DOI : 10.35124/bca.2019.19.1.1361

Biochem. Cell. Arch. Vol. 19, No. 1, pp. 1361-1367, 2019 | www.connectjournals.com/bca | *ISSN 0972-5075*

ESTIMATION OF IMIDACLOPRID RESIDUES ON HONEYBEES USING THE QUECHERS METHOD BY HPLC

Suzan D. Hadi[1], Kameela W. Shaher[1] and Abdulkareem J. Ali[2]

[1]Collage of Agricultural Engineering Sciences, University of Baghdad, Iraq.
[2]Ministry of Agriculture, Iraq.

(Accepted 10 February 2019)

ABSTRACT : The honeybee *Apis mellifera* is known to be an ecologically and economically important pollinator worldwide. Recent declines in bee populations have prompted numerous studies on the factors that influence their vitality. The major factor contributing to current declines in bee populations is insecticides, the most importantly is the neonicotinoids, and has been singled out due to its extensive use in crop protection. The most commonly used pesticide in this group is the imidacloprid; therefore, it has been studied.

Residues of imidacloprid were measured in honeybees in Baghdad, Iraq. Imidacloprid was applied at the recommended rate (25 ml ai 100^{-1}). Samples were collected at (48 h, 1, 2, 3, 4, 5) weeks after application. Analysis was carried out by the QuECHERS method using HPLC. The mean recoveries obtained for spiked samples of honeybees at two fortification levels (0.1 and 0.5 mg/kg) ranged 104-96%. Limits of detection (LOD) were 0.1 ng/g and limit of quantification (LOQ) were 0.01 ng/g. The mean residues for samples collected at (48 h , 1 ,2, 3, 4 ,5) weeks after application is 1.06, 0.26, 1.14, 0.47, 1.47, B.O.D, respectively.

According to our results, it is deduced that the residue of the pesticide in honey bee workers in the fifth week is less than MRL. The increase and reduction in pesticide residues in honeybees is likely due to several factors. The most important of which is the method and time of spraying, the time of the pesticide permanence as well as the age of the bee, the younger the bee, the less tolerant to the pesticide due to the few proteins responsible for the destruction of the pesticide unlike the older workers. As well as storage of the pesticide in fatty bodies, which are characterized by a high level of fat, which affects the polarity and non-degradation of the pesticide that will lead to the pesticide accumulation. Moreover, the workers might be exposed to the pesticide directly in addition to its feeding inside the beehive on food containing the pesticide.

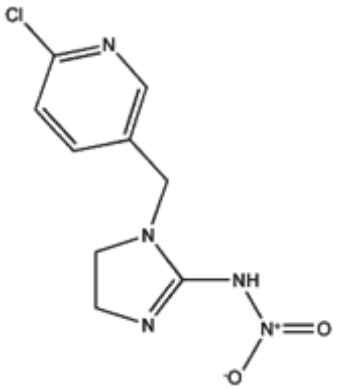
Key words : Honeybees, Imidacloprid residue, HPLC, QuECHERS method.

INTRODUCTION

Honey bees are an environmentally and economically beneficial insect because their role in pollinating a wide range of plants in addition to benefiting from it and its products as an indicator and guide to environmental pollution, industrial or agricultural (FAO, 2008). The pesticide reaches honeybees and its products through pollen, nectar and water (Chauzat and Faucon, 2007) during volatilization during plowing (Greatti *et al*, 2003) and through plant secretions (Girolami *et al*, 2009). Reports in recent years in some European and American countries have revealed the disappearance of some honeybee colonies. After several studies, several factors have been found to cause the decline of honeybee colonies, including viral diseases, mobile phone networks, food stress, environmental conditions, Varroa, heavy metals and pesticides. The most toxic pesticides were divided into 5 groups: organic phosphorus pesticides, carbamate pesticides, pyrothroids and nicotinic pesticides, the latter being the most dangerous and most toxic group for honeybee colonies (Laurino *et al*, 2011; Suchail *et al*, 2001; Lwasa *et al*, 2004). This group was first introduced in the 1990s and became the most widely used pesticide in various countries (Jeschke *et al*, 2011; Kathleen and Casida, 2013). Its use was increased early in the century because it is a systemic pesticide that enters through the roots and travels through tissue vessels to the leaves, which makes the plant protected against insects that feed on it. This precautionary system has been used very widely. In 2011, in the United States of America, this group of pesticides was used in maize plants, ranging from 79-100% ha (Douglas and Tooker, 2015) only about 5% of these pesticides are taken by the plants, while the other proportion is spread in the environment (Sur and Stork, 2003).

In Germany and Italy, concerns about the impact of this group of pesticides on non-target organisms are increasing because of its spread in the dust from the seed drilling machines, leading to poisoning of honey bee colonies (Pistorius *et al*, 2009; Bortolotti *et al*, 2003). The residues of these pesticides were detected in agricultural soils, pollen and nectar for crops treated in particularly with imidacloprid (Bonmatin *et al*, 2007). In another study, samples were collected from a variety of potential exposure methods near agricultural fields and analyzed to determine whether pesticides existed, which included pesticide residues from nectar, pollen, or dust from uncultivated tillage. Bees may be exposed to these compounds and other agricultural pesticides in several ways throughout the search for food (Krupke *et al*, 2012).

Imidacloprid is one of the most important pesticides in the nicotine group and its chemical name is *N*-{1-[(6-Chloro-3-pyridyl) methyl]-4, 5-dihydroimidazol-2-yl} nitramide. Its chemical composition is:



Its average field persistence is estimated to be 21-26 days and is characterized by its efficacy against insects of Homoptera, Diptera, Coleoptera and Lepidoptera (Mullins *et al*, 1993). It is used by spraying the leaves of the plants, spraying the soil and seed treating (Elbert *et al*, 1998). This pesticide was widely used because it is considered safe for non-target organisms as well as for permanent use in the IPM system (James *et al*, 2011). In addition, imidacloprid pesticide was used as an alternative to other pesticides such as the group of organic phosphorus and pyrothroids as a more specialized specialty for targeting insects (Lagadic, 1993).

This pesticide is highly specialized as it is found to have low effectiveness against insects such as *Heliothis virscens* and *Spodoptera littoralis*, both of which are multi-feed insects. The effect of the pesticide after 48 hours with LD50 at a concentration of 350 mg / ng and 650 mg / ng respectively (Lagadic, 1993). On the other hand, it was found to have a strong effect on absorbent insects such as *Myzus persicae* at a concentration of 30 ng/mg within 48 hours of exposure (Elbert, 1991). A study of imidacloprid residues with an average of 2- 3.9 ìg/ kg in pollen and less than 2 ìg/kg in treated corn nectar, sunflower and cabbage (Bonmatin *et al*, 2003).

MATERIALS AND METHODS

Preparation of the imidacloprid active ingredient standard solution

A total of 0.005 g of the active ingredient of the imidacloprid was dissolved in 100 ml of acetonitrile. A dilute solution was prepared at 10 ppm concentration by taking 1 ml of the standard solution and complementing the volume to 100 mL with acetonitrile. The solutions were stored at 4°C and in the dark.

Calibration solutions

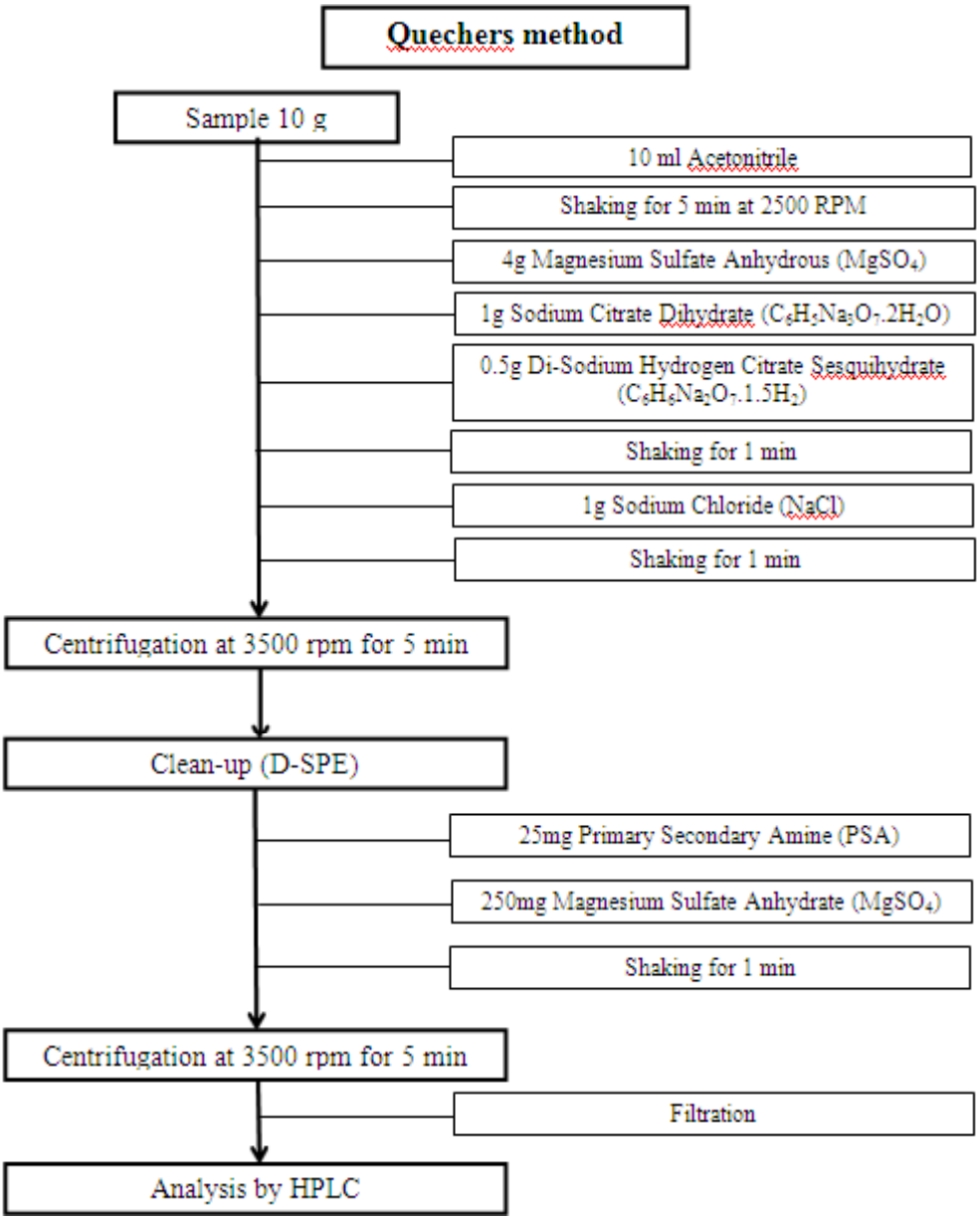
A spiked control solution was prepared of honey bees containing 1 mg/kg of active ingredient by taking 10 mL of standard solution and drying it with N_2 and dissolved with 10 ml blank extract of honeybees. The solutions were stored at 4°C and in the dark.

Honeybees' colony and sampling

Hives were placed at the University of Baghdad overlooking the Tigris River. Three Hives were randomly distributed in the orchard, which contained various plants such as papaya, Lupus, sunflower, cucumber and eucalyptus trees as well as flowering bushes. Comparative samples were taken for the honeybee before spraying the pesticide, and then, the imidacloprid pesticide was sprayed on 19/5/2018, with mixing ratio of 25 ml per 100 liters on the cultivated plants and the neighboring fields. The samples of dead bees were collected after spraying the pesticide, which is characterized by the symptoms of poisoning and by extending the parts of the mouth forward. The samples were collected during 48 hours, 1, 2, 3, 4 and 5 weeks) with three replicates each time. All samples were stored at -18°C in the dark until extraction.

Sample preparation

The method CEN 15662 QuECHERS (Anastassiades *et al*, 2013) was used. As in the scheme (1), 5 grams of dead bees' samples were taken and mixed vigorously with 7.5 ml water to ensure consistency. 10 ml of ACN were placed in 50 ml Teflon tubes and vortexed for 5 minutes. Anhydrous Magnesium Sulphate, Trisodium Citrate and Disodium Hydrogen Citrate) salts were added and vortexed again for 1 minute and then sodium salt was added and shacked by hand for 1 minute.Samples were centrifuged for 3500 rpmfor 5 minutes to separate the water layer from the organic layer. Aliquot of upper layer were transported to d-SPE columns containing (PSA sorbent and anhydrous $MgSO_4$) for cleanup to remove



Schem 1 : Queechers method of extraction.

polar substances present in the matrix. Extracts were centrifuged for 1 min at 6000 rpm to separate solids from solution. The leachate were purified and placed in the 1 ml tubes and kept at a temperature of -18°C in a dark place.

Method validation

In order to verify the accuracy of the analysis method, it is preferable to specify the LOQ limit of quantification and limits of detection (LOD). Extraction efficiency was determined by recovery test by using concentrations of 0.5 and 0.1 mg/kg of honeybee samples, extracted and purified separately with the use of a known standard pesticide sample to compare it with the two concentrations.

Calibration curve

The calibration curve was prepared using concentrations of 0.01, 0.02, 0.05, 0.1, 0.2 and 0.5 mg / kg by preparing them from calibration solutions and bee samples.

Optimaization

A standard solution of 1 mg/kg was prepared, as well as a blank honeybee spiked at 0.5 kg/mg concentration, analyzed by HPLC and the retention time (Rt) of the pesticide and its behavior in the honey bee sample were determined.

HPLC instrumentation

The HPLC system used for the determination of imidacloprid (Alyaseri *et al*, 2012; Rao *et al*, 2012) in

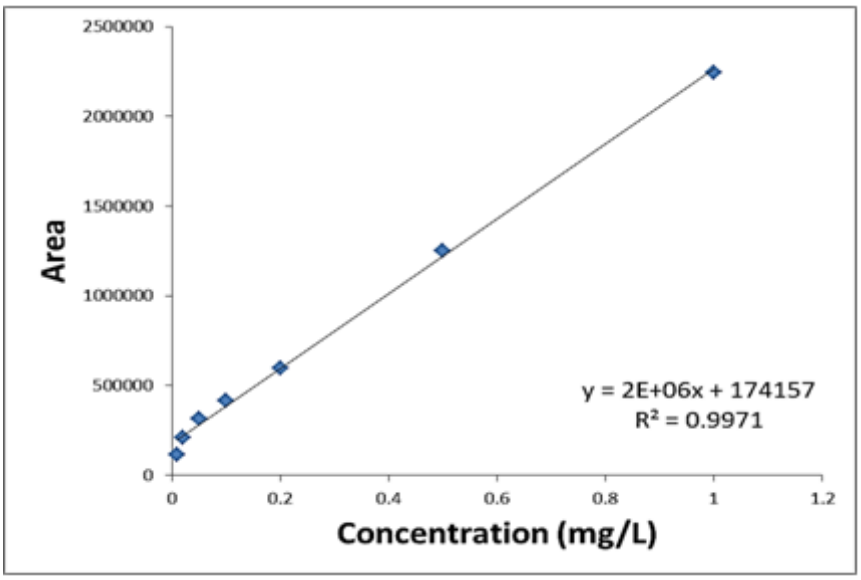


Fig. 1 : Linearity curve for imidacloprid.

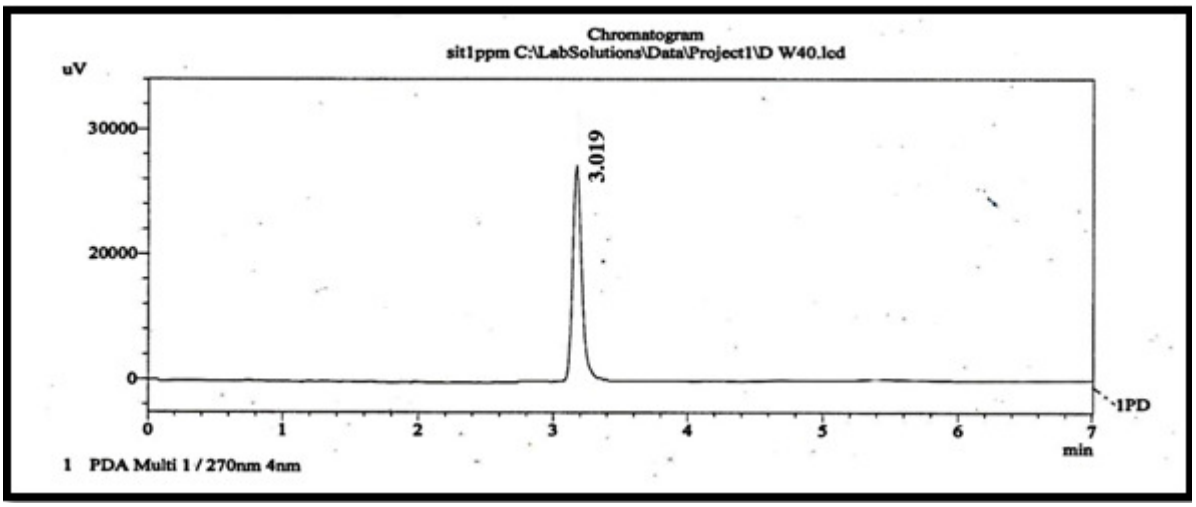


Fig. 2 : A typical HPLC chromatogram for imidacloprid.

honeybees consisted of Shimadzu HPLC with LC-20AT pump and SPD- 20A and a reversed Phase C-18 analytical column of 250 mm×4.6 mm and particle size 5.0 ì m (Phenomenex). Column temperature was maintained at 30°C. The injected sample volume was 10 ìL. Mobile Phases were a (CAN) and Milli-Q water (70:30(v/v)). The flow-rate used was kept at 1 mL/min. The detector wavelength was 270 nm. The external standard method was used for these analyses.

RESULTS AND DISCUSSION

Method validation and performance

It was found that the efficiency of the Recovery when using the Quechers method was a rapid, accurate and effective way to determine imidacloprid residues. samples of honey bees containing the pesticide with 0.1 and 0.5 kg/ìg concentrations were 89 and 104% respectively and are acceptable within the limits set by SANCO which should be 20-120% (SANCO, 2009).

The purpose of the validation of the extraction method is to confirm the analysis and the exact methodology, which is repeatable, which has been confirmed by using the limit of detection (LOD) criteria, which is the measurement of the least concentration that gives a response sensed by the apparatus. in addition, the LOQ limit of quantification, which is known as the lowest concentration that gives a quantitative response that can be measured at a standard deviation of less than 20% (Vryzas and Papadopoulou-Mourkidou, 2002). It was found that the LOD was 0.1 g/ng is the lowest concentration sensed by the device. While, LOQ was (0.01 g/ng) which was less than 20%, considerably below the European Food Safety Authority (EFSA, 2013).

R2 found that the linear relationship of the concentrations used in Table 1 was for all concentrations

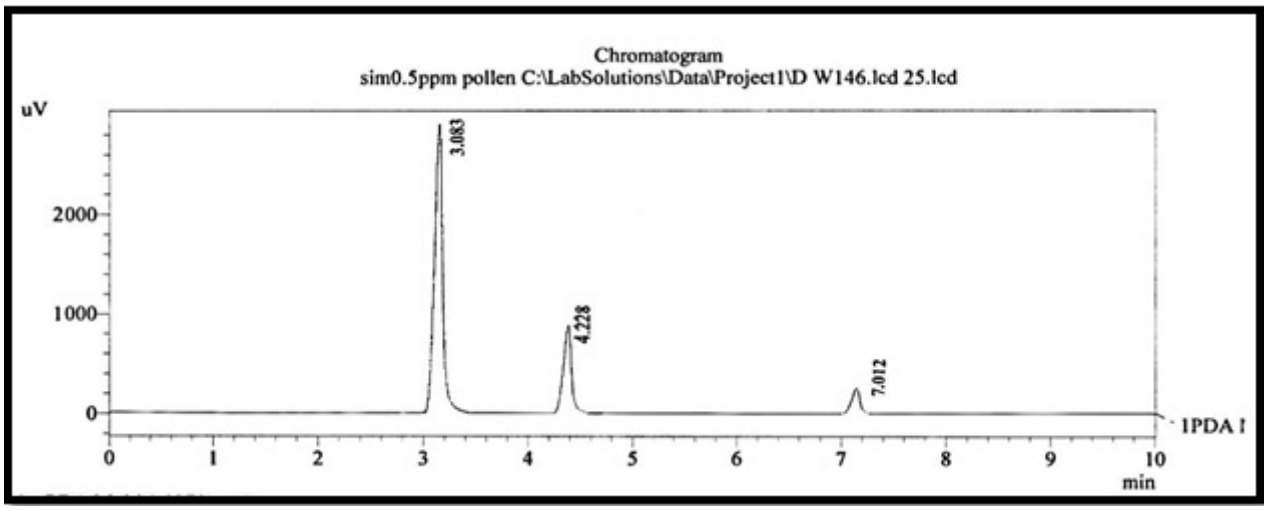


Fig. 2 : A typical HPLC chromatogram for honeybees and imidacloprid.

Table 1 : Linearity of imidacloprid reference standard.

Conc. (mg/L)	Replication	Area	Mean area
0.01	R1	118372	114310
	R2	110248	
0.02	R1	212345	212233
	R2	212121	
0.05	R1	316821	313638.5
	R2	310456	
0.1	R1	417345	415954.5
	R2	414564	
0.2	R1	598739	598739
	R2	598739	
0.5	R1	1245343	1249940
	R2	1254537	
1	R1	2243545	2242889.5
	R2	2242234	
		Intercept	2E+06x
		Slope	174157
		Correlation Coefficient	0.9971

used and the correlation coefficient was 0.9971 and is consistent with the results of SANCO (2009).

It was observed that the retention time of imidacloprid is around 3.01 in all treatments where Fig. 2 represents the analysis of the imidacloprid standard solution, and Fig. 3 represents the treatment of honey bees containing pesticide residues.

Imidacloprid Residue Levels in Honeybees

It was found that the Imidacloprid residues at different periods in honey bee samples as shown in Table 2 that the average of residues after 48 hours of spraying was1.06 mg/kg. While after 7 days, a decrease in pesticide residues at an average of 0.26 kg/mg was observed. On the other hand, after 14 days a rise in the level of residues in the body of honeybees at the rate of 1.14 kg/mg was observed. While after 21 days, the pesticide residues decreased by an average of 0.47 kg/mg after 28 days, residues was the highest in honey bees workers at the rate of 1.47 kg \ mg. additionally, after 35 days, it was noted that no residues was found.

It is concluded from the above that the reason for the presence of high concentrations of pesticide residues in honey bee workers' samples after 48 hours of treatment is that the pesticide is not degraded or dissolved in the treated plants flowers. Moreover, the reason for the residues decrease in the samples after the first week is due to its absorption by plants in addition to the degradation and the emergence of new flowers as well as individual differences of bees and its' age, as when the bees were young, it is less tolerant and resistant to the pesticide and therefore its death in short periods of time (Smirle, 1993). On the other hand, we note from the results shown in Table 2 that there is an increase in pesticide residues after 14 days of treatment. This is due to the fact that the flying bees are not exposed to the pesticide during flight and search for food only, but during feeding on the bread of bees inside the hives containing honey and pollen treated with pesticide and this corresponds to what is mentioned in Crailsheim *et al* (1992).

While the decrease in pesticide residues concentrations after 21 days is likely due to the young flying honeybees' workers, when they werein larva age, they fed on food containing pesticide and when they fly in search of food, they will be exposed to the pesticide with low concentrations, but adequate to cause death and this result corresponds to Wolschin *et al* (2007), Smirle (1993). After

Table 2 : Method performance and validation: Limits of Detection (LOD) and Quantification (LOQ), recoveries (%), repeatability (RSD%) and Coefficient of correlation(r^2) in honeybees.

Recovery N=3		MRL (ng/g)	LOD (ng/g)	LOQ (ng/g)	Time	RSD%	r^2
*l*g 10ng	*l*g 50ng						
104%	96%	50	0.1	0.01	48 h	1.06±0.75	0.9971
					1 week	0.26±0.06	
					2 week	1.14±0.07	
					3 week	0.47±0.15	
					4 week	1.47±0.40	
					5 week	N.F	

28 days, the presence of pesticide residues in high concentrations is due to its accumulation in fatty bodies due to the high fat levels and thus reducing the polarity, which prevents the degradation of the pesticide and accumulation in the body. In addition to the individual and age differences between honeybee colonies in tolerating pesticides as mentioned above, as well as its feeding inside the hives on food containing pesticides. These findings corresponds to the results from Schmehl *et al* (2014), Rand *et al* (2015).

REFERENCES

Food and Agriculture Organization of the United Nations [FAO] (2008) A contribution to the international initiative for the conservation and sustainable use of pollinators: rapid assessment of pollinators status. Food and Agriculture Organization of the United Nations, Rome, Italy.

The South African National Civic Organization [SANCO] (2009) Method Validation And Quality Control Procedures For Pesticide Residues Analysis in Food And Feed. Document No. 10684/2009.

Bonmatin J M, Moineau I, Charvet R, Fleche C, Colin M E and Bengsch E R (2003) A LC/APCI-MS/MS method for analysis of imidacloprid in soils, in plants and in pollens. *Anal Chem*. **75**, 2027–2033.

Bonmatin J M, Marchand P A, Cotte J F, Aajoud A, Casabianca H, Goutailler G and Courtiade M (2007) Bees and systemic insecticides (imidacloprid, fipronil) in pollen: subnano-quantification by HPLC/MS/MS and GC/MS. In: Del Re AAM, Capri E, Fragoulis, Trevisan M (eds) *Environmental fate and ecological effects of pesticide*. La Goliardica Pavese, Pavia, (It), pp. 827–824.

Bortolotti L, Montanari R, Marcelino J, Medrzycki P, Maini S and Porrini C (2003) Effects of sub-lethal imidacloprid doses on the homing rate and foraging activity of honey bees. *Bulletin of Insectology* **56**(1), 63-67.

Chauzat M P and Faucon J (2007) Pesticide residues in beeswax samples collected from honey bee colonies (*Apis mellifera* L.) in France. *Pest Manag Sci*. **63**, 1100–1106.

Crailsheim K (1992) The flow of jelly within a honeybee colony. *J. Comp. Physiol. B* **162**, 681–689.

Crailsheim K, Schneider L H W, Hrassnigg N, Bühlmann G, Brosch U, Gmeinbauer R and Schöffmann B (1992) Pollen consumption and utilization in worker honeybees (*Apis mellifera carnica*): dependence on individual age and function. *J. Insect Physiol.* **38**, 409–419.

Douglas M R and Tooker J F (2015) Large-Scale Deployment of Seed Treatments Has Driven Rapid Increase in Use of Neonicotinoid Insecticides and Preemptive Pest Management in U.S. Field Crops. *Environmental Science & Technology* **6** (2), 13-25.

Elbert A, Overbeck H, Booker B, Hertwig J and Erdelen G (1991) Imidacloprid a new systemic insecticide. *Pflanzenschutz Nachrichten Bayer* **11**, 113-136.

Elbert A, Nauen R and Leicht W (1998) Imidacloprid, a novel chloronicotinyl insecticide: biological activity and agricultural importance.- In: Insecticides with novel modes of action: Mechanism and application (Ishaaya I and Degheele D eds). Springer Verlag, Berlin Heidelberg, Germany, 50-74.

Girolami V, Mazzon L, Squartini A, Mori N, Marzaro M and Bernardo A (2009) Translocation of neonicotinoid insecticides from coated seeds to seedling guttation drops: a novel way of intoxication for bees. *J. Econ. Entomol*. **102**, 1808–1815.

Greatti M, Sabatini A, Barbattini R, Rossi S and Stravisi A (2003) Risk of environmental contamination by the active ingredient imidacloprid used for corn seed dressing. Preliminary results. *Bull Insectology* **56**, 69–72.

James R and Xu J (2011) Mechanisms by which pesticides affect insect immunity. *J. of Invert. Path*. **109**(2), 175-182.

Jeschke P, Nauen R, Schindler M and Elbert A (2011) Overview of the status and global strategy for neonicotinoids. *J. Agri. Food Chem*. **59**, 2897-2908.

Kathleen A D and Casida J (2013) Neuroactive Insecticides: argets, Selectivity, Resistance, and Secondary Effects. *Annual Review of Entomology* **58**(1), 99-117.

Krupke C H, Hunt G J, Eitzer B D, Andino G and Given K (2012) Multiple Routes of Pesticide Exposure for Honey Bees Living Near Agricultural Fields. *PLoS ONE* **7**(1), e29268. doi:10.1371/journal.pone.0029268.

Lagadic L and Bernardm L (1993) Topical and oral activities of imidacloprid and cyfluthrin aginst susceptible laboratory strains of *Heliothis virescens* and *Spodoptera littoralis*. *Pestic Sci*. **38**, 323-328.

Laurino D, Porporato M, Patetta A and Manino A (2011) Toxicity of neonicotinoid insecticides to honey bees: laboratory tests. *Bulletin of Insectology* **64**(1), 107-113.

Lwasa S and Nyakaana J B (2004) Effectiveness of Development Planning in Reduction of Poverty; Kawempe Division, Kampala. Kampala, Makerere University: 89.

Mullins J (1993) Imidacloprid a new mitrogunidin insecticide, pest control with enhanced environmental safety American Chemical Society Symposium, series 524, pp. 183-198 American Chemical Society, Washington, DC.

Pistorius J, Bischoff G, Heimbach U and Stähler M (2009) Bee poisoning incidents in Germany in spring 2008 caused by abrasion of active substance from treated seeds during sowing of maize. *Julius-Kühn Archiv*. **423**, 118-126.

Rand E S E, Smit S, Beukes M, Apostolides Z, Pirk C W W and Nicolson S W (2015) Detoxication mechanisms of honey bees

(*Apis mellifera*) resulting in tolerance of dietary nicotine. *1SCIENTIFIC RepoRts* | **5**, 11779 | DOI: 10.1038/srep11779

Schmehl D R, Teal P E A, Frazier J L and Grozinger C M (2014) Genomic analysis of the interaction between pesticide exposure and nutrition in honey bees (*Apis mellifera*). *J. Insect Physiol*. **71**, 177–190.

Smirle M J (1993) The influence of colony population and brood rearing intensity on the activity of detoxifying enzymes in worker honey bees. *Physiol. Entomol*. **18**, 420–424.

Suchail S, Guez D and Belzunces L P (2001) Discrep-ancy between acute and chronic toxicity induced by imidacloprid and its metabolites in *Apis mellifera*. *Environmental Toxicology and Chemistry* **20**(11), 2482- 2486.

Sur R and Stork A (2003) Uptake, translocation and metabolism of imidacloprid in plants. *Bull. Insectol*. **56**, 35-40.

Vryzas Z and Papadopoulou-Mourkidou E (2002) Determination of Triazine and Chloroacetanilide Herbicides in Soils by Microwave-Assisted Extraction (MAE) Coupled to Gas Chromatographic Analysis with Either GC–NPD or GC–MS. *J. Agr. Food Chem*. **50**, 5026–5033.

Wolschin F and Amdam G V (2007) Comparative proteomics reveal characteristics of life-history transitions in a social insect. *Proteome Sci*. **5**, 10.