Spectroscopy

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 3000 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 -20 eV for Imidacloprid,

-23 eV for Hydroxy-Metabolite and -13 eV for Olefin-Metabolite at an approximate collision gas thickness of 1.56×10^{15} atoms/cm^2.

Nebulization gas is set at 1.48 l/min, curtain gas is set at 0.95 l/min and turbo gas is set at 6 l/min.

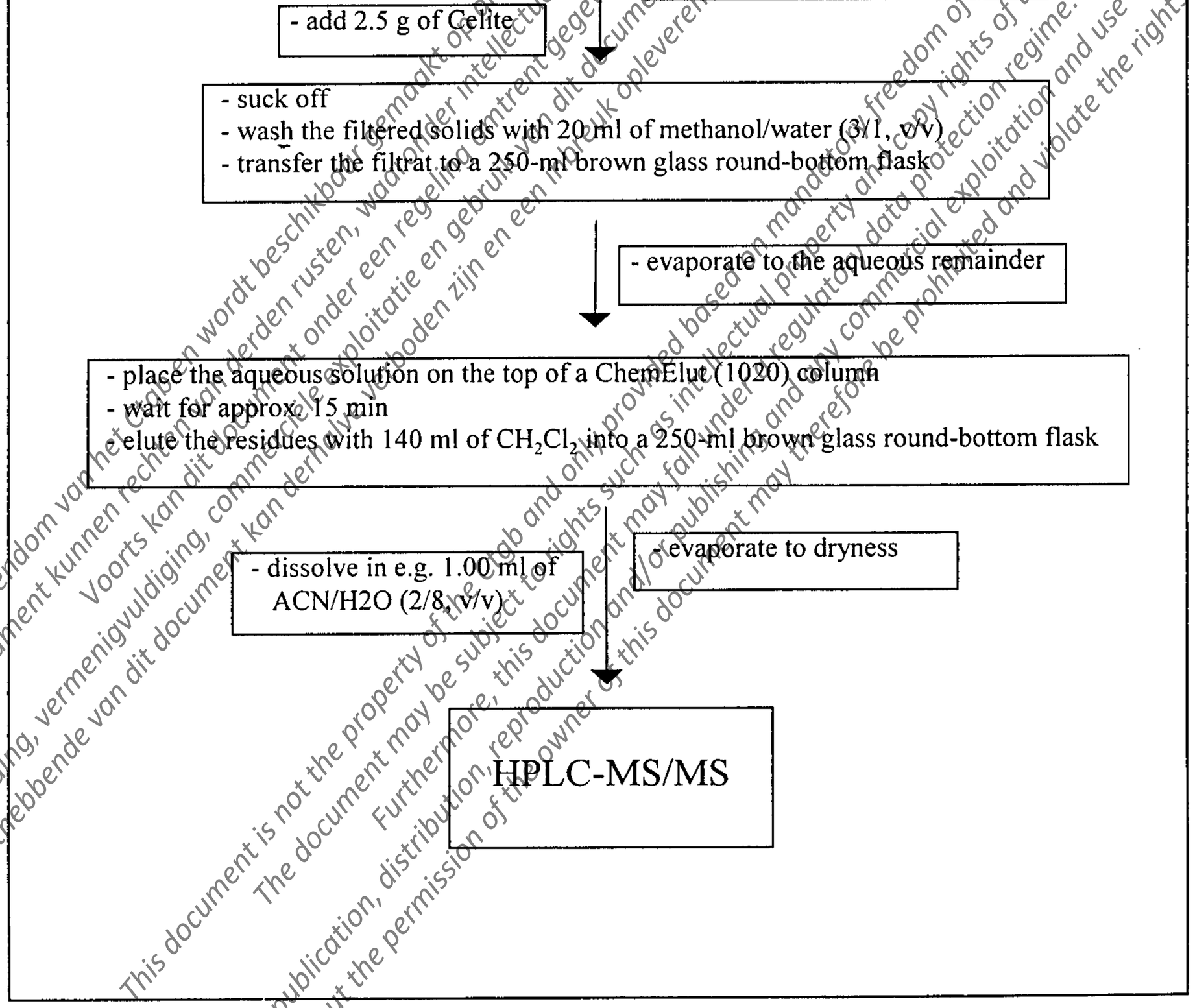
Detector:	e.g. Triple Quadrupol LC/MS/MS Mass Spectrometer, Perkin-Elmer Sciex Instruments API 300, Apple™ Macintosh® System 8.0
Interface:	Electrospray, TurboIon Spray Potential: +4900 V Temperature: 300 °C Nebulizer gas: Nitrogen 5.0 (99.999% purity), 1.48 l/min
Scan type:	MRM (Multiple Reaktion Monitoring 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)#	258.0	210.9	500	-20
Imidacloprid (Cl 35)	256.0	208.9	500	-20
Hydroxy-Metabolite (Cl 37)#	274.0	190.8	250	-23
Hydroxy-Metabolite (Cl 35)	272.0	190.8	250	-23
Olefin-Metabolite (Cl 37)#	256.0	237.8	250	-13
Olefin-Metabolite (Cl 35)	254.0	235.8	250	-13

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

4.) Flow Diagram Method for the Determination of Residues of Imidacloprid and Metabolites in/on Nectar and Honey Samples



5.) Flow Diagram Method for the Determination of Residues of Imidacloprid and Metabolites in/on Rape Pollen, Rape Flower and Bee Samples:

Flow Diagram Method for the Determination of Residues of Imidacloprid, Hydroxy-and Olefin-Metabolite in/on Rape Pollen, Rape Flower and Bee Samples

- place e.g. 2.0 g sample material in a 150-ml beaker
- add approx. 30 ml of methanol/water (3/1, v/v)

- homogenisation with an Ultra Turrax for 1 min

- add 2.5 g Celite

- suck off
- wash the filtered solids with 30 ml of methanol/water (3/1, v/v)
- determine the total volume of the samples
- transfer an aliquot of e.g. 30 ml (1 g sample) to a 250-ml brown glass round-bottom flask

- concentrate to the aqueous remainder

- place the aqueous solution on the tope of a ChemElut (1020) column
- wait for approx. 15 min
- elute the residues with 140 ml of CH_2Cl_2 into a 250-ml brown glass round-bottom flask

- dissolve the residues in 2 ml of toluene/ethyl acetate (85/15, v/v)

- evaporate to dryness

- apply the organic solution onto a 0.5 g (3 ml) Silica gel (SiOH) column
- rinse the round-bottom flask with 10 ml of toluene/ethyl acetate (70/30, v/v)
- apply the solution to the column, too
- elute the residues with 5 ml of acetonitrile into a 25-ml brown glass pear-shaped flask

- evaporate to dryness

- dissolve in e.g. 1.00 ml of ACN/H2O (2/8, v/v)

HPLC-MS/MS

6.) Results of Bee Samples, Nectar Samples of Bees, Rape Flower and Rape Pollen Samples.

A) Bee Samples:

Sample code	Sample description	Sample weight	Residues		
			Hydroxy-NTN [mg/kg]	Olefin-NTN [mg/kg]	Imidacloprid [mg/kg]
Samples taken before exposure					
A Bees Control 06/22/98	Bees from hives (before transport to testfield) 22.06.98 Great Britain	34.4 g	< 0.01	< 0.01	< 0.01
Samples taken during exposure					
B Bees Control 06/23,24/98	Bees from flower 23 + 24.06.98 Great Britain	8.7 g	< 0.01	< 0.01	< 0.01
C Bees Poncho 06/23,24/98	Bees from flower 23 + 24.06.98 Great Britain	6.6 g	< 0.01	< 0.01	< 0.01

B) Nectar Samples:

Sample Name	Sample description	Sample weight	Residues		
			Hydroxy-NTN [mg/kg]	Olefin-NTN [mg/kg]	Imidacloprid [mg/kg]
Nectar harvested from the honeybulb of bees which were collected before exposure					
A Nectar Control	A Elm Farm N Great Britain	1.66 g	< 0.01	< 0.01	< 0.01
Nectar harvested from the honeybulb of bees which were collected during exposure					
B Nectar Control	B Elm Farm N Great Britain	0.92 g	< 0.01	< 0.01	< 0.01
C Nectar Poncho	C Elm Farm N Great Britain	0.49 g	< 0.01	< 0.01	< 0.01
Nectar harvested directly from the plants via micro-capillaries					
Nectar Control 06/23/24/98	Control Nectar Sample from Micro Capillary Great Britain		< 0.01	< 0.01	< 0.01
Nectar Poncho 06/23/98	Treated Nectar Sample from Micro Capillary Great Britain		< 0.01	< 0.01	< 0.01

C) Rape Flower Samples:

Sample Name	Sample description	Sample weight	Residues		
			Hydroxy-NTN [mg/kg]	Olefin-NTN [mg/kg]	Imidacloprid [mg/kg]
Rape Flower Control	Control Flower Sample 23.06.98 Great Britain	ca. 20 g	< 0.01	< 0.01	< 0.01
Rape Flower Poncho	Treated Flower Sample 23.06.98 Great Britain	ca. 20 g	< 0.01	< 0.01	< 0.01

D) Rape Pollen Samples:

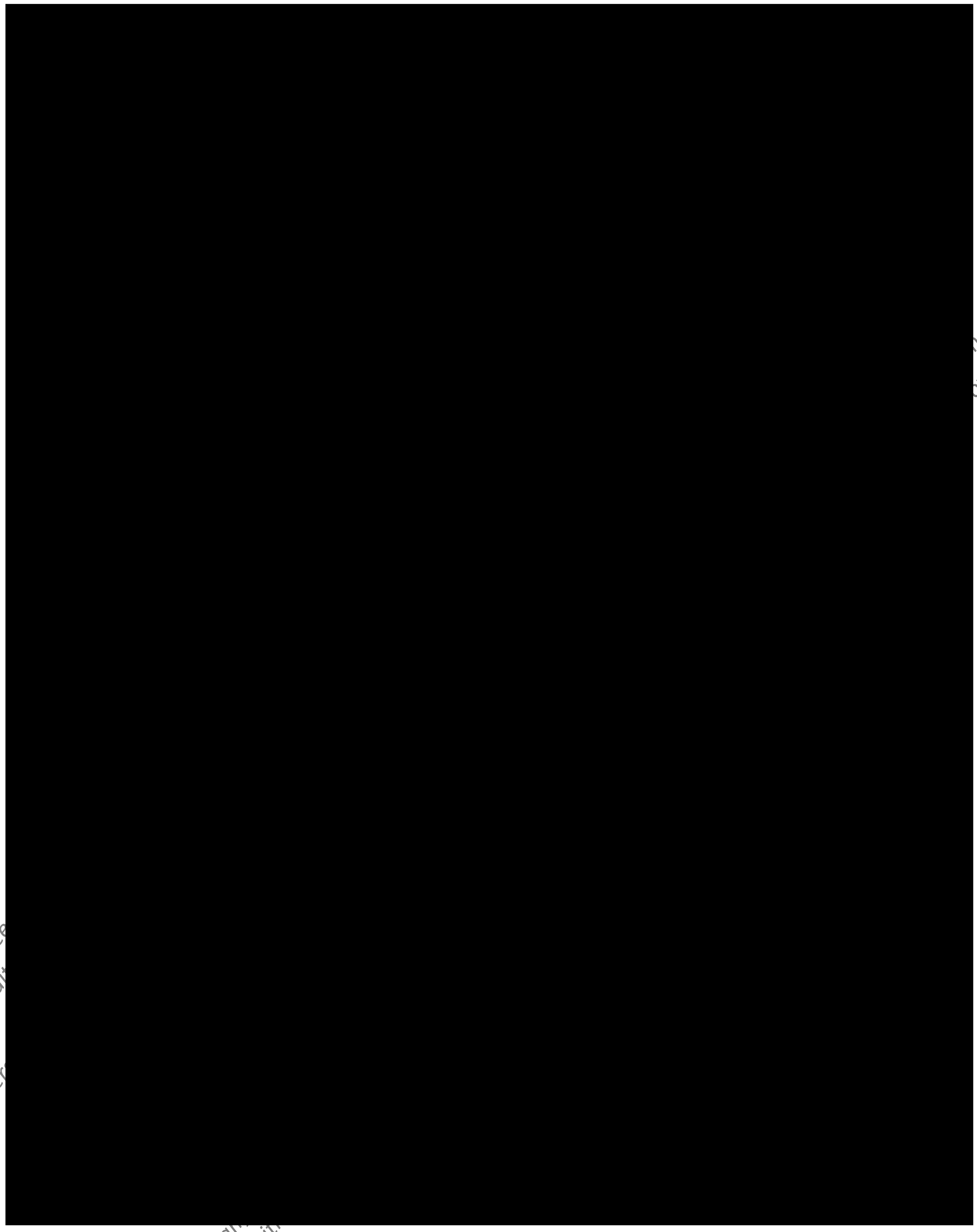
Sample Name	Sample description	Sample weight	Residues		
			Hydroxy-NTN [mg/kg]	Olefin-NTN [mg/kg]	Imidacloprid [mg/kg]
B Pollen Control	Elm Farm Great Britain	0.54 g	< 0.01	< 0.01	< 0.01
C Pollen Poncho	Elm Farm Great Britain	0.62 g	< 0.01	< 0.01	< 0.01

7.) Results of Water Control Samples

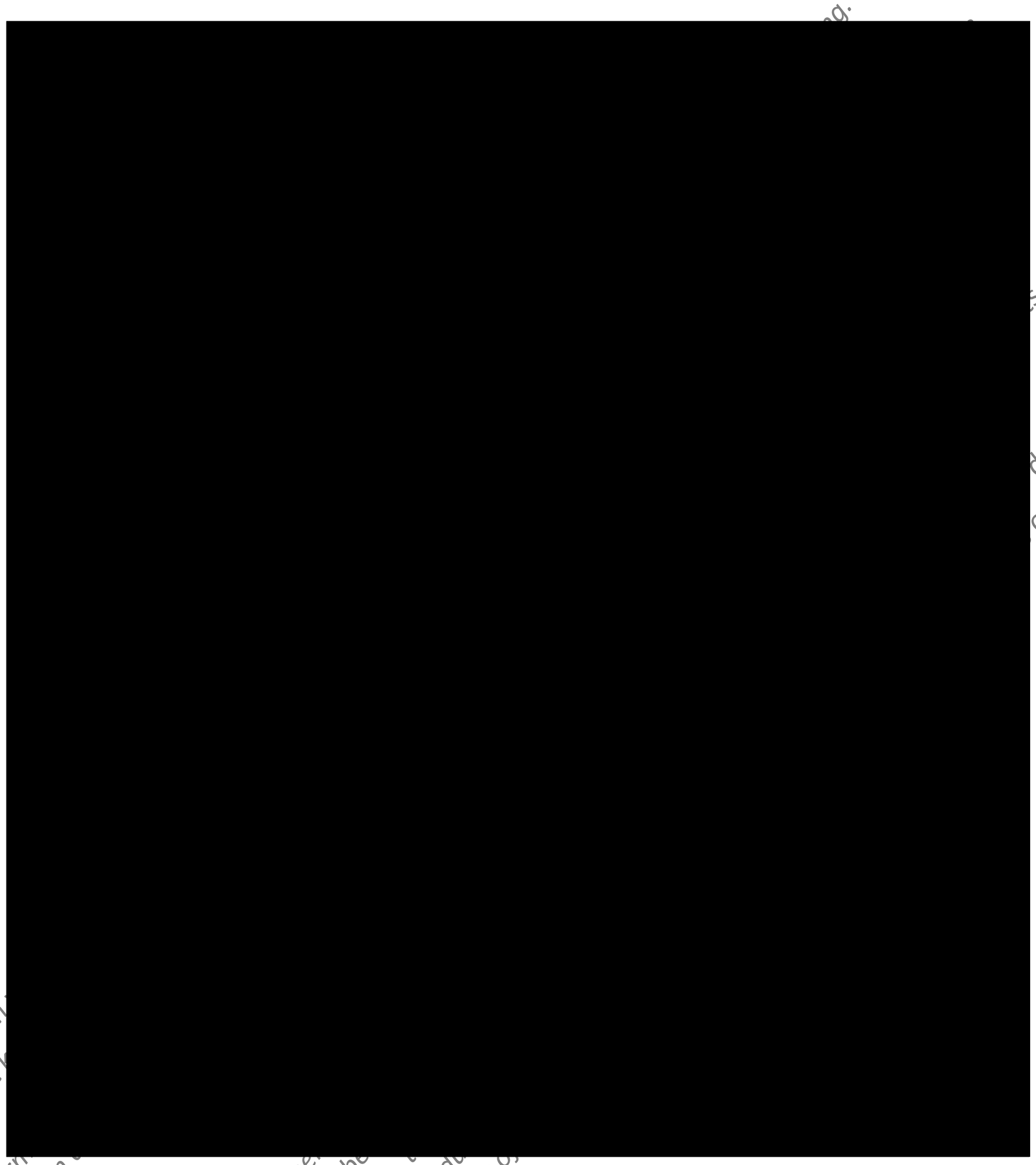
Sample Name	Sample description	Sample weight	Residues		
			Hydroxy-NTN [mg/kg]	Olefin-NTN [mg/kg]	Imidacloprid [mg/kg]
Water UK E 3701356-5 Blank Control Sample Technican	Great Britain	1 g	< 0.01	< 0.01	< 0.01
Water UK E 3701356-5 Blank Control Sample Tent	Great Britain	1 g	< 0.01	< 0.01	< 0.01
Water UK E 3701356-5 Poncho FS 500 Blank Sample Tent	Great Britain	1 g	< 0.01	< 0.01	< 0.01
Water UK E 3701356-5 Poncho FS 500 Blank Sample Technican	Great Britain	1 g	< 0.01	< 0.01	< 0.01

Limit of Quantitation= 0.01 mg/kg

Appendix II: Copy of the GLP Certificate



Appendix II (continued):



Appendix III: Quality Assurance Statement

Referat GLP

[redacted]