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Assessment of Insecticide Residues in Raw Honey by High Performance Liquid Chromatography with Ultraviolet Detection

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Abstract.- Insecticides residues are of serious concern due to their presence in many food stuffs. In the current investigations, an effort was made to evaluate and determine residues of six insecticides (imidacloprid, acetamaprid, cypermethrin, deltamethrin, chlorpyrifos and endosulfan) in raw honey samples of *Apis mellifera* L. from the central multi-cropped districts of Punjab Province, Pakistan. The honey samples were analyzed for insecticides residues using multiresidue analysis by High Performance Liquid Chromatography Ultraviolet detection. The mean recoveries obtained for spiked samples of honey at two fortification levels (0.1 and 0.01 mg/kg) ranged 77-94% with relative standard deviations less than 12%, for most of the insecticides. The residues of imidacloprid were detected in maximum samples, however the overall results for real honey samples showed that residues concentration detected, were below maximum residue limits and did not show a threat for human health.

Key words: Raw honey, HPLC, Contamination, Insecticides residues.

INTRODUCTION

Insecticides improve the nutritional value and safety of food by protecting it from different insect pests (Narayanasamy, 2006) and thus considered economic and effective substances for insect pest management in agricultural production (Damalas, 2009). In Pakistan, there is a tremendous use of insecticides on fruits, vegetable and field crops (Hussain *et al*., 2002) that pose hazards to human due to their slow degradation in the environment through different food chains (Hamilton *et al*., 2004). The presence of insecticide residues in different food stuffs has been considered a serious problem to human health because of their slow degradation, high bio-accumulation and high mammalian toxicity *e.g.*, organochlorines (Wang *et al*., 2010). Insecticide residues are present in almost all compartments of agro-ecosystems, but the most real risk of human is through consumption of residues in different food stuffs (Price, 2008). Many types of insecticides have potential to enter into food chain through fatty products (Qu *et al*., 2010) and non-fatty products such as in honey (Blasco *et al*., 2004; Erdogrul, 2007). The detection of insecticide residues in honey has become a serious concern (Bogdanov, 2006) and can impact the quality of honey and result in serious problems to human health when present in large quantities.

There are different routes of exposure of insecticides to honey (Rial-Otero *et al*., 2007) such as from plant source via pollens and nectars, direct accumulation of insecticides into hives, through water as it is a requirement to maintain the temperature of the colony during summer and in winter to break the crystal form of honey. Incidental poisoning of bees is another source of contamination of honey while flying over insecticides applications areas; by bringing back the contaminated dust particles they intoxicate the whole colony and honey. Every day, 10,000-25,000 honeybee workers make different trips to explore roughly 7 km^2 for collection of pollens and nectars (Devillers and Pham-Delegue, 2002). Due to increasing attention of public to the quality of honey, the control of insecticide residues in honey is a vital task for primary health around the world as insecticide residues in honey are increasingly present (Raghunandan and Basavarajappa, 2013).

Honey is a natural, nutritious, healthy, and popular food produced by honey bees from nectars of plants and is used by children, old and ill people as food and medicine (Tewari and Irudayaraj, 2004). It has a variation in taste, color and smell depending on nectars of flowers (Cenet *et al*., 2015). Honey must be free of any chemical contamination for the

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safe use of human's (Tsipi *et al*., 1999). Insecticide residues level determination in bee products is critical to monitor contamination for safe consumer health (Fernandez *et al*., 2002) and to assess the potential risk of honey from insecticide residues. The objectives of this study were to determine residual levels of different insecticides in raw honey of *Apis mellifera* L. from various floral origins collected from Central Punjab, Pakistan.

Table I.- List of insecticides with common names, trade names, chemical group and toxicity classification.

Trade name	Common name	Chemical group	Toxicity
Confidor @200SL	Imidacloprid	Nicotinoid	II/WHO
Mospilan @20 SP	Acetamaprid	Nicotinoid	II/WHO
Arrivo @10 EC	Cypermethrin	Pyrethyroid	II/WHO
Decis @2.5 EC	Deltamethrin	Pyrethyroid	II/WHO
Lorsban @40 EC	Chlorpyrifos	Organophosphate	II/WHO
Thiodan @35 EC	Endosulfan	Organochlorin	II/WHO

MATERIALS AND METHODS

Insecticide standards, reagents and solvents

Certified analytical standards of imidacloprid, actamaprid, cypermethrin, deltamethrin, chlorpyrifos and endosulfan (Table I) were purchased from their respective manufacturing companies with >98% purity. Distal water was obtained with the help of glass-distilled and further purified with the help of a Millipore Milli-Q water purifier. HPLC grade acetonitrile, ethyl acetate, sodium chloride and anhydrous sodium sulphate were purchased from local market. Merck limited C18-bonded silica (50 µm) and florisil (60-100 mesh) were also purchased. The insecticides standard stock solutions were individually prepared in acetone by dissolving 20 mg in 25 ml of solvent and were stored in a freezer at -18°C. The stock standard solutions were used up to 3 months. Suitable concentrations of working standards were prepared from the stock solutions by dilution using acetonitrile, immediately prior to sample preparation.

Sampling

Sixteen samples of multi-flower raw honey of *Apis mellifera* L. (domestic bees) were collected from the Central Districts of Punjab, Pakistan from four Districts (Faisalabad, Sargodha, Chiniot and Sahiwal). After collection, these samples were brought to the laboratory and were stored in a dark place at 10°C until analysis.

Extraction procedure and cleaned up

The residues were extracted using a previous method with some modifications (Alyaseri *et al*., 2012; Rissato *et al*., 2004). A 50g of honey sample portion was weighed in a flask. The sample was mixed with 5 ml of water and homogenized by shaking to reduce its viscosity and to facilitate handling. The sample was then mixed with 50 ml of solvent (ethyl acetate) and was submitted to extraction by agitating for 20 min. In a separator funnel the organic phase was separated by centrifugation at 2500g for 10 min. The supernant was collected and the residues were re-extracted with 40 ml of solvent. The solvent was evaporated in rotary evaporator under reduced pressure at 65°C. Finally the residues were dissolved in 5 ml of ethyl acetate and passed through a 0.50 µm sized pore PTFE filter. The samples were then cleaned by adding 0.5 g silica gel, 1g anhydrous sodium sulphate, 5g mixture of activated carbon and silica gel or florisil. These were then passed through a chromatographic column and then the filtered extracts received, were concentrated under a gentle N_2 stream. Then 1 ml of ethyl acetate was added to this eluate and was submitted to analysis by High Performance Liquid Chromatography equipped with ultraviolet (HPLC-UV).

Method validation

Method validation ensured analysis credibility. In these studies, the parameters accuracy, precision, linearity and limits of detection (LOD) and limit of quantification (LOQ) were considered. The accuracy of the method was determined by recovery tests, using samples spiked at two different levels of 0.1 and 0.01 mg/kg. Known concentration of the pure insecticides standard solution of each type and extraction and cleaned up were performed as described above. The concentration of each insecticide in the final extracts was calculated. Recovery studies were performed to examine the efficacy of extraction and cleaned up.

Linearity was determined by different known concentrations which were prepared by diluting the stock solutions.

Liquid chromatography - ultraviolet

The HPLC-UV system was used for the determination of insecticide residues (Alyaseri *et al*., 2012; Rao *et al*., 2012) in honey. The system consisted of Shimadzu HPLC with LC-20AT pump and SPD- 20A and was interfaced with LC solution software and equipped with a reversed Phase C-18 analytical column of 250 mm×4.6 mm and particle size 5.0 µm (Phenomenex). Column temperature was maintained at 30°C. The injected sample volume was 20 µL. Mobile Phases A and B were acetonitrile and Milli-Q water (75:25(v/v)). The flow-rate used was kept at 1.2 mL/min. The detector wave length was 230 nm. The external standard method was used for these analyses.

Identification and calculation

The compounds were identified by comparing the retention times of the samples peaks with that of the standard peaks. Then the amount of residues (mg/kg) was calculated using a method by Kumari *et al*. (2003).

RESULTS AND DISCUSSION

Spike recoveries and methods validation

The spike recoveries tests of different insecticides were performed by the analysis of honey samples fortified at two different concentration levels (0.1 and 0.01 mg/kg) with pure insecticide standards. The mean recoveries of these insecticides in the spiked samples ranged 77-94% for most of the insecticides with a relative standard deviation of < 12% and were deemed satisfactory according to the requirements of the European Commission (SANCO, 2000). The method was validated because it is necessary for any reliable chromatographic analysis (Levison *et al*., 1995). In validation, linearity of calibration curve, sensitivity and selectivity of the solute detection, reproducibility, instrument precision, detection limit and quantitation limit were determined (Lee *et al*., 1995). All results obtained were satisfactory (Table II).

Table II.- Percentage recoveries of insecticide residues in spiked samples of honey at 0.1 and 0.01mg/kg with RSD %.

Insecticides	H.S. Rec. % (RSD)	L.S. Rec. % (RSD)
Imidacloprid	92 (5.6)	86 (5.8)
Acetamaprid	94 (6.3)	85 (4.7)
Deltamethrin	84 (11.3)	77 (6.1)
Cypermethrin	86 (7.5)	79 (7.2)
Endosulfan	81 (7.6)	77 (3.8)
Chlorpyrifos	86 (6.3)	79 (5.2)

Mean values are converted into percentage; H.S., high recovery; L.S., low recovery; RSD, for relative standard deviation.

Results of monitoring studies

Table III shows the results obtained after analyzing 16 multi-flower raw honey samples of *Apis mellifera* L. by HPLC-UV previously reported (Rao *et al*., 2012) for the determination of insecticides residues in honey. Out of sixteen honey samples, 50% were contaminated with the residues of different insecticides. Imidacloprid was the most frequent insecticide, detected in 37.5% of samples analyzed. The mean concentrations of imidacloprid detected ranged, 0.003-0.017 mg/kg, while the residues of deltamethrin, chlorpyrifos and endosulfan detected with a quantity of 0.013, 0.012 and 0.005 mg/kg, respectively. Residues of cypermethrin and acetamaprid were not detected in any samples. The detected residues of these insecticides were below maximum residues limits (MRLs) as permitted by European Commission (EC) Regulation (European Commission, 2006) in honey (Table IV).

The previous investigations from many researchers have reported the contamination of honey from many parts of the world with different pesticides residues during their monitoring programs. However, it is difficult to compare our results with the results of other monitoring programs of the world, because the concentrations of insecticide residues detected, is different across the world (Blasco *et al*., 2004; Herrera *et al*., 2005). The previous investigations (Anju *et al*., 1999) reported the presence of different insecticide residues in marketed honey from India. The residues of different organochlorine (OC) pesticides detected, were between 0.01 and 6 mg/kg and the residues of different (OP) and carbamates pesticides detected in

Table III. - Insecticide residues detected in raw honey (mg/kg) of *Apis mellifera* L. from Central Districts of Punjab-Pakistan.

S. No	Sample code	Imidacloprid	Acetamaprid	Cypermethrin	Deltamethrin	Endosulfan	Chlorpyrifos
1	Faisalabad 1	Nd	Nd	Nd	0.013±0.001	Nd	Nd
2	Faisalabad 2	0.017±0.015	Nd	Nd	Nd	Nd	Nd
3	Faisalabad 3	Nd	Nd	Nd	Nd	Nd	Nd
4	Faisalabad 4	Nd	Nd	Nd	Nd	Nd	Nd
5	Sargodha 1	0.005±0.002	Nd	Nd	Nd	Nd	Nd
6	Sargodha 2	0.003±0.01	Nd	Nd	Nd	Nd	Nd
7	Sargodha 3	Nd	Nd	Nd	Nd	Nd	Nd
8	Sargodha 4	Nd	Nd	Nd	Nd	Nd	Nd
9	Chiniot 1	0.008±0.005	Nd	Nd	Nd	Nd	Nd
10	Chiniot 2	0.01±0.016	Nd	Nd	Nd	Nd	Nd
11	Chiniot 3	Nd	Nd	Nd	Nd	Nd	Nd
12	Chiniot 4	Nd	Nd	Nd	Nd	Nd	Nd
13	Sahiwal 1	Nd	Nd	Nd	Nd	Nd	0.012±0.08
14	Sahiwal 2	0.004±0.003	Nd	Nd	Nd	0.005±0.02	Nd
15	Sahiwal 3	Nd	Nd	Nd	Nd	Nd	Nd
16	Sahiwal 4	Nd	Nd	Nd	Nd	Nd	Nd

Values are expressed as means ± standard deviation, Nd, insecticides not detected.

Table IV.- Maximum residues limits of insecticides in mg/kg, studied in honey.

Insecticides	MRL
Imidacloprid	0.05
Acetamaprid	0.05
Deltamethrin	0.03
Cypermethrin	0.05
Endosulfan	0.01
Chlorpyrifos	0.03

Regulation (EC) No 396/2005, updated on 08/10/2013

honey varied between 0.1 and 9 mg/kg. Similarly different OC pesticide residues have been reported in honey and their limit of detection was 0.05 and 0.20 µg/kg (Antonescu and Mateescu, 2001). In another previous study, 50% of the honey samples were contaminated from Romania with residues of OC pesticides (Blasco *et al*., 2003). The residues of OCs, OPs and carbamates reported from Spain and Portugal, and their mean concentrations were 0.03 and 4.31 mg/kg (Fidente *et al*., 2005). The different concentrations of nicotinoid insecticide residues have also been reported in honey in previous studies (Chauzat and Faucon, 2007).

The previous findings (Rissato *et al*., 2007) show the contamination of bee products with different pesticides with a range of 0.126 mg/kg to 0.265 mg/kg from France. Presence of different pesticide residues in honey above MRLs have been reported from Brazil (Fell and Cobb, 2009), the residues of endosulfan detected, were 0.027 mg/kg and 0.024 mg/kg, chlorpyrifos residues detected in a quantity of 0.01 and 0.015 mg/kg and residues of cypermethrin were detected with a quantity of 0.092 mg/kg. Many other studies also showed the presence of pesticide residues in honey samples and bee products (Bermejo *et al*., 2010; Ivana *et al*., 2010; Johnson *et al*., 2010; Peres *et al*., 2010). In most cases, contamination of honey is caused by insecticides application in the surrounding area which markedly influences the kind and quality of honey, therefore the residues determination of insecticides could be helpful to maintain the safety and quality of the honey.

CONCLUSION

The results obtained show that imidacloprid was the most frequently detected insecticide in *Apis mellifera* L. honey from Central Punjab, Pakistan followed by deltamethrin, endosulfan and chlorpyrifos, but their residues levels were below Maximum Residue limits (MRLs). These results show the importance of insecticide residues monitoring programs in honey to minimize its contamination.

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