The impact of Gaucho 70 WS seed treated sunflower seeds on honey bees

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REPORT NUMBER

BF 1/98

NUMBER OF PAGES IN REPORT

22

COMPLETION DATE

16 December 1998

SIGNATURE SHEET

[redacted]

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Position Date

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1.0 SUMMARY

Sunflower seeds were dressed with Gaucho 70 WS (0.7 mg a.i. per seed) and sown on 8.5.98. Four bee colonies were introduced to the 1.25 ha trial field 75 days later when the plants were in flower. The same process was carried out using undressed seed on a control field of the same size 4 kilometres away, where the same parameters were measured.

The use of Gaucho seed dressing did not lead to increased bee mortality.

Treatment with Gaucho did not reduce foraging visits to sunflowers.

Bees collected large amounts of pollen from both sunflower fields.

Colony weights remained almost unchanged at both sites. This is not unusual as weight depends on the site, variety and weather conditions.

A bee counter allowed us to accurately determine the number of bees returning to the hive from the treated field. No evidence of bee disorientation was found.

No residues of imidacloprid or its main metabolites were found in the honey bladders after preparation or in the remaining bees.

Our final conclusion is that at the time when sunflowers are in flower no relevant residues of the treatment product remain in the nectar that could affect bees.

2.0 INTRODUCTION

Gaucho 70 WS is used in France to control wireworms and aphids in sunflower crops. A trial of Gaucho 70 WS as a sunflower seed dressing was conducted in 1998 to assess the risk to honeybees.

3.0 MATERIAL AND METHODS

3.1 Trial substance

Name of the trial substance:	Gaucho 70 WS
Active ingredients:	Imidacloprid
Chemical name of the active ingredients:	1-((6-Chloro-3-pyridinyl)methyl)-N-nitro-2-imidazolidinimine
Indication:	Insecticidal dressing
Concentration tested:	157.5 g/U [1 U = 150,000 seeds] (= 0.7 mg a.i./seed)
Sunflower variety tested:	Fleury (thousand grain weight: 49.86 g)

3.2 Reference substance

No reference substance has yet been specified for this application form.

3.3 Trial dates and details of participants

Seed was sown on the trial plots on 08.05.1998. The final field measurements were conducted on 06.08.1998.

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Trial director:	[redacted]
Technicians:	[redacted]
Trial report number:	BF 1/98

3.4 Sunflower seed dressing procedure

On 15.4.1998 a part-batch of 7.48 kg of Fleury sunflower seed was stirred in a dressing drum with 218.1 g of the preparation (Gaucho 70 WS + Tutan 500 FS + Degranil) and 122 ml of tap water for 12 seconds. The reference seeds were mixed with 63.3 g of Tutan FS 500 + Degranil and 198.5 ml of tap water. The seeds were dried, packed in paper bags and stored at room temperature until they were transported to the trial plots.

3.5 Trial location and position of trial plots

Fleury sunflower seeds were sown on 8 May 1998 on two plots, each 1.25 ha in size, located 4 km apart in the Euskirchen district (North Rhine-Westphalia). The treated plot was located in Metternich and the control plot in Hausweiler. 89,000 seeds were sown, corresponding to an application volume of 59 g a.i. per ha. The fields were in a purely arable farming district, where the main crops were cereals and sugar beet. The control field in Hausweiler had sandy, gravelly soil and was therefore of poorer quality than the treatment field in Metternich. 56,167 plants per hectare emerged in Metternich (treatment field) compared with 45,250 in Hausweiler (control field). The sunflowers started to flower on 22.7.98, 75 days after sowing.

Four bee colonies comprising 20 combs were introduced to each field on the morning of 27.7.98. Each hive was equipped with a bee counter (BeeSCAN) that automatically recorded the number of bees leaving and entering the hive. The sunflowers remained in flower until 6.8.98. The colonies were removed from the fields on 10.8.98 when the plants had stopped flowering.

3.6 Trial parameters

The following trial parameters were recorded:

- Changes in hive weight
- Foraging visits to flowers
- Number of bees entering and leaving the hive (using the BeeSCAN device)
- Number of bees with pollen
- Mortality
- Residue of trial substance and metabolites in bees and honey stomachs.

3.7 Description of the trial

Recording hive weights:

Hive weights were recorded before the start of the trial, i.e. before the bees were introduced to the plots, and on days 4 and 10 of the trial.

Foraging visits to flowers

The number of bees foraging on 6 x 100 flowering sunflower discs was recorded. The average figures based on daily assessments at six spots in the field are shown in the results section of this report.

Number of bees returning to the hive

The number of bees leaving and entering the hive was recorded using BeeSCAN devices for one colony per site. During the course of the trial, the device in the control field was found to be faulty and therefore we can only present the results from the treatment field.

Number of bees with pollen

The number of bees returning to the hive with pollen on their limbs in one minute was recorded by monitoring bees on the landing board.

Mortality

Dead bee traps made of wire mesh were placed in front of the flight holes of three colonies. Bees were able to crawl through the mesh but could not drag dead bees out with them. This allowed us to record the number of bees dying in the hive and being caught in the dead bee traps. We also counted all the dead bees on a 2m x 3m grid laid out on the ground in front of the four hives.

Trial substance and metabolite residue in bees and honey stomachs

100 foraging bees were collected from sunflower discs in both fields on 5.8.98. The bees were killed using ether and then frozen until preparation, when the abdomen was separated from the thorax and the honey bladder was removed from the abdomen. The body parts were placed in a 60:20 [sic] water/methanol mixture.

4.0 RESULTS

4.1 Changes in hive weights during the trial

The table below shows how hive weights evolved during the course of the trial. Hive weights remained more or less unchanged on both sites throughout the trial.

Treatment	27.7.	31.7.	6.8.
Control	49.9 kg	50.1 kg	50.2 kg
Gaucho	52.7 kg	52.8 kg	51.5 kg

It is not unusual to see no increase in hive weights during trials of this kind, as weight change is affected by factors such as location, species, weather conditions and competition for food by bumblebees. Other sources confirm absence of weight gain by bees feeding on sunflowers in particular (see Hedtke; article in Deutsches Bienenjournal, 11/98). The slightly better weight evolution in the control group may be due to the fact that this colony visited a field of dwarf bush beans near to the control plot which started flowering at the end of the trial.

4.2 Foraging visits to flowers

The table below shows the level of foraging activity (average number of bees foraging on 100 sunflower discs, six counts performed).

Treatment	27.7.	28.7.	29.7.	30.7.	31.7.	1.8.	3.8.	4.8.	5.8.	Average
Control	76.8	55.8	95.5	47.5	42.8	43.3	57.5	67.5	27.5	57.2
Gaucho	260.5	218.5	113.3	66.8	83.3	56.2	168.2	208.0	110.3	142.8

Bee visits were generally higher on the treatment field because the sunflowers growing there were in better condition. Foraging activity was very intense immediately after the bee colonies were introduced to the treatment field on 27.7.98 (more than 200 bees on 100 discs). The first two counts took place while the bees were exploring their surroundings and collecting large amounts of the good source of food suddenly offered to them. Very high numbers of bees were observed on the sunflower discs particularly close to the hives. The sunflowers growing on the control plot were not in such good condition and so less attractive to the bees. Consequently, foraging activity was less intense than on the treatment field.

Weather conditions worsened towards the middle of the trial period. Temperatures fell to 18 degrees, and it was windy and rainy. Foraging activity declined temporarily on both trial plots as a result, but picked up again towards the end of the trial period as the weather improved. Our observations do not point to Gaucho used as a seed dressing having any effect on foraging activity.

A strikingly high level of foraging activity by bumblebees on both fields was noted. A spot-check count on 27.7.98 on the control field showed that 95.7 bumblebees were visiting 100 sunflower discs, a figure well in excess of that recorded for honeybees (55.8). Throughout the trial bumblebees were foraging in about the same numbers as honeybees. Again, we could not detect any effect of Gaucho used as a seed dressing on bumblebee foraging activity.

4.3 Number of bees returning to the hive

Diagrams 1 to 5 show the data recorded by BeeSCAN on the treatment field for the period 27.07.-31.07.98. The diagrams clearly show that all the bees leaving the hive return after a short time, as the curves for departing and returning bees are very close together. The response of foraging bees to weather changes (rain and wind) is also clear to see. The night of 27.7. to 28.7. was very warm, and as a result many bees spent the night in the field rather than returning to the hive. On subsequent days the number of bees entering the hive was higher than the number leaving. These might be returning bees from that day and/or bees from neighbouring colonies carried along by the wind, a phenomenon which is often observed if serial population counts are performed. The maximum number of departures per day was recorded at 123,000; with an estimated population size of 25,000 bees and around 10,000 foraging bees in a colony, this equates to more than 12 departures per bee.

4.4 Number of bees returning to the hive with pollen

The results of this observation are shown in the table below. The figures represent the number of bees returning to the hive with pollen on their limbs on the basis of a one-minute observation of the landing board (total figures for 4 colonies).

Treatment	27.7.	28.7.	29.7.	30.7.	31.7.	1.8.	3.8.	4.8.	5.8.	Average
Control	11	22	25	8	10	13	30	43	13	20.0
Gaucho	10	1	2	7	11	16	58	23	48	24.2

We were unable to determine the origin of the pollen as the bees did not leave behind any pollen in the pollen traps that we had set up. As no alternative food sources or wild plants were present, we assume that most of the pollen was sunflower pollen. On average, the bees in the treatment field collected more pollen that the bees in the control field. Gaucho was not found to affect this parameter either.

4.5 Mortality

Very few dead bees were found in the dead bee traps and on the grid (see table below). We can conclude from this that foraging on sunflowers grown from seed dressed with Gaucho did not lead to increased bee mortality.

Treatment	27.7.	28.7.	29.7.	30.7.	31.7.	1.8.	3.8.	4.8.	5.8.	6.8.	total
Gaucho	19	17	13	10	8	13	29	15	55	22	201
Control	12	17	6	16	6	21	17	25	8	19	147

4.6 Trial substance and metabolite residue in bees and honey stomachs

Honey bladders and remaining bees were investigated to determine levels of imidacloprid and the metabolites olefinimidacloprid and hydroxyimidacloprid. As demonstrated in the attached analytical report, no residues could be determined in either matrix (limit of determination 0.01 mg/kg). Analysis of honey bladder content allows us to conclude that sunflower nectar contains no discernible residues of imidacloprid or toxicologically relevant metabolites.

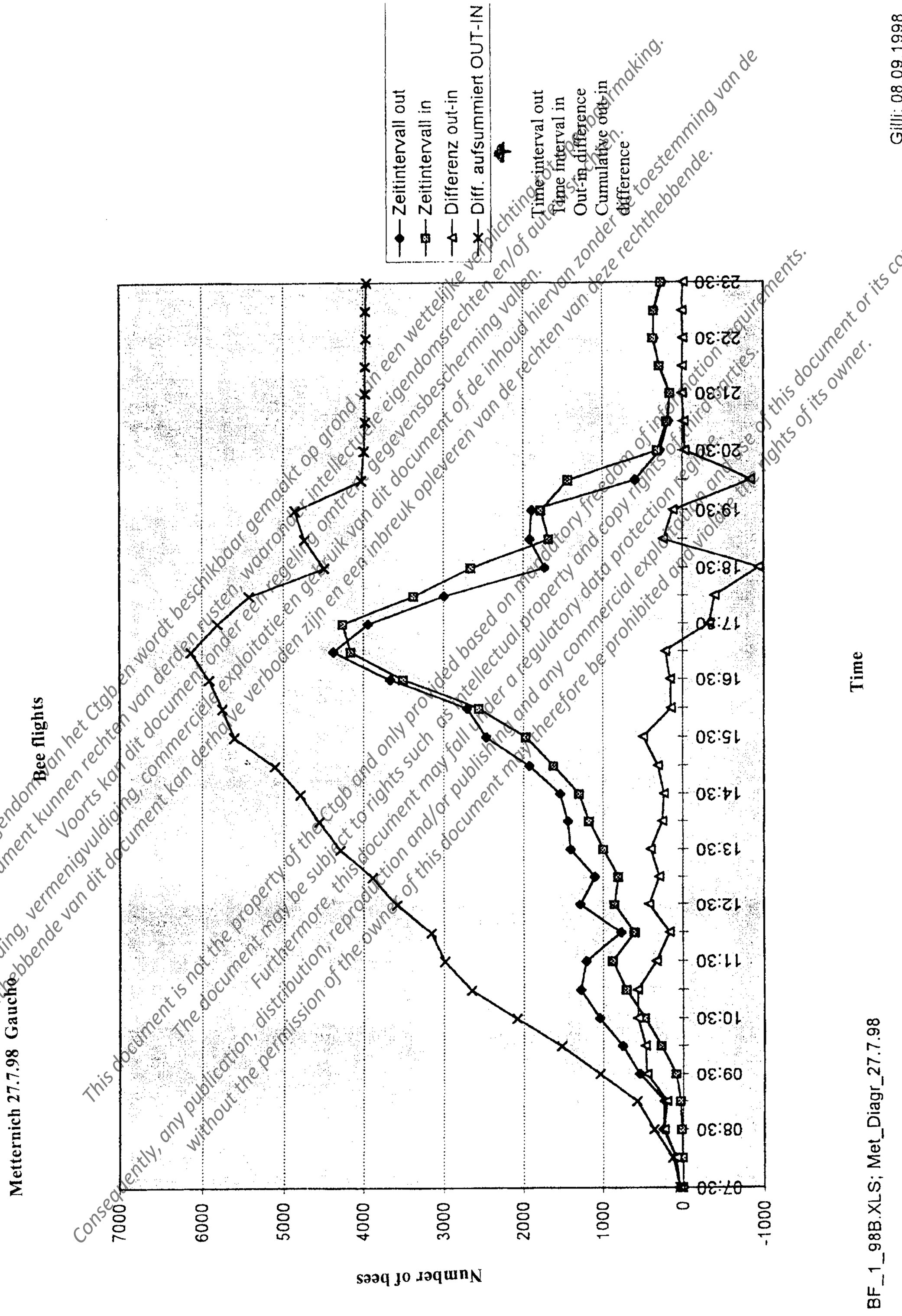

Diagram 1: Automatic record of flight movements using the BeeSCAN device
This diagram shows flight movements for the treated field on 27.7.1998.

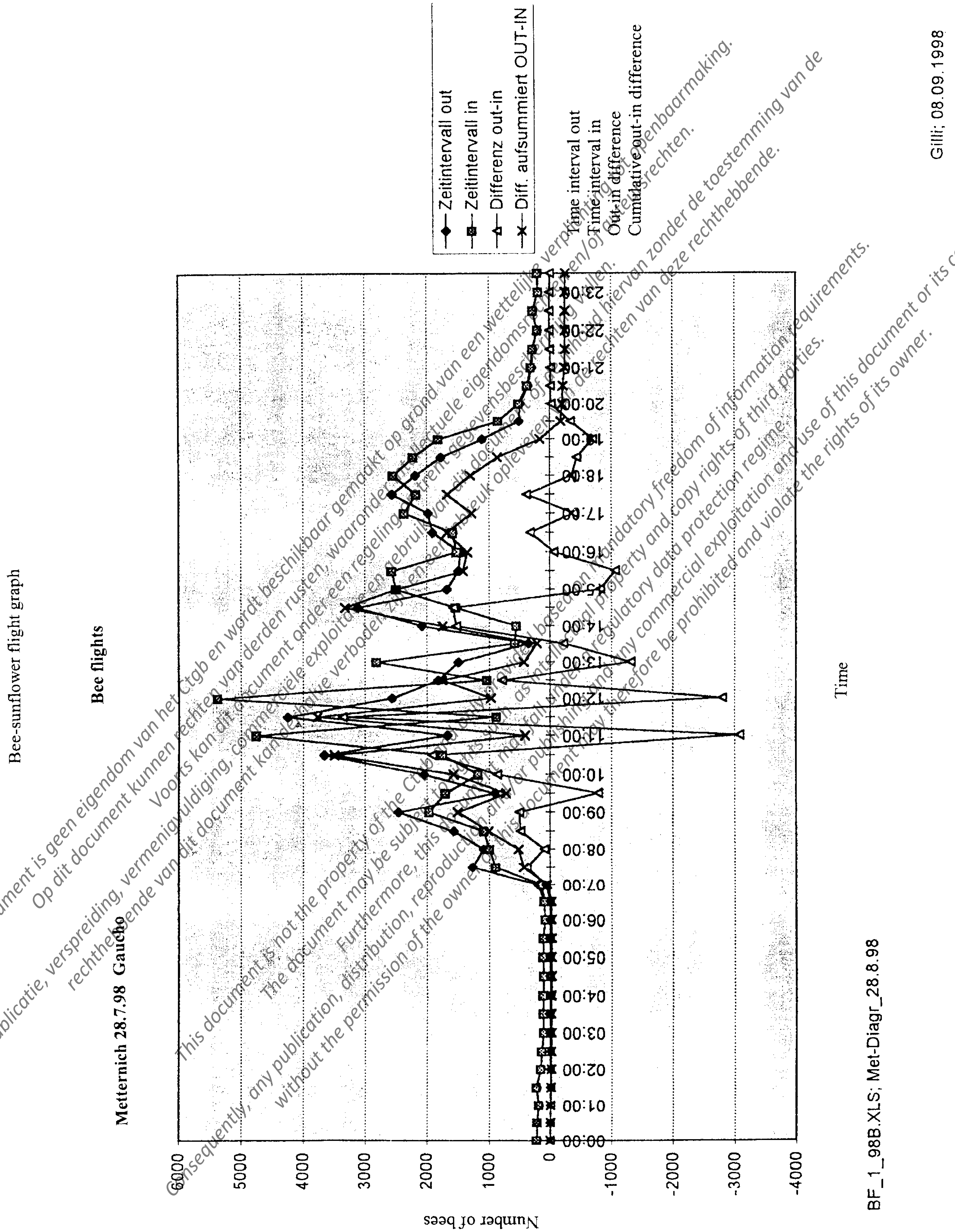

Diagram 2: Automatic record of flight movements using the BeeSCAN device
This diagram shows flight movements for the treated field on 28.7.1998.

Bee-sunflower flight graph

Metternich 29.7.98 Gaucho

Bee flights

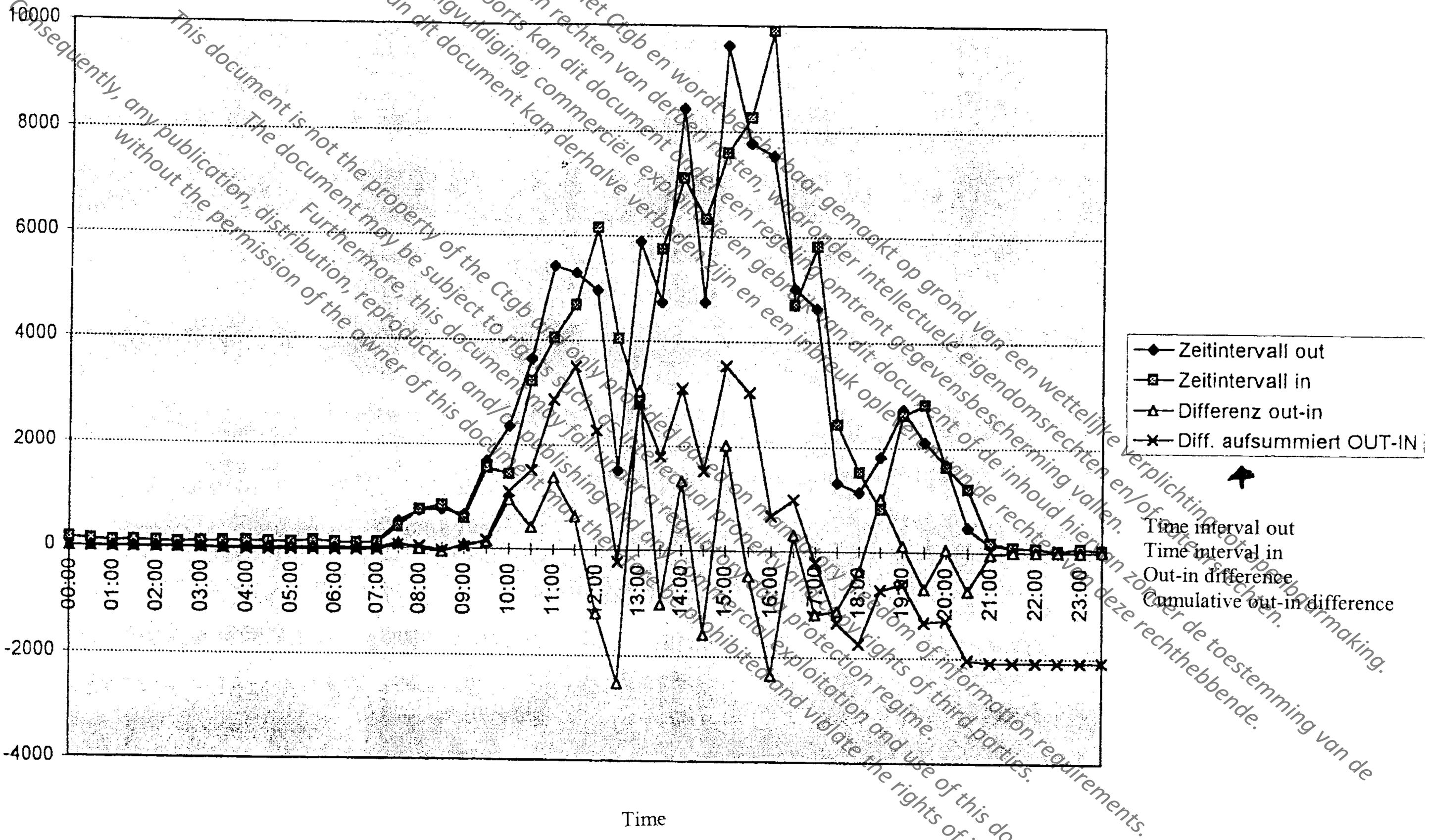

Diagram 3: Automatic record of flight movements using the BeeSCAN device.
This diagram shows flight movements for the treated field on 29.7.1998

Gilli; 08.09.1998

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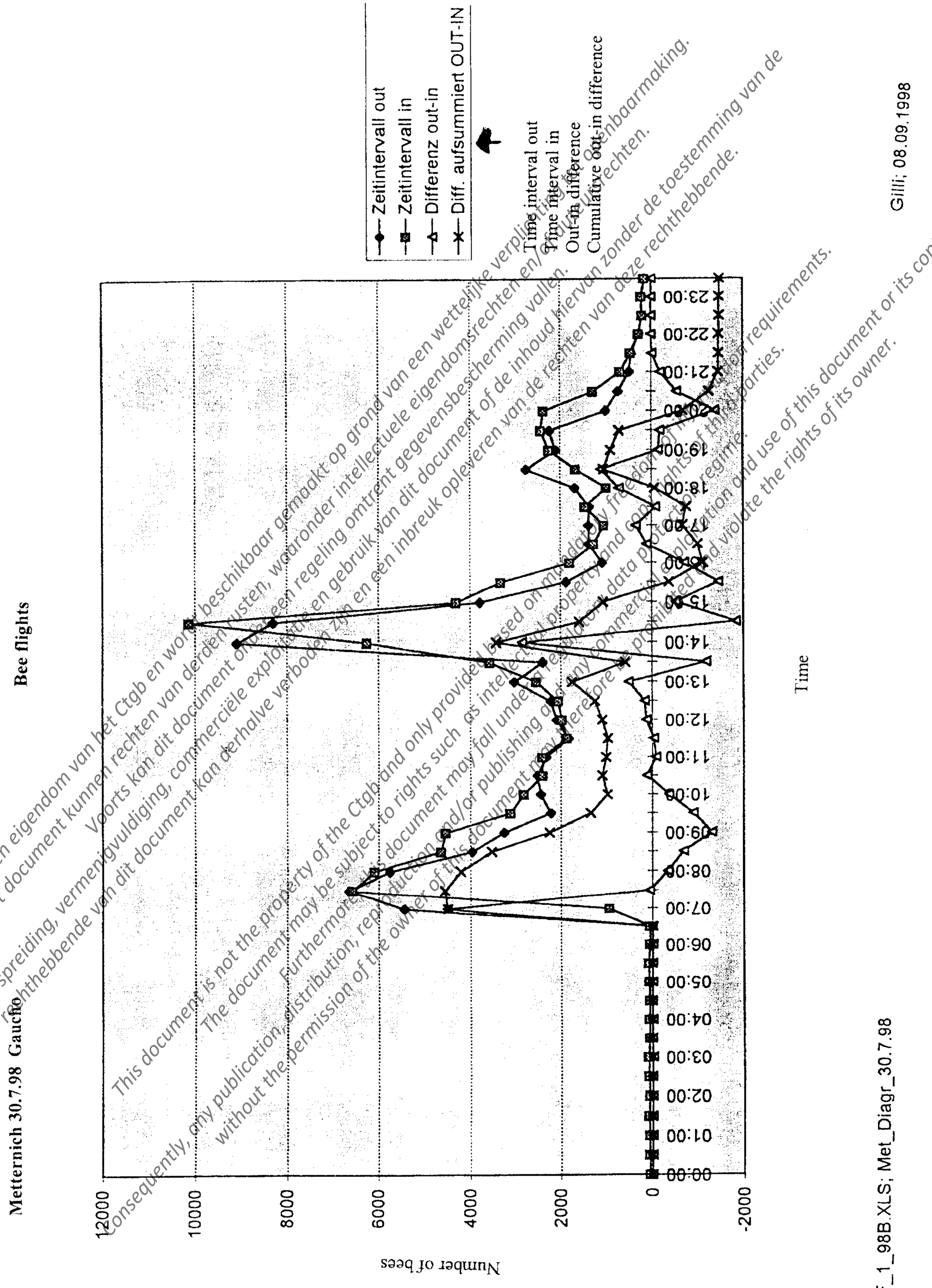
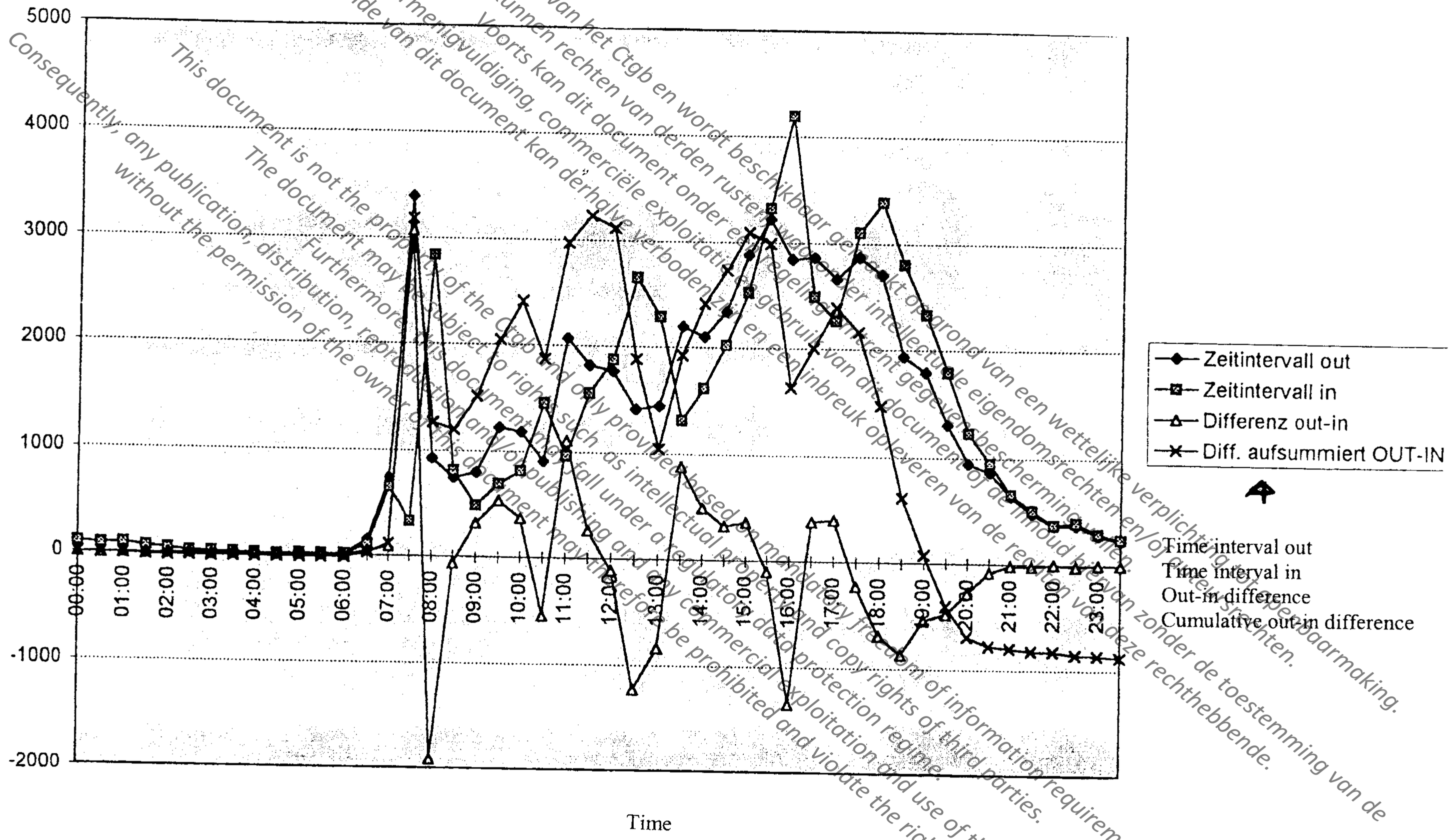


Diagram 4: Automatic record of flight movements using the BeeSCAN device
This diagram shows flight movements for the treated field on 30.7.1998.

Bee-sunflower flight graph

Bee flights

Metternich 31.7.98 Gaucho



Number of bees

Time

Diagram 5: Automatic record of flight movements using the BeeSCAN device
This diagram shows flight movements for the treated field on 31.7.1998.

BF_1_98B.XLS; Met_Diagr_31.7.98

Gilli; 08.09.1998

Anhang I: Analytikbericht

Bee-Samples from Germany (Euskirchen) in/on Sunflower (Honeybees and nectar from the bee bulbs)

Residue Analytical Method for the Determination of Imidacloprid, Hydroxy- and Olefin-Metabolite in Sunflower, Bees 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:

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.

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.) 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

b) Retention Times:

Olefin-NTN	ca. 4.5 min
Hydroxy-NTN	ca. 5.3 min
Imidacloprid	ca. 8.3 min

c) 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 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, 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 x 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 at6 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

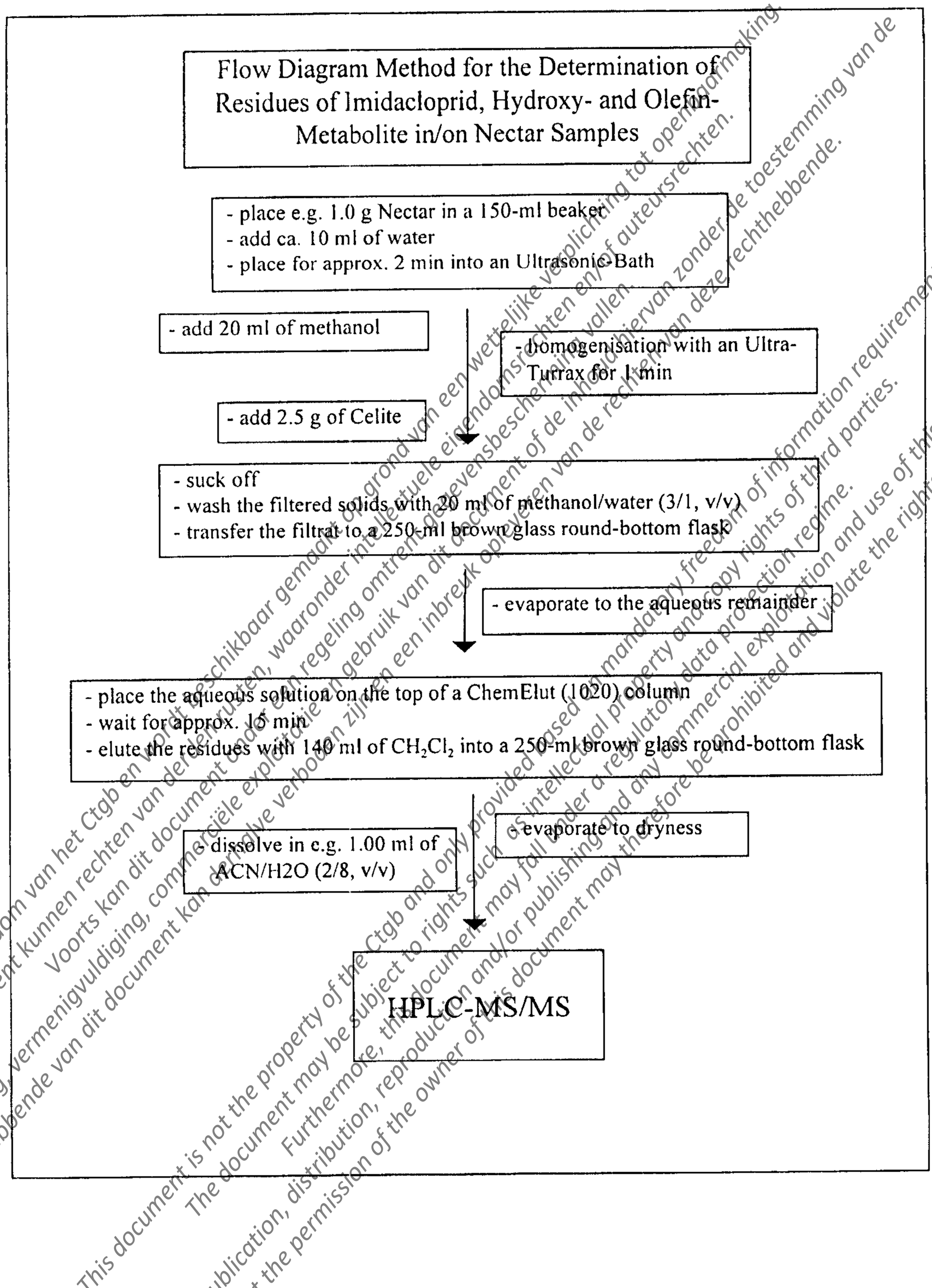
Aquisition mode: Profile

Mass spectrometer operating parameters:

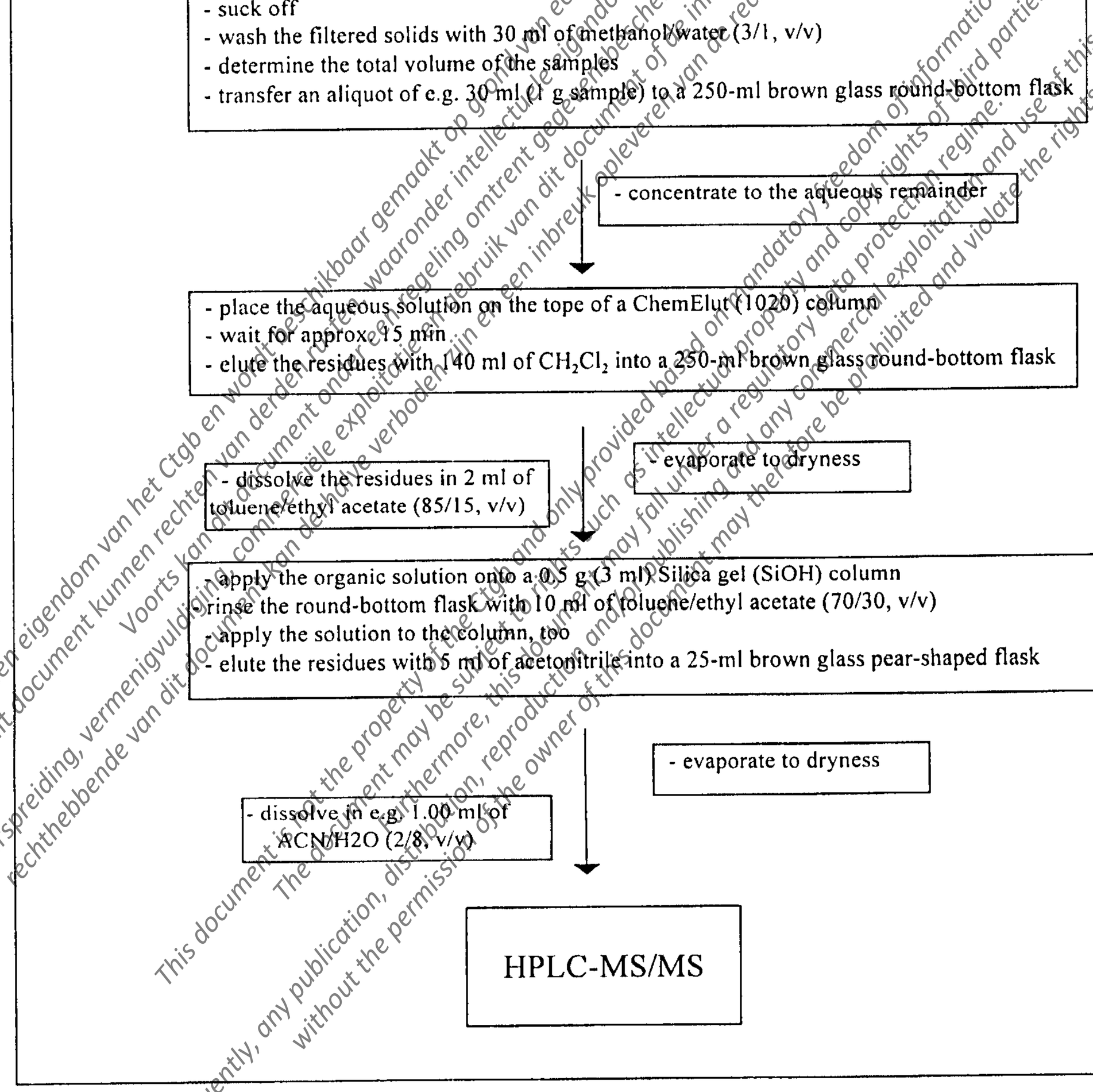
Compound	Precursor Ion Q1 Mass (amu)	Product Ion Q3 Mass (amu)	Dwell Time (msec)	Collision Energy (eV)
Imidacloprid (Cl 37)#	258.0	210.9	500	-20
Imidacloprid (Cl 35)	256.0	208.9	500	-20
Hydroxy-Metabolite (Cl 37)#	274.0	190.8	250	-23
Hydroxy-Metabolite (Cl 35)	272.0	190.8	250	-23
Olefin-Metabolite (Cl 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 Samples from Honeybee Bulbs.



5.) Flow Diagram Method for the Determination of Residues of Imidacloprid and metabolites in/on Bee Samples.



5.) Results of Bee Samples and Nectar Samples of Bees (prepared).

A) Bee Samples:

Sample label	Sample description	Sample weight	Hydroxy-NTN [mg/kg]	Olefin-NTN [mg/kg]	Imidacloprid [mg/kg]
Euskirchen Biene Kontrolle	A Euskirchen (Untreated)	12.5 g	< 0.01	< 0.01	< 0.01
Euskirchen Biene Gaucho	B Euskirchen (Gaucho WS 70 0.7 mg a.i./Korn)	14.9 g	< 0.01	< 0.01	< 0.01

B) Nectar Samples:

Sample label	Sample description	Sample weight	Hydroxy-NTN [mg/kg]	Olefin-NTN [mg/kg]	Imidacloprid [mg/kg]
Euskirchen Nektar Kontrolle	A Euskirchen (Untreated)	0.65 g	< 0.01	< 0.01	< 0.01
Euskirchen Nektar Gaucho	B Euskirchen (Gaucho WS 70 0.7 mg a.i./Korn)	0.58 g	< 0.01	< 0.01	< 0.01

ATTACHMENT

Signature page(s) from original German report

UNTERSCHRIFTENSEITE

[redacted]

Prüfleiter Feld

6.12.98

Funktion Datum

Prüfleiter Rückstandsproben

16 Dezember 1998

Funktion Datum

Prüfleiter Rückstandsanalytik

16 December 1998

Funktion Datum

Leiter der Prüfeinrichtung

21.12.98

Funktion Datum

ANFRAGEN

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