REPORT

HONEYBEE (*APIS MELLIFERA* L.) ORAL TOXICITY STUDY IN THE LABORATORY WITH IMIDACLOPRID TECHN.

Study Director [redacted]

Test facility **Ambrosiushoeve**
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Sponsor **Bayer AG**
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REPORT APPROVAL

Study Director

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date: 22-09-1999

Principal Investigator

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date: 22-09-1999

STATEMENT OF GLP COMPLIANCE (Quality Assurance)

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Quality Assurance Officer [redacted]

Date: 1999-09-22

ANNEX to STATEMENT OF GLP COMPLIANCE

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Quality Assurance Officer [redacted]

Date: 1999-09-22

STATEMENT OF GLP COMPLIANCE (Study Director)

[redacted]

Study Director

[redacted]

Date: 22-09-1999

SUMMARY

The purpose of the toxicity study was to examine the effects of imidacloprid techn. on honeybees when applied in the laboratory. Per concentration 10 honeybees were fed with 100 µl sucrose solution 50% containing a range of concentrations of imidacloprid techn. By sharing the food (trophallaxis), each honeybee gets about 10 µl.

The sponsor indicated that the oral LD_{50} was between 5 and 20 ng / honeybee. That is why the concentration range of about 4 ng to 20 ng Imidacloprid techn. / honeybee was tested.

Per concentration 10 honeybees were fed with 100 µl sucrose solution 50% containing respectively: 21.22 ng imidacloprid techn., 16.78 ng imidacloprid techn, 13.18 ng imidacloprid techn, 8.63 ng imidacloprid techn. and 4.09 ng imidacloprid techn. per 10 µl.
The treatment was compared to a 50 % sucrose-solution (negative control) and a Dimethoate positive control.

The concentrations of imidacloprid techn. fed to the bees in this test, did not cause mortality of the honeybees. However effects were observed. The most significant effect was the "frozen behaviour" at which the honeybees are motionless except for a little trembling of body parts like abdomen, antennae or tarsus. Some honeybees, which had taken in about 20 ng, showed spasms and were paralysed.
As there are no data on mortality, the LD_{50} of imidacloprid techn. could not be determinated. The lethal concentration is more than 21 ng/bee
The ED_{50} of imidacloprid techn. after 24 hours, calculated with the linear regression is 34 ng / honeybee. ($r^2 = 0.50$).
The data on effect vary a lot but the effect is clear. Amounts of 4.39 ng / honeybee or less do not result in any effect. Amounts of 6.83 ng imidacloprid techn. or higher result more or less in the described frozen behaviour.

ARCHIVING AND STORAGE

The original signed protocol, the original signed report and a copy of the raw data are archived by the sponsor.
A copy of the protocol, a copy of the report a reference sample of the test substance and the raw data are archived by Ambrosiushoeve.

PREFACE

GENERAL

Title	Honeybee (*Apis mellifera mellifera* L.) oral toxicity study in the laboratory with imidacloprid techn.
Project nr	AH99.4.22.4
Sponsor	Bayer AG Agricultural Centre Institute for Environmental Biology [redacted]
Test facility	Ambrosiushoeve Ambrosiusweg 1 5081 NV Hilvarenbeek The Netherlands
Study Director	[redacted] Ambrosiushoeve Ambrosiusweg 1 5081 NV Hilvarenbeek The Netherlands
Principal Investigator	[redacted] Ambrosiushoeve Ambrosiusweg 1 5081 NV Hilvarenbeek The Netherlands

SCHEDULE

Start experiments	05-07-1999
End observations	08-07-1999

QUALITY ASSURANCE

This study is performed in compliance with the Ambrosiushoeve Standard Operating Procedures and with the most recent edition of:
OECD Principles of Good Laboratory Practise (as revised in 1997) ENV/MC/CHEM(98)17 (OECD Paris 1998)

GUIDELINE

Guideline on test methods for evaluating the side effects of plant protection products on honeybees.
European and Mediterranean plant protection organisation Bulletin EPPO 22, 203-215 (1992).

DEFINITIONS

LD_{50} is the lethal dose value in ng product per honeybee, which kills 50 % of the honeybees within in a defined period of time.
LD_{50} is the dose value in ng product per honeybee, which brings about an effect on 50 % of the honeybees within in a defined period of time.
Mortality: honeybees are considered to be dead when they don't respond on stimuli like touching with a pair of tweezers.
Effect: Effect is mortality + paralysis + spasm + "frozen behaviour" at which the honeybees are motionless except a little trembling of body parts like abdomen, antennae or tarsus.
A replicate is the same test substance in a certain concentration applied to 10 honeybees from another hive.

OBJECTIVE

PURPOSE AND RATIONALE

The purpose of the toxicity study was to examine the effects of imidacloprid techn. on honeybees when applied in the laboratory. Per concentration 10 honeybees were fed with 100 µl sucrose solution 50% containing respectively: 21.22 ng imidacloprid techn., 16.78 ng imidacloprid techn, 13.18 ng imidacloprid techn, 8.63 ng imidacloprid techn and 4.09 ng imidacloprid techn per 10 µl.
By sharing the food (trophallaxis), each honeybee gets about 10 µl.
The treatment was compared to a 50 % sucrose-solution (negative control) and a Dimethoate positive control.
This study provides a rational basis for the assessment of the toxicological risk to honeybees.

MATERIAL AND METHODS

TEST SYSTEM

Test system	Honeybees, *Apis mellifera mellifera* L. (Hymenoptera: Apidae).
Rationale	Recognised by the cited EPPO guideline as the recommended test system.
Source	Research Centre for Insect Pollination and Beekeeping. "Ambrosiushoeve"; responsible beekeeper: [redacted]
Sampling	Bees were collected from combs without brood.
Number of animals per cage used for testing	10 worker honeybees.
Number of animals treated per concentration	3 x 10 worker honeybees (three cages of 10 honeybees = 3 replicates).
Randomisation	Allocation of the honeybees to any particular treatment was by chance.

TEST SUBSTANCE

Identification:	imidacloprid techn.
Description:	solid powder colour: beige
Batch:	PT 230824088
Composition:	1-{(6-chloro-3-pyridinyl)=methyl}-N-nitro-2-=imidazolidinimine
CAS no.:	138261-41-3
Article no.:	04145852
Tox. no.:	4941-00
Purity:	98.6%,
Analytical method:	HPLC, ext. Std.
Expiry date:	03-09-1999
Stability for at least 48 Hours in vehicle:	water stable acetone stable

REFERENCE SUBSTANCE

Identification	Dimethoate
Description	blue liquid
Batch	92511812
Composition	400 g dimethoate /litre
Expiry date	December 2000

HUSBANDERY / ENVIRONMENTAL CONDITIONS

The honeybees were kept in groups of 10, in 10 cm wide, 5.5 cm deep and 8.5 cm high stainless steel cages with front walls made of glass for observation of the honeybees and perforated bottoms for ventilation. The stainless steel parts were lined with white paper (No. 68 Macher-Nagel & Co. D-5165 Düren).
Apart from oral dosing and during starvation, a 50% aqueous sucrose-solution was provided ad libitum for food.
The honeybees were kept in a dark room with a continuously monitored environment of 25±2°C. During feeding, the honeybees were in the same room in the light. Observations were carried out using red light.

TEST ARTICLE PREPARATION

imidacloprid techn.
On 05-07-1999. 0.0109 gram imidacloprid techn was dissolved in 122.94 gram 50% sucrose-solution (1063.93 ng / 10 µl). The solution was stirred overnight. On 06-07-1999) 0.9975 gram of the clear solution was diluted with 9.0348 50% sucrose-solution to a concentration of 105.79 ng / 10 µl, the stock solution. From this stock solution a dilution range was made:

- 1.0047 gram stock solution + 4.004 gram sucrose-solution 50% resulted in an imidacloprid techn. concentration of 21.22 ng / 10 µl,
- 1.0033 gram stock solution + 5.3212 gram sucrose-solution 50% resulted in an imidacloprid techn. concentration of 16.78 ng / 10 µl,
- 1.0396 gram stock solution + 7.3058 gram sucrose-solution 50% resulted in an imidacloprid techn. concentration of 13.18 ng / 10 µl,
- 0.5115 gram stock solution + 5.7617 gram sucrose-solution 50% resulted in an imidacloprid techn. concentration of 8.63 ng / 10 µl,
- 0.4823 gram stock solution + 11.979 gram sucrose-solution 50% resulted in an imidacloprid techn. concentration of 4.09 ng / 10 µl.

Dimethoate 40%:
The LD_{50} of Dimethoate is about 0.2 µg a.i. / honeybee. A concentration range of approximately 0.1, 0.2 and 0.3 µg dimethoate a.i./ honeybee = 0.75, 0.5 and 0.25 µg Dimethoate formulation was tested.

- 0.0826 gram Dimethoate 40% was dissolved in 120.03 gram 50% sucrose-solution. This resulted in the concentration of 8.3 µg Dimethoate 40% / 10 µl sucrose-solution 50%.
- 0.9952 gram Dimethoate 40% (concentration 8.3 µg / 10 µl) + 8.9979 gram sucrose-solution 50% resulted in a Dimethoate 40 % concentration of 0.82 µg / 10 µl.
- 2.0283 gram Dimethoate 40% (concentration 0.82 µg / 10 µl) + 0.999 gram sucrose-solution 50% resulted in a Dimethoate 40 % concentration of 0.55 µg / 10 µl.
- 0.9957 gram Dimethoate 40% (concentration 0.82 µg / 10 µl) + 1.9736 gram sucrose-solution 50% resulted in a Dimethoate 40 % concentration of 0.28 µg / 10 µl.

Sucrose-solution 50%:

- 153.90 gram sucrose was dissolved in 154.81 gram tapwater. This resulted in a sucrose-solution 50%. The density of the sucrose-solution was 1.2 g/ml.

TREATMENT

Naive honeybees were taken from combs without brood and transferred to a 50 x 40 x 40 cm flight cage in the laboratory at room temperature in the light. In this flight cage they stayed, deprived of food, for 1 hour to 1 hour 30 minutes. After this starvation period, the honeybees were transferred to the test cages (10 honeybees per cage) and fed with the test substance. Per cage 100 mm^3 sucrose-solution with the test substance (test solution) in different concentrations was offered (10 mm^3 / honeybee). By sharing the food (trophallaxis), each honeybee gets about 10 µl.
After intake of the test solution, the honeybees were provided with sucrose-solution 50%. The sucrose-solution was changed daily.
The sponsor indicated that the oral LD_{50} was between 5 and 20 ng / honeybee. That is why the concentration range of about 4 ng to 20 ng Imidacloprid / honeybee was tested.
In the test, 5 concentrations of imidacloprid techn.: 21.22, 16.78, 13.18, 8.63 and 4.09 ng/10µl were fed to the honeybees. Each concentration was offered to 3 cages (3 x 10 honeybees = 3 replicates).
In the positive control 3 concentrations: 0.82, 0.55 and 0.28 µg / 1 µl were fed to the honeybees. Each concentration was offered to 3 cages (3 x 10 honeybees).
In the negative control the 50% sucrose solution was offered to the honeybees.
The amount of test solution taken in, has been determined by re-weighing the feeding system the honeybees were fed with.
The percentage of mortality and effect per cage of both the test substance and the positive control were calculated per cage.
The test was carried out once.

OBSERVATIONS

Behaviour abnormalities, e.g. paralysis, uncoordinated movements or any locomotor disabilities as well as mortality were recorded every 30 minutes during intake and 24 hours and 48 hours after feeding started.

RESULTS

DATA / TABLES

Table 1
Amounts of imidacloprid techn. taken in and mortality and other effects during the observation period of the honeybees offered 21.22 ng / 10μl sucrose-solution 50%.

		Mortality (n bees)			paralysed / spasm (n bees)			frozen behaviour (n bees)		
		Replicate Taken in / bee			Replicate taken in / bee			Replicate taken in / bee		
Date	observation after (hours)	**1** 20.90 ng	**2** 19.58 ng	**3** 20.67 ng	**1** 20.90 ng	**2** 19.58 ng	**3** 20.67 ng	**1** 20.90 ng	**2** 19.58 ng	**3** 20.67 ng
06-07-1999	4:40	0	0	0	2	0	0	4	5	4
07-07-1999	26:26	0	0	0	0	0	0	6	3	1
08-07-1999	49:55	0	0	0	0	0	0	1	0	1

Table 2
Imidacloprid techn.: Percentages mortality and percentages effect of the honeybees, offered 21.22 ng / 10μl sucrose-solution 50%.

		Percentage mortality			percentage effect		
		Replicate			Replicate		
Date	observation after (hours)	**1**	**2**	**3**	**1**	**2**	**3**
06-07-1999	4:40	0%	0%	0%	60%	50%	40%
07-07-1999	26:26	0%	0%	0%	60%	30%	10%
08-07-1999	49:55	0%	0%	0%	10%	0%	10%

Table 3
Amounts of imidacloprid techn. taken in and mortality and other effects during the observation period of the honeybees offered 16.78 ng / 10μl sucrose-solution 50%.

		Mortality (n bees)			paralysed / spasm (n bees)			frozen behaviour (n bees)		
		Replicate Taken in / bee			Replicate taken in / bee			Replicate taken in / bee		
Date	observation after (hours)	**1** 15.75 ng	**2** 11.09 ng	**3** 16.18 ng	**1** 15.75 ng	**2** 11.09 ng	**3** 16.18 ng	**1** 15.75 ng	**2** 11.09 ng	**3** 16.18 ng
06-07-1999	4:40	0	0	0	0	0	0	3	3	4
07-07-1999	26:23	0	0	0	0	0	0	1	2	2
08-07-1999	49:55	0	0	0	0	0	0	1	0	0

Table 4
Imidacloprid techn.: Percentages mortality and percentages effect of the honeybees, offered 16.78 ng / 10μl sucrose-solution 50%.

		Percentage mortality			percentage effect		
		Replicate			Replicate		
Date	Observation after (hours)	**1**	**2**	**3**	**1**	**2**	**3**
06-07-1999	4:40	0%	0%	0%	30%	30%	40%
07-07-1999	26:23	0%	0%	0%	10%	20%	20%
08-07-1999	49:55	0%	0%	0%	10%	0%	0%

Table 5
Amounts of imidacloprid techn. taken in and mortality and other effects during the observation period of the honeybees offered 13.18 ng / 10µl sucrose-solution 50%.

		mortality (n bees)			paralysed / spasm (n bees)			frozen behaviour (n bees)		
		Replicate taken in / bee in µg			Replicate Taken in / bee			Replicate taken in / bee		
Date	observation after (hours)	**1** 9.36 ng	**2** 12.97 ng	**3** 12.91 ng	**1** 9.36 ng	**2** 12.97 ng	**3** 12.91 ng	**1** 9.36 ng	**2** 12.97 ng	**3** 12.91 ng
06-07-1999	4:39	0	0	0	0	0	0	0	4	6
07-07-1999	26:22	0	0	0	0	0	0	1	1	1
08-07-1999	49:55	0	0	0	0	0	0	0	0	1

Table 6
Imidacloprid techn.: Percentages mortality and percentages effect of the honeybees, offered 13.18 ng / 10µl sucrose-solution 50%.

		percentage mortality			percentage effect		
		Replicate			Replicate		
Date	Observation after (hours)	**1**	**2**	**3**	**1**	**2**	**3**
06-07-1999	4:39	0%	0%	0%	0%	40%	60%
07-07-1999	26:22	0%	0%	0%	10%	10%	10%
08-07-1999	49:55	0%	0%	0%	0%	0%	10%

Table 7
Amounts of imidacloprid techn. taken in and mortality and other effects during the observation period of the honeybees offered 8.63 ng / 10µl sucrose-solution 50%.

		mortality (n bees)			Paralysed / spasm (n bees)			frozen behaviour (n bees)		
		Replicate taken in / bee in µg			Replicate taken in / bee			Replicate taken in / bee		
Date	observation after (hours)	**1** 7.59 ng	**2** 6.83 ng	**3** 8.53 ng	**1** 7.59 ng	**2** 6.83 ng	**3** 8.53 ng	**1** 7.59 ng	**2** 6.83 ng	**3** 8.53 ng
06-07-1999	4:38	0	0	1	0	0	0	1	6	2
07-07-1999	26:22	0	0	1	0	0	0	1	1	0
08-07-1999	49:55	0	0	1	0	0	0	1	3	0

Table 8
Imidacloprid techn.: Percentages mortality and percentages effect of the honeybees, offered 8.63 ng / 10µl sucrose-solution 50%.

		percentage mortality			percentage effect		
		Replicate			Replicate		
Date	Observation after (hours)	**1**	**2**	**3**	**1**	**2**	**3**
06-07-1999	4:38	0%	0%	10%	10%	60%	30%
07-07-1999	26:22	0%	0%	10%	10%	10%	10%
08-07-1999	49:55	0%	0%	10%	10%	30%	0%

Table 9
Amounts of imidacloprid techn. taken in and mortality and other effects during the observation period of the honeybees offered 4.09 ng / 10µl sucrose-solution 50%.

		Mortality (n bees)			paralysed / spasm (n bees)			frozen behaviour (n bees)		
		Replicate taken in / bee in µg			Replicate taken in / bee			Replicate taken in / bee		
Date	observation after (hours)	1 4.19 ng	2 3.86 ng	3 4.39 ng	1 4.19 ng	2 3.86 ng	3 4.39 ng	1 4.19 ng	2 3.86 ng	3 4.39 ng
06-07-1999	4:40	0	0	0	0	0	0	0	0	0
07-07-1999	26:26	0	0	0	0	0	0	0	0	0
08-07-1999	49:55	0	0	0	0	0	0	0	0	0

Table 10
Imidacloprid techn.: Percentages mortality and percentages effect of the honeybees, offered 4.09 ng / 10µl sucrose-solution 50%.

		percentage mortality			percentage effect		
		Replicate			Replicate		
Date	observation after (hours)	1	2	3	1	2	3
06-07-1999	4:40	0%	0%	0%	0%	0%	0%
07-07-1999	26:26	0%	0%	0%	0%	0%	0%
08-07-1999	49:55	0%	0%	0%	0%	0%	0%

Table 11
Amounts of Dimethoate 40% taken in and mortality and other effects during the observation period of the honeybees offered 0.82 µg / 10µl sucrose-solution 50%.

		mortality (n bees)			paralysed / spasm (n bees)			frozen behaviour (n bees)		
		Replicate taken in / bee in µg			Replicate taken in / bee			Replicate taken in / bee		
Date	observation after (hours)	1 0.76 µg	2 0.76 µg	3 0.77 µg	1 0.76 µg	2 0.76 µg	3 0.77 µg	1 0.76 µg	2 0.76 µg	3 0.77 µg
06-07-1999	4:37	2	5	1	2	1	1	0	0	0
07-07-1999	26:21	8	8	7	0	0	0	0	0	0
08-07-1999	49:56	8	8	8	0	0	0	0	0	0

Table 12
Dimethoate 40%: Percentages mortality and percentages effect of the honeybees, offered 0.82 µg / 10µl sucrose-solution 50%.

		percentage mortality			percentage effect		
		Replicate			Replicate		
Date	observation after (hours)	1	2	3	1	2	3
06-07-1999	4:40	20%	50%	10%	40%	60%	20%
07-07-1999	26:26	80%	80%	70%	80%	80%	70%
08-07-1999	49:55	80%	80%	80%	80%	80%	80%

Table 13
Amounts of Dimethoate 40% taken in and mortality and other effects during the observation period of the honeybees offered 0.55 µg / 10µl sucrose-solution 50%.

		mortality (n bees)			paralysed / spasm (n bees)			frozen behaviour (n bees)		
		Replicate taken in / bee in µg			Replicate taken in / bee			Replicate taken in / bee		
Date	observation after (hours)	1 0.50 µg	2 0.59 µg	3 0.51 µg	1 0.50 µg	2 0.59 µg	3 0.51 µg	1 0.50 µg	2 0.59 µg	3 0.51 µg
06-07-1999	4:45	0	0	0	0	1	0	0	0	0
07-07-1999	26:24	3	5	2	0	0	0	0	0	0
08-07-1999	49:45	3	6	3	0	0	0	0	0	0

Table 14
Dimethoate 40%: Percentages mortality and percentages effect of the honeybees, offered 0.55 µg / 10µl sucrose-solution 50%.

		percentage mortality			Percentage effect		
		Replicate			Replicate		
Date	observation after (hours)	1	2	3	1	2	3
06-07-1999	4:45	0%	0%	0%	0%	10%	0%
07-07-1999	26:24	30%	50%	20%	30%	50%	20%
08-07-1999	49:45	30%	60%	30%	30%	60%	10%

Table 15
Amounts of Dimethoate 40% taken in and mortality and other effects during the observation period of the honeybees offered 0.28 µg / 10µl sucrose-solution 50%.

		Mortality (n bees)			Paralysed / spasm (n bees)			frozen behaviour (n bees)		
		Replicate taken in / bee in µg			Replicate taken in / bee			Replicate taken in / bee		
Date	observation after (hours)	1 0.22µg	2 0.26 µg	3 0.27 µg	1 0.22µg	2 0.26 µg	3 0.27 µg	1 0.22µg	2 0.26 µg	3 0.27 µg
06-07-1999	4:46	0	1	0	0	0	0	0	0	0
07-07-1999	26:26	1	3	1	0	0	0	0	0	0
08-07-1999	49:55	1	4	1	0	0	0	0	0	0

Table 16
Dimethoate 40%: Percentages mortality and percentages effect of the honeybees, offered 0.28 µg / 10µl

		Percentage mortality			percentage effect		
		Replicate			Replicate		
Date	observation after (hours)	1	2	3	1	2	3
06-07-1999	4:46	0%	10%	0%	0%	10%	0%
07-07-1999	26:26	10%	30%	10%	10%	30%	10%
08-07-1999	49:55	10%	40%	10%	10%	40%	10%

Table 17
Sucrose-solution 50%: Amounts of test substance taken in and mortality and other effects during the observation period.

		Mortality (n bees)			paralysed / spasm (n bees)			frozen behaviour (n bees)		
		Replicate taken in / bee in µg			Replicate taken in / bee			Replicate taken in / bee		
Date	observation after (hours)	1	2	3	1	2	3	1	2	3
06-07-1999	4:46	1	0	0	0	0	0	0	0	0
07-07-1999	26:28	1	0	0	0	0	0	0	0	0
08-07-1999	49:59	1	1	0	0	0	0	0	0	0

Table 18
Sucrose-solution 50%: Percentages mortality and percentages effect of the honeybees.

		percentage mortality			percentage effect		
		Replicate			Replicate		
Date	Observation after (hours)	1	2	3	1	2	3
06-07-1999	4:46	10%	0%	0%	10%	0%	0%
07-07-1999	26:28	10%	0%	0%	10%	0%	0%
08-07-1999	49:59	10%	10%	0%	10%	10%	0%

MORTALITY AND OTHER EFFECTS

The concentrations Imidacloprid techn. offered and taken in per honeybee, do not cause mortality of the honeybees. However effects are observed. The most significant effect is the "frozen behaviour" at which the honeybees are motionless except for a little trembling of body parts like abdomen, antennae or tarsus. Some honeybees, which have taken in about 20 ng, show spasms and are paralysed.

STATISTICAL ANALYSIS

The data, used for the calculation of the ED_{50} (24 hours) of imidacloprid techn. and the LD_{50} (24 hours) of Dimethoate were corrected for control mortality (mean mortality after 24 hours).
For the correction, the formula of Schneider Orelli: % effect = ((b-k) / (100-k)) x 100
b = percentage mortality / test cage
k = percentage mortality of negative control (mean of the 3 test cages).
As there are no data on mortality, the LD_{50} of Imidacloprid techn. could not be calculated,.
The ED_{50} after 24 hours of Imidacloprid techn. calculated with the linear regression is 34 ng / honeybee. ($r^2 = 0.50$).

EVALUATION OF THE RESULTS

The data on effect vary a lot but the effect is clear. Amounts of 4.39 ng / honeybee or less do not result in any effect. Amounts of 6.83 ng imidacloprid techn. or higher result more or less in the described frozen behaviour.
It should be kept in mind that honeybees that would suffer these kind of effects in the field, would have little chance of survival.

VALIDY OF THE TEST

The LD_{50} of Dimethoate based on the data obtained in this test is 0.61 µg / honeybee (= 0.24 µg a.i. / honeybee). The acceptable range of the LD_{50} of Dimethoate in 24 hours is 0.05 to 0.4 µg a.i. / honeybee. The mortality in the control does not exceed 15%. The results in the positive and negative control meet the standards. The test can be considered as valid and the effects observed in the honeybees, fed with imidacloprid can be considered as caused by imidacloprid techn.