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# Determination of Thiamethoxam residues in honeybees by high performance liquid chromatography with an electrochemical detector and post-column photochemical reactor

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**Abstract**

A method for the determination of Thiamethoxam in bee samples was set up by means of high performance liquid chromatography with an electrochemical detector and post-column photochemical reactor (HPLC–h$\nu$–ED). Analytical method was based on a rapid sample extraction procedure with acetone, followed by chromatographic separation into a C18-RP column isocratically operated by 60 mM phosphate buffer/acetonitrile (75/25) mobile phase at pH 2.7. A photochemical reactor was used as a tool to verify and eventually quantify the presence of Thiamethoxam in the samples by distinguishing it from interference contribution. Detection was performed with a potential of 880 mV after a photoactivation with a 254 nm light. The least detectable dose was 0.002 mg kg$^{-1}$. Recovery rates ranged between 59.88 and 71.62%.


*Keywords:* Thiamethoxam; Neonicotinoid; Photoactivation; Electrochemical detector; Honeybee; HPLC; Pesticide

## 1. Introduction

The research development about the neonicotinoids toxicology aims to create molecules with a greater activity against sucking and chewing pests combined with a lower toxicity against bees and other handy insects. Therefore, the first generation, dominated by Imidacloprid® (Bayer, Leverkusen), evolved into the second one [1–5] which was inaugurated in 1998 by the introduction of Thiamethoxam® (Novartis, Switzerland). Thiamethoxam, (*EZ*)-3-(2-chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazinan-4-ylidene(nitro)amine, is a nitro-substituted neonicotinoid that acts agonistically on insect nicotinic acetylcholine receptors (nAChR) [6]. The molecule mimics the acetylcholine chemical messenger and it binds to the receptor site. This binding site is more abundant in insects than in warm-blooded animals making the chemical selectively more toxic to insects than warm-blooded animals. This blockage leads to the accumulation of acetylcholine resulting in the insect's paralysis and eventually in death. The oral $LD_{50}$ in bees, the dose that kills half (50%) of the tested bees, increased from 18 ng bee$^{-1}$ for Imidacloprid to 30 ng bee$^{-1}$ for Thiamethoxam [7–11]. Laboratory and field studies indicate that Thiamethoxam is highly toxic to honeybees by either contact or ingestion. Despite its high root systemicity, semi-field studies, where bees were exposed to plants grown from Thiamethoxam treated seeds, showed no effect such as mortality, foraging activity, flight intensities or behaviour. But because some decline in hive quality was observed [12], some doubt arose about the safety of Thiamethoxam. The lower toxicity with respect to other neonicotinoids could probably be not enough to completely wipe out the risk for apiculture.

The Thiamethoxam physicochemical properties render it useful for a wide range of application techniques, including foliar, seed treatment, soil drench and stem application.

The honeybee (*Apis mellifera*) is an important insect worldwide: its pollinate activity is important for the production of high-quality commercial seeds and fruits. The study of pesticide effects on the honeybee is, therefore, vital because of the need to control a wide variety of agricultural pests with insecticides without hurting bees that inadvertently come into contact with pesticides when foraging [13,14].

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Some authors' studies dealt with the determination of Thiamethoxam in various real samples by using different methods: LC–MS technique was used by Obana et al. [15] for vegetables and fruits and the detection limit was 0.01 mg $kg^{-1}$. A LC/ESI(+)/MS system was used for quantitative analysis of fruits and vegetables [16], drinking water [17] and honey [18]. Detection limits were 0.02 mg $kg^{-1}$ in fruits and vegetables, 0.01 μg $l^{-1}$ in drinking water and 0.01 mg $kg^{-1}$ in honey. The LC/MS/MS technique was applied by Zywitz et al. [19] for analysing fruits and vegetables with a detection limit of 0.003 mg $kg^{-1}$. The HPLC–UV system was applied to fresh and cooked vegetable samples by Singh et al. [20] with a detection limit of 0.04 mg $kg^{-1}$.

In our paper we present chromatographic and instrumental conditions, sample extraction procedure in case of bees, linearity range, repeatability and recovery rates of a new method based on reversed-phase HPLC with an electrochemical detector.

The detection of Thiamethoxam is preceded by photochemical activation to enhance sensitivity of the method. Moreover, the photoreactor allows the subtraction of some possible interferences which may lead to overestimation of Thiamethoxam in bad conditions bee samples [21,22].

## 2. Experimental

### 2.1. Materials and method

All solvents were HPLC grade (Carlo Erba Reagenti, Milano, Italia). Thiamethoxam stock solution was prepared by dissolving 0.014 g of the powder (97.0% certificated, Dr. Ehrenstorfer GmbH, Augsburg, Germany) in 10 ml of acetone and it was stored in freezer at −20 °C. Calibration curve was built by analysing seven different fresh dilutions in mobile phase of the stock solution: 0.01358, 0.02716, 0.05432, 0.13580, 0.27160, 0.54320, and 1.358 mg $l^{-1}$. The regression data were obtained from the average of three curve replicates. Three replicates per concentration were done.

The HPLC system was composed of an ESA Bioscence Inc. (Chelmsford, MA, USA) model 582 Solvent Delivery System pump, a pulse damper, a Rheodyne (Rohnert Park, CA, USA) 9725i injector, a reversed-phase Phenomenex® (Torrance, CA, USA) Luna C18(2) column (150 mm × 4.6 mm i.d., 5 μm particle size), an ICT BeamBoost® (Wien, Austria) photochemical reactor, equipped with a 254 nm UV lamp (Osram Sylvania ultraviolet G8W, Danvers, MA, USA) and a 5 m × 0.25 mm i.d. Teflon reaction coil.

Detection was performed with a model 5600A ESA Bioscence Inc. (Chelmsford, MA, USA) CoulArray® electrochemical detector. The first three electrodes were set, respectively, at 100, 125 and 150 mV and the fourth, the dominant channel, was set at 880 mV.

The column was operated at room temperature, isocratically, at a flow rate of 0.8 ml $min^{-1}$, using phosphate buffer/acetonitrile (75/25, v/v) at pH 2.70 adjusted by $H_3PO_4$ (85%, RPE, ACS-iso, Carlo Erba). Phosphate buffer was a 0.060 M aqueous solution of di-sodium hydrogen phosphate dihydrate (RPE, Carlo Erba, Milano, Italy). The mobile phase was filtered before using through a 0.22 μm Phenex nylon filter membrane under water pump suction.

All the bee samples used in the experiment were collected from the Institute apiary and they were stored in the refrigerator (−40 °C).

### 2.2. Real samples extraction

An amount of 3.5 g of bees was placed in a 50 ml beaker together with 40 ml of acetone. Sample was allowed to soak for 30 min in an ultrasonic bath (ultrasound power 120%) (Elma, CA, USA) at room temperature. The solution was then filtered through Celite® 545 (10 g ca, 0.02–0.1 mm particle size, Merck, Darmstadt, Germany) directly in a 150 ml round bottomed flask. After concentration in a small volume by means of a rotary evaporator under vacuum (bath temperature 40 °C, max 200 mbar), the residue was evaporated by $N_2$ current gas and redissolved in 1.0 ml of the mobile phase. Before injecting into the HPLC system, the residue was filtered through a Filtek filter syringe (RC 15 mm, 0.22 μm pore size). The injected volume was 0.020 ml.

### 2.3. Recovery tests

Bee samples in good state of conservation, definitely free of Thiamethoxam, were used for recovery tests. Before the extraction, a measured amount of a 10 mg $l^{-1}$ dilution in acetone of Thiamethoxam standard stock solution was added to bees after the thawing out. The rest of 15 min was left to let acetone evaporate before the extractive procedure.

Percentage recoveries were calculated at three different concentrations: 0.687, 0.343 and 0.172 mg $kg^{-1}$. Every test was repeated three times. The lowest concentration for the recovery test was calculated so that it was smaller than the $LD_{50}$ of 30 ng $bee^{-1}$ which corresponds to 0.200 mg $kg^{-1}$ ca.

## 3. Results and discussion

The electrochemical properties of the active ingredient were first investigated. For this purpose a Thiamethoxam standard solution of 1 mg $l^{-1}$ was injected into a Luna-C18 column operated with the same mobile phase as that used in the determination of Imidacloprid in our laboratory [21]. The flow rate was set at 1.0 ml $min^{-1}$ and a potential of 900 mV was used to ensure the possible result. In spite of the high potential, without the photochemical reactor, the detection limit (680 mg $l^{-1}$) was not satisfactory for the purpose of this study because of the low electroactivity of Thiamethoxam chemical structure.

Structural feature of the Thiamethoxam molecule shows a useful similitude with the Imidacloprid structure in the nitro group (Fig. 1): in the case of Imidacloprid such group is pointed as the site of the photochemical reaction that makes the molecule electrochemically detectable. Such behaviour is explainable with the transformation of the nitro group to hydroxy group as shown in the Fig. 1 [23,24]. The knowledge of this photochemical reaction let us foresee an improvement in sensitivity by the irradiation with a 254 nm light in Thiamethoxam determination too. Really the Thiamethoxam electrochemical activity was

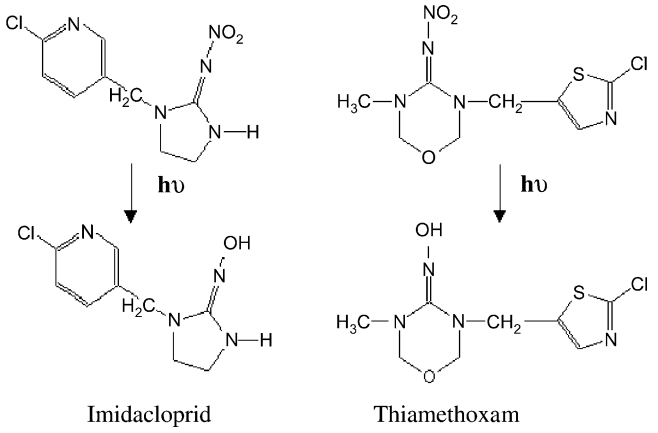


Fig. 1. Photochemical reactions on Imidacloprid (on the left) and on Thiamethoxam (on the right) caused by irradiation with 254 nm light.

increased by photoactivation and the sensitivity was so remarkably improved. Although it is not the aim of this work but it would be very interesting to verify the photochemical product of this molecule.

Hydrodynamic voltammogram (Fig. 2) was recorded for Thiamethoxam, using a standard of 0.6 mg $l^{-1}$, in order to determine the best voltage for the detection. The choice of the proper potentials entails a compromise between sensitivity, enhanced by increasing voltage, and selectivity, reduced by increasing voltage. In view of these considerations a dominant potential of 880 mV was chosen for the fourth electrode whereas the first three channels were set at 100, 125 and 150 mV. The low voltage of the first three channels is the consequence of a verified advantage: By concentrating the oxidation of the whole amount of Thiamethoxam in a single channel, the sensitivity was in fact maximized.

Afterwards the chromatographic conditions were optimized to avoid tailing peaks and to improve the peak shape: Mobile phase pH was, therefore, adjusted to 2.7 and the flow rate was decreased to 0.8 ml $min^{-1}$.

Calibration curve was obtained by the injection of seven different standard levels in the concentration range between 0.01358 and 1.358 mg $l^{-1}$: The response was linear and the regression equation was $y = 0.7436x - 2.1735$ where

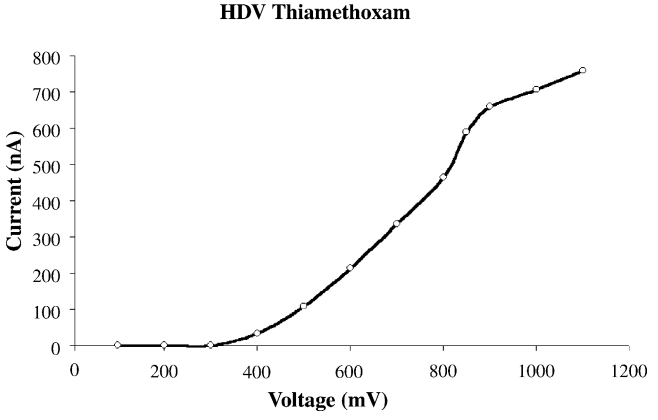


Fig. 2. Hydrodynamic voltammogram got by injecting at increasing potential (ranged from 100 to 1100 mV) a 0.6 mg $l^{-1}$ Thiamethoxam standard.

Table 1
Instrumental reproducibility by standard deviations (SD) at different Thiamethoxam concentrations

| Concentration level (mg $l^{-1}$) | Average peak height (nA) | SD (nA) |
|---|---|---|
| 0.01358 | 19.65 | 1.1 |
| 0.02716 | 36.50 | 2.1 |
| 0.05432 | 60.30 | 4.1 |
| 0.13580 | 203.00 | 8.5 |
| 0.27160 | 384.00 | 2.8 |
| 0.54320 | 719.50 | 0.7 |
| 1.35800 | 1830.00 | 0.0 |

Means of three replicates.

$y$ = concentration (μg $l^{-1}$) and $x$ = peak height (nA), with a slope standard deviation of 0.0014 ($N = 3$), intercept standard deviation of 2.1044 ($N = 3$), linear correlation coefficient of 0.9998 and a $yx$ standard error of 10.5491.

The reproducibility of the quantitative measurements was studied at every level of the calibration curve as shown in Table 1: results were satisfactory for all the considered concentration range.

The $LD_{50}$ for Thiamethoxam is 30 ng $bee^{-1}$ which is equivalent to 0.200 mg $kg^{-1}$ but in the case of neonicotinoids, subletal doses were often suspected to cause some threats to bees at concentrations as low as a few ng $g^{-1}$ [25–28]. That is the reason as to why the first goal of the present work was to reach a detection limit as low as possible. Such limit was investigated accounting as minimum peak height the value calculated as three times the standard deviation of the noise, resulting from injection of the mobile phase, plus the average height of all the noise peaks. This value was experimentally established to correspond to 0.0075 mg $l^{-1}$. Calculating the bee amount considered in the proposed analysis, the instrumental detection limit corresponds to 2.1 ng $g^{-1}$ in bee sample which abundantly fulfills the expectations of this study.

The detector involved in the present method often allows a very fast and easy procedure for preparing the sample. The first attempt was, therefore, very raw: The extraction by acetone and the filteration through a 0.22 μm membrane were proved to be enough for determining the active ingredient without any doubt of interferences. The extraction procedure was applied to many bee samples of different origin and in different driven states (mould, putrefaction). In the case of non-degraded bee samples the chromatogram was free of interference peaks. For bad state bee samples it was instead capitalized as an additional tool: the knowledge about the different photochemical properties of Thiamethoxam with respect to coeluted interferences depending on whether the BeamBoost® reactor is on or off. As long as the active compound concentration is lower than the detec-

Table 2
Recovery rates on bee samples; conditions are described in the text

| Sample | Fortification (mg $kg^{-1}$) | Recovery (%) | DS |
|---|---|---|---|
| Bees | 0.687 | 71.18 | 5.37 |
| Bees | 0.343 | 71.62 | 8.55 |
| Bees | 0.172 | 59.88 | 2.68 |

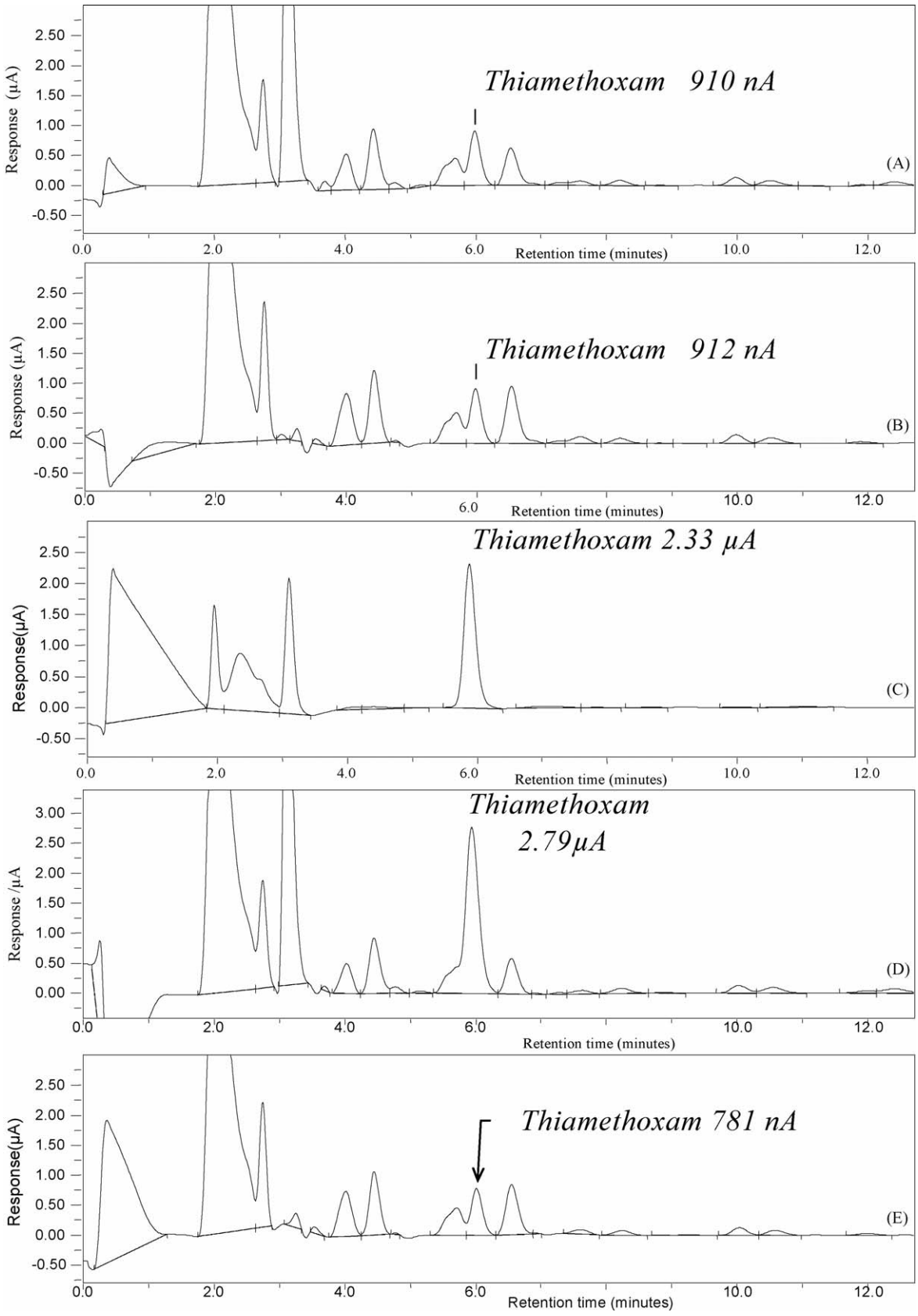


Fig. 3. Photoactivation effects on bee samples: control bad state bee sample acquired with BeamBoost® on (A) and off (B); Thiamethoxam 2.716 mg $l^{-1}$ standard detected with the BeamBoost® on (C); control bad condition bee sample after fortification with the active ingredient (2.716 mg $l^{-1}$, equivalent to 0.776 mg $kg^{-1}$ bee) acquired with the BeamBoost® on (D) and off (E). Conditions and discussion are in the text.

tion limit without photoactivation (680 mg $l^{-1}$), Thiamethoxam peak completely disappears when the photochemical reactor is switched off, whereas no variation occurs if the only contribution to peak arises from interferences. This is a clear consequence of the nature of degradation products: Ketones, aldehydes, esters, amines and alcohols, deriving from putrefaction process in bees, are in fact electroactive in both the presence and the absence of photoactivation.

If a peak appears in BeamBoost® on condition at Thiamethoxam retention time, the second injection of the same sample but in BeamBoost® off condition will confirm the presence of Thiamethoxam only if the peak height decreases or disappears. The difference in the height of peaks detected with the BeamBoost® on and off gives a correct quantification of Thiamethoxam sample contamination. In Fig. 3A, a control bad state bee sample analysed with the BeamBoost® on is shown; Fig. 3B shows the chromatogram of the same control sample analysed with the BeamBoost® off: Peak height was 910 nA in A and it only increased to 912 nA in B as it was expected.

Fig. 3D shows the chromatogram of a control bad condition bee sample after fortification with the active ingredient (2.716 mg $l^{-1}$, equivalent to 0.776 mg $kg_{bee}^{-1}$) acquired with the BeamBoost® on, whereas Fig. 3E shows the BeamBoost® off acquisition. In this case the peak height only decreased but it did not disappear and the interference contribution was separated from the total peak height. Fig. 3C shows a Thiamethoxam standard at 2.716 mg $l^{-1}$.

The use of photochemical reactor as a quantification tool is allowed also by the excellent repeatability of the chromatographic system: The percentage error was calculated with respect to the average peak area values ($N = 3$) of each point of calibration curve (Table 1). Results were in the range between 0.12 and 5.82%.

Finally the recovery rates of Thiamethoxam in bees were determined through fortification tests, in which measured amounts of the active ingredient at three different levels (0.687 mg $kg_{bee}^{-1}$, 0.343 mg $kg_{bee}^{-1}$ and 0.172 mg $kg_{bee}^{-1}$) were added to control samples, prior to extraction. Every test was repeated three times. The results could be considered suitable for the detection of Thiamethoxam in bees (Table 2).

## 4. Conclusion

The photochemical reactor has resulted in an essential tool both to reach a satisfactory sensitivity and to confirm Thiamethoxam contamination in degraded samples. Photoactivation furthermore allows the exact quantification of the active ingredient by subtracting the interference contribution from the peak total height.

The nonoccurrence of a clean-up procedure turns in recovery's advantage and permits an economical analysis, a low environmental impact and a faster result with respect to other analytical procedure.

The detection limit of the new proposed method is the lowest compared with the methods mentioned as references: 0.002 mg $kg^{-1}$ against 0.003 mg $kg^{-1}$ which is the lowest between the reported papers.

Linearity range and recovery rates are suitable for the detection of a low Thiamethoxam quantity in honeybee samples.

The Thiamethoxam detection limit without photoactivation corresponds to the highest quantifiable concentration without any doubt about the interference contribution. Such limit is, however, equivalent to a quantity of active compound (194 mg $kg_{bee}^{-1}$) largely higher than the quantity usually found by our tests in real bee samples: even though the samples came from apiary where mortality was high or complete, they contained indeed at most 1–2 mg $kg^{-1}$ of Thiamethoxam.

The results obtained in this study lead us to conclude that the proposed HPLC–h$\nu$–ED method may be conveniently applied for bee health control projects as well for biomonitoring projects.

## References

[1] P. Maienfish, H. Huerlimann, A. Rindlisbacher, L. Gsell, H. Dettwiler, J. Haettenschwier, E. Sieger, M. Walti, Pestic. Sci. 57 (2001) 165.
[2] S.B. Soloway, A.C. Henry, W.D. Kollmeyer, W.M. Padgett, J.E. Powell, S.A. Roman, C.H. Tieman, R.A. Corey, C.A. Home, in: H. Geissbuhler, G.T. Brooks, P.C. Kearney (Eds.), Advances in Pesticides Sciences, Part 2, Pergamon Press, New York, USA, 1979, p. 206.
[3] I. Minamida, K. Iwanaga, T. Tabuchi, I. Aoki, T. Fusaka, H. Ishizuka, T. Okauchi, J. Pestic. Sci. 18 (1993) 8.
[4] H. Takahashi, J. Mitsui, N. Takakusa, M. Matsuda, H. Suzuki, K. Ishimitzu, T. Kishimoto, Proceeding Bringhton Crop protection Conference—Pest and Diseases, Farnham, Surrey, UK, 1992, p. 89.
[5] P. Maienfisch, F. Brandl, W. Kobel, A. Rindlisbacher, R. Senn, in: I. Yamamoto, J.E. Casida (Eds.), Nicotinoid Insecticides and the Nicotinic Acetylcholine Receptor, Springer-Verlag, Tokio, Japan, 1999, p. 177.
[6] R. Nauen, U. Ebbinghaus-Kintscher, V.L. Salgado, M. Kaussmann, Pestic. Biochem. Phys. 76 (2003) 55.
[7] S. Suchail, D. Guez, L.P. Belzunces, Environ. Toxicol. Chem. 19 (2000) 1901.
[8] S. Suchail, D. Guez, L.P. Belzunces, Environ. Toxicol. Chem. 20 (2001) 2482.
[9] R. Steffens, H. Lin, Proceeding of XXI International Congress of Entomology. Abstract book, vol. 1, Iguaçu Falls, Brazil, 2000, p. 322.
[10] C. Elbert, C. Erdelen, J. Kuehnhold, R. Nauen, H.W. Schmidt, Y. Hattori, Proceeding Bringhton Crop protection Conference—Pest and Diseases, vol. 2A-1, Bringhton, UK, 2000, p. 21.
[11] T. Iwasa, N. Motoyama, J.T. Ambrose, R.M. Roe, Crop Prot. 23 (2004) 371.
[12] National Registration Authority for Agricultural and Veterinary Chemicals (NRA), Commonwealth of Australia Gazette NRA 1, 2001, 13.
[13] A. Rortais, G. Arnold, M.P. Halm, F. Touffet-Briens, Apidologie 36 (2005) 71.
[14] E.L. Atkins, in: J.E. Graham (Ed.), The Hive and the Honeybee, Dadant and sons, Hamilton, 1992, p. 1153.
[15] H. Obana, M. Okihashi, K. Akutsu, Y. Kitagawa, S. Hori, J. Agric. Food Chem. 51 (2003) 2501.
[16] A. Di Muccio, P. Fidente, D. Attard Barbini, R. Dommarco, S. Seccia, P. Morrica, J. Chromatogr. A 1108 (2006) 1.
[17] S. Seccia, P. Fidente, D. Attard Barbini, P. Morrica, Anal. Chim. Acta 553 (2005) 21.
[18] P. Fidente, S. Seccia, F. Vanni, P. Morrica, J. Chromatogr. A 1094 (2005) 175.
[19] D. Zywitz, M. Anastassiades, E. Scherbaum, Proceeding of of EPRW 2004-V European Pesticide Residues Workshop, Stockholm, Sweden, 2004, p. 77.
[20] S.B. Singh, G.D. Foster, S.U. Khan, J. Agric. Food Chem. 52 (2004) 105.
[21] M. Rancan, A.G. Sabatini, G. Achilli, G.C. Galletti, Anal. Chim. Acta 555 (2006) 20.

[22] P. Bocchini, C. Andalò, R. Pozzi, G.C. Galletti, A. Antonelli, Anal. Chim. Acta 441 (2001) 37.
[23] J.L. Vilchez, R. El-Khattabi, R. Blanc, A. Navalón, Anal. Chim. Acta 371 (1998) 247.
[24] J.L. Vilchez, M.C. Valencia, A. Navalon, B. Molinero-Morales, L.F. Capitan-Vallvey, Anal. Chim. Acta 439 (2001) 299.
[25] P. Bonnafe, Lapis 8 (2000) 19.
[26] L. Bortolotti, C. Porrini, G. Sbrenna, Informatore Fitopatologico 3 (2002) 66.
[27] P. Bonnafe, Lapis 8 (2000) 19.
[28] C. Porrini, L. Monaco, Lapis 9 (2001) 8.