Introduction

The aim of this chapter is to describe to develop analytical method for analysis of imidacloprid in vegetable samples. Fruits and vegetables are important components of the human diet since they provide essential nutrients that are required for most of the reactions occurring in the body. Pesticide residue analysis in vegetable and food has traditionally been performed using gas chromatography (GC), but there is increasing use of liquid chromatography (LC) with tandem mass spectrometry (MS/MS). LC is favored for polar, less thermally-stable, less volatile, compounds. GC-MS is preferred for volatile, thermally-stable species. The data quality can be improved through better retention and separation of components, especially for structurally similar pesticides and high-level matrix co- extractives. QuEChERS (Quick–Easy–Cheap–Effective–Rugged–Safe) is a sample preparation approach developed by Anastassiades et al. (2003) as a simple, rapid, effective, yet inexpensive, way to extract pesticide residues from fruits and vegetables, followed by a dispersive solid phase extraction (dSPE) cleanup of the extract. It is well established that QuEChERS can result in good recovery values not only for a large number of pesticides, but also for a wide variety of commodities.

The extraction procedure for method used depends on the nature of matrix. The vegetable samples are simply extracted by shaking with suitable solvent. Kapoor U**.** et.al (2013) have reported QuEChERS (quick, easy, cheap, effective, rugged, and safe) method of extraction procedure for imidacloprid in fruits, fruit juices, and baby foods followed by high-performance liquid chromatographic analysis, and imidacloprid residues were qualitatively confirmed by liquid chromatography-mass spectrometry. Proietto et al. (2013) performed the rapid and reliable multiresidue analysis wherein the analytical methods were developed and validated for the determination of six neonicotinoids pesticides (acetamiprid, clothianidin, imidacloprid, nitenpyram, thiacloprid, and thiamethoxam) in honey. The modified QuEChERS method allowed a very rapid and efficient single-step extraction, while the detection was performed by UHPLC/MS-MS. The recovery studies were carried out by spiking the samples at two concentration levels 10 and 40 μg/kg fortification levels.

A number of methods have been employed to measure imidacloprid residues: Photo chemical fluorimetric method (Vilchez *et al.* 1996), electrochemical method, enzyme-linked immune sorbent assays (Wantatable, *et al.* 2004), capillary electrophoresis, gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography HPLC (Zhou Q *et al.* 2003). Among these methods GC and LC are the most suitable methods. However, GC cannot be used directly to determine imidacloprid due to the poor volatility and polarity. In contrast to GC, HPLC is more effective and appropriate for the residual analysis of imidacloprid, and it has been successfully employed for assaying imidacloprid in the soils, water as well as in the vegetables (Sajjad Ahmad Baig *et al*. 2012). Imidacloprid residues can be analyzed by derivatization and gas chromatography. High performance liquid chromatography (HPLC) has been already used and appears to be a suitable alternative because of the thermolability and polarity of imidacloprid. The LC method gave good results for imidacloprid in groundwater, soils, and fruits and vegetables but the limit of detection (LOD) was still too high for the present purpose (Srivastava Ashutosh K et al. 2012). Furthermore, the required method must satisfy strict quality criteria to be classified as a trace levels determination and low levels extraction procedure. The QuEChERS method commonly uses GC–MS and LC–MS/MS to cover the wide range of pesticides for analysis**.** The QuEChERS method was applied to sample preparation in this study, because it has several advantages over most of the traditional extraction techniques.

The ultra fast liquid chromatography (UFLC) is quite popular for enhancing the laboratory's productivity. The UFLC yields good resolution, low sensitivity, fast analysis with area repeatability better than conventional HPLC. The improvement in analysis is largely due to use of smaller particles used in column (3.5µ, 2.2µ or sub-2 micron columns) and associated hardware to achieve higher pumping pressures, supported by faster injections and detection.

We developed a new extraction scheme with high recovery rates, coupled to a UFLC methodology in order to extract and quantify imidacloprid in soils, vegetables cabbage and spinach. The present study will concern extensively to trace level determination of imidacloprid in vegetables (cabbage and spinach) and soil by validating and using Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) and solid phase extraction method followed by Ultra Fast Liquid Chromatography (UFLC). Comparison between (QuEChERS) extraction method and Solid phase extraction for its

efficiency and sensitivity were carried out. More generally, such a method can be easily adapted for the analysis of fruits and vegetables.

Materials and methods

Analytical reference standards of imidacloprid (98.5% purity) were obtained from Dr. Ehrenstorfer, Germany. All the other chemicals and solvents used were analytical and HPLC grade.

Samples

Different types of agricultural products (e.g. cabbage and spinach) were purchased from local markets in Hyderabad, India. Samples were grinded with high speed grinder in 2 litter capacity jar with lid and stored at −20 ±2 ^{o}C.

Preparation of Standard Solutions

Stock solution

Reference standard solutions (1000 µg/mL) of imidacloprid prepared in acetonitrile and were kept at −20 ± 2 °C as stock solution.

Calibration mixture solutions

Calibration mixtures of concentration levels 0.005, 0.01, 0.05, 0.1, 0.5 and 1.0 µg/mL were prepared in acetonitrile and were stored at −20 ± 2 ^{o}C.

Validation Parameters

The specificity of the analytical method was studied by injecting solvent, reference standard solution, control soil and vegetable samples (cabbage and spinach sample extracts) injected into Ultra Fast Liquid Chromatography (UFLC) instrument. There was no interference of the components with each other. The linearity was established by injecting five different concentrations, viz. 0.005 to 1.0 µg/mL and determining the response of imidacloprid, these were fitted by linear regression to assess the linearity. The limit of detection was determined to be 0.005 µg/mL at a level of approximately three times the background of control injection around the retention time of the peak of interest. Detection Limit (signal-to-noise ratio = 3 ± 0.5:1) was established S/N Ratio approach. This method can only be applied to analytical procedures which exhibit baseline noise. It is determined by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing minimum concentration at which the analyte can be reliably detected. A S/N ratio of 3:1 is considered acceptable for estimating LOD (with Relative Standard Deviation

(RSD) ≤ 10%) whereas for LOQ, S/N ratio of 10:1 is considered appropriate (with Relative Standard Deviation (RSD) ≤ 3%).

Standard deviation of the response and slope

The LOD and LOQ may be expressed as: LOD = 3.3 × σ/S and LOD = 10 × σ/S where σ = the standard deviation of the response, S = the slope of the calibration curve of analyte. The slope S may be estimated from the calibration curve of the analyte. The value of σ may be taken from as standard deviation of analytical background responses of an appropriate number of blank samples. The linear dynamic range of imidacloprid is shown in **Figure D.**

The precision (% RSD) of the analytical method was determined by five replications in duplicate injection of fortified substrate soil and (cabbage and spinach sample extracts) at LOQ, 10 × LOQ and 50 × LOQ levels. The accuracy (% recovery) of the method was determined by five replications in duplicate injection of fortified substrate soil and (cabbage and spinach sample extracts) at LOQ, 10 times and 50 times LOQ levels. Precision (% RSD) should not exceed 20% at lower levels.

Sample fortification

A representative sample (10g) of soil and vegetables cabbage and spinach was transferred to Polyethylene (PFTE) 50 mL tubes. The soil and vegetables cabbage and spinach sample was fortified with imidacloprid at three different fortification levels: LOQ and 10 × LOQ and 50 LOQ levels separately. The control samples were processed similarly where in acetonitrile was added.

Extraction procedure

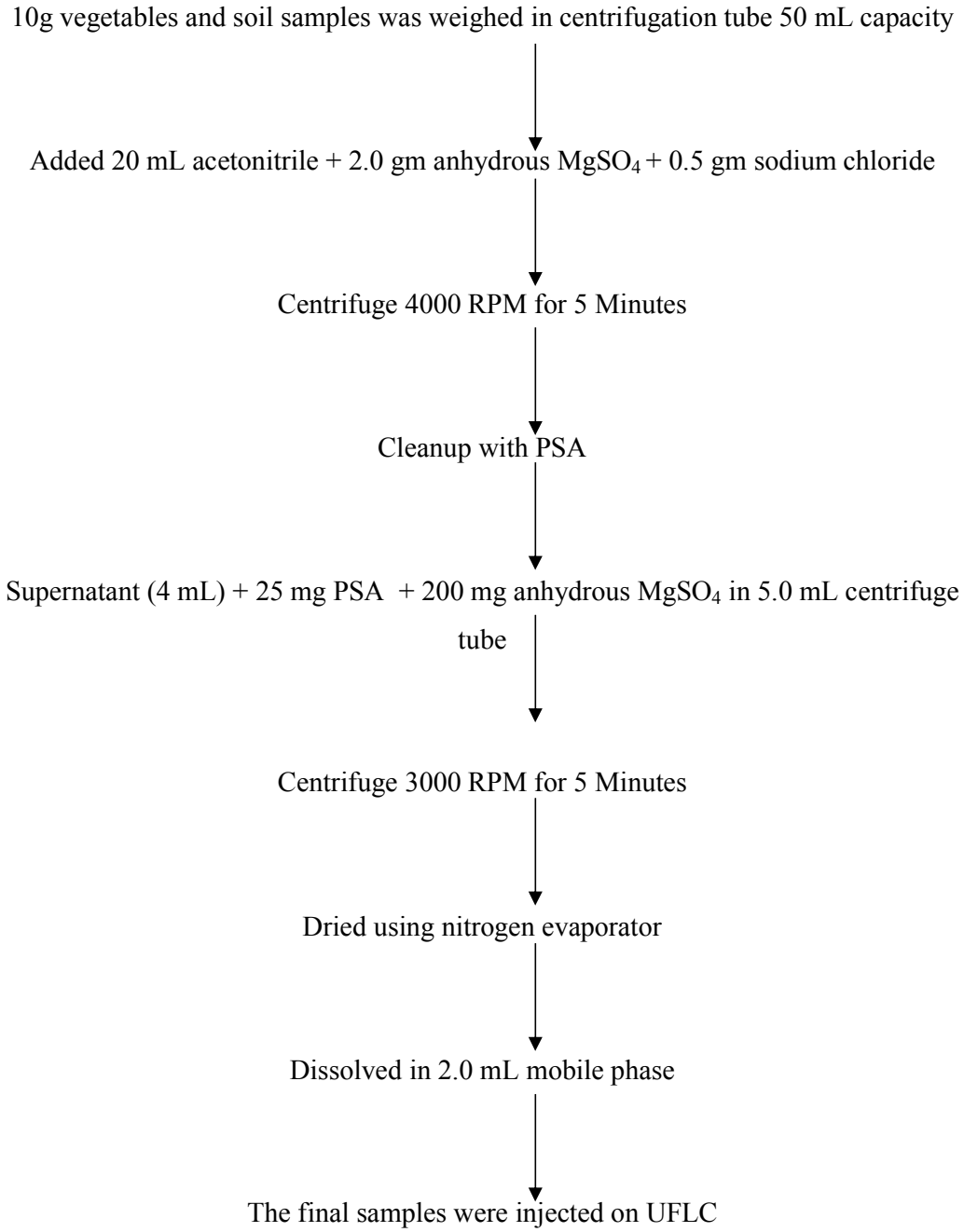
Extraction procedures used in our study for analysis of imidacloprid in vegetables (cabbage and spinach) and soil using solid phase extraction (SPE) and QuEChERS Method described as follows:

QuEChERS method as described by Anastassiades, et al., (2008)

A quantity of 10g soil and vegetables samples (cabbage and spinach) was weighed and transferred in Polyethylene (PFTE) 50 mL tube then 20 mL acetonitrile was added and shaken vigorously for one minute, then added 2.0 gm anhydrous $MgSO_4$ and 0.5 gm sodium chloride and shaken immediately for 1.0 minute. Centrifugation was carried out at 4000 rpm for 5 minutes and cleaned up with 25 mg PSA. Supernatant (4 mL) of the clear solution was transferred to 5.0 mL centrifuge tube, followed by 25 mg PSA, 25

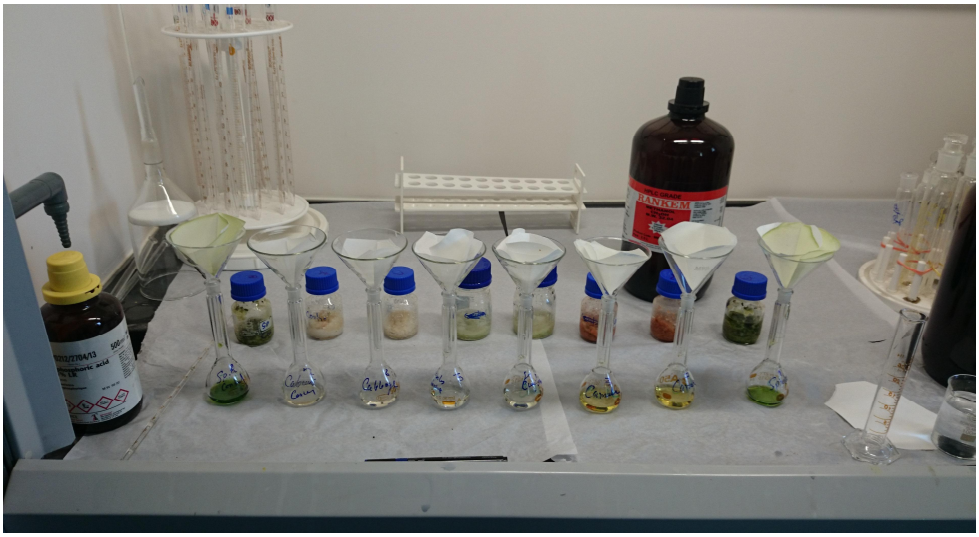
mg and 200 mg anhydrous $MgSO_4$, mixture was shaken well and centrifuged at 3000 rpm. An aliquot was taken out for dryness using nitrogen evaporator and residue dissolved in 2.0 mL mobile phase. The final samples were injected on UFLC.

Flow Chart for QuEChERS method as described by Anastassiades, et al., (2008)

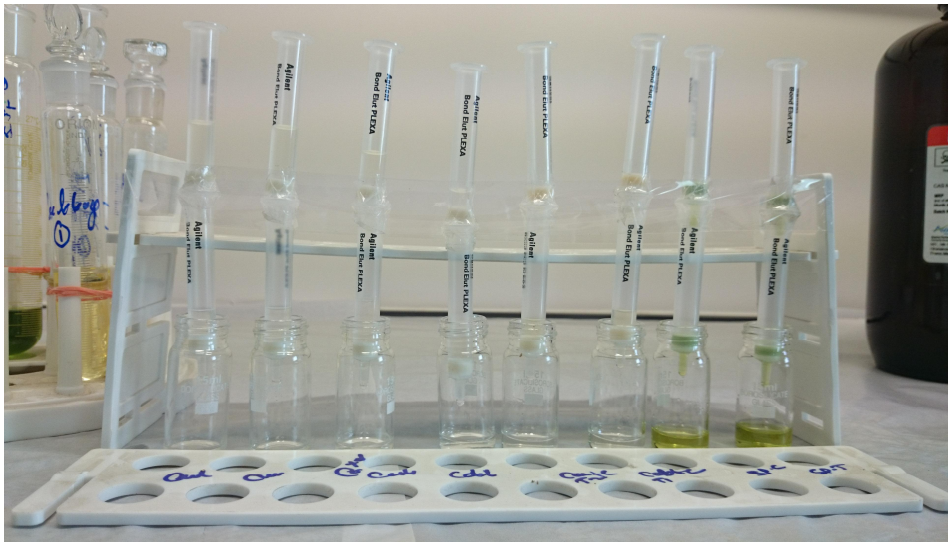


Solid phase Extraction method (SPE)

10 grams of soil sample was weighed in reagent bottles of 50 mL capacity. 20 mL acetonitrile was added and tube shaken with orbital shaker for 10 minutes. This process was repeated twice. The extract was filtered through Whatman filter paper No.1 into 100 mL graduated tube. The image below shows one of the steps of the experimental set up (**Extraction procedure from Vegetables Samples).**

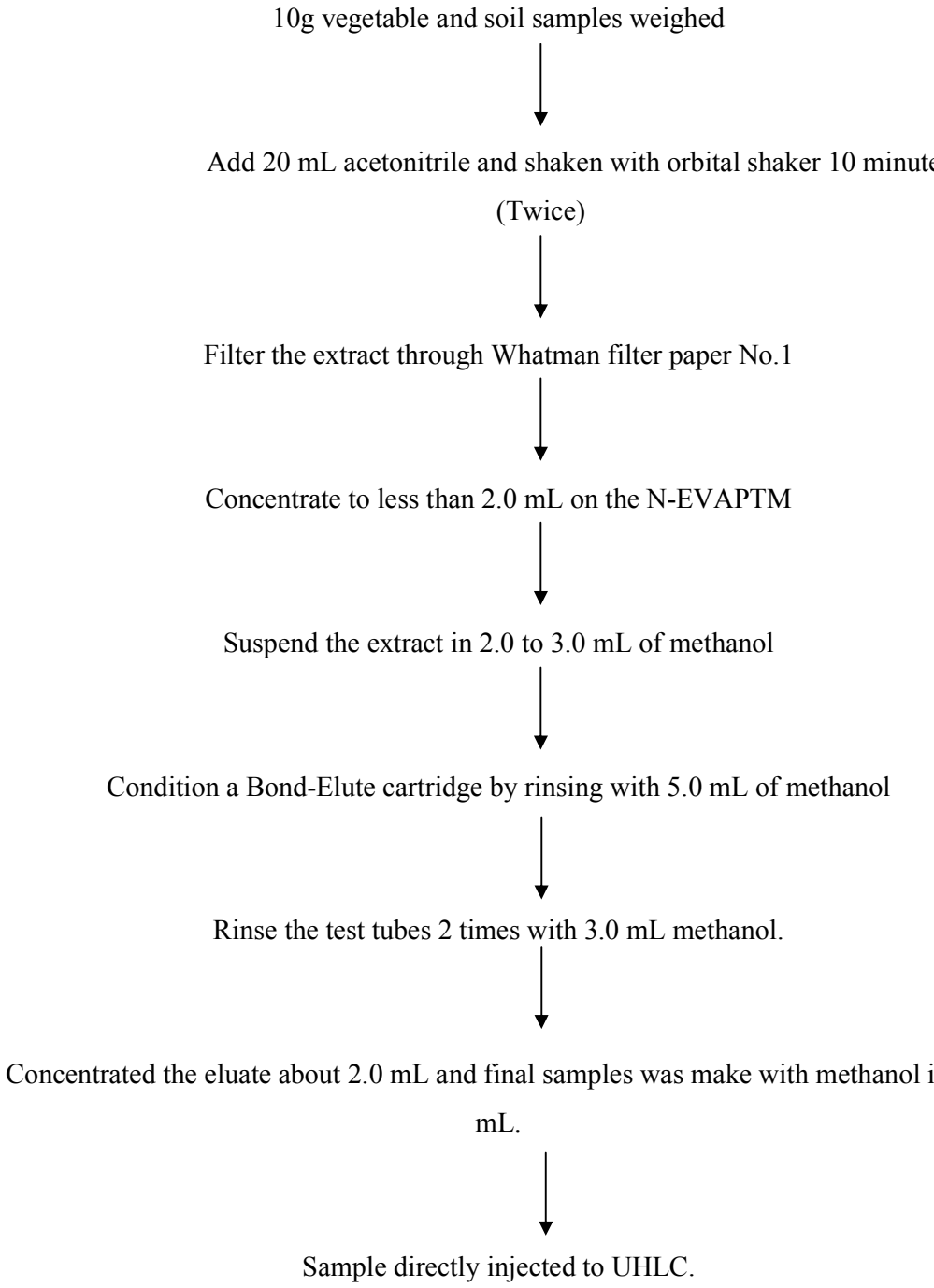


From this, 20 mL aliquot of extract was concentrated to less than 2.0 mL on the N-EVAPTM. The concentrate was suspended in 2 to 3 mL of methanol. A Bond-Elute cartridge was conditioned by rinsing with 5.0 mL of methanol on a vacuum and the extract suspension was loaded on the cartridge and the eluant was collected in a test tube (as shown in the image below). **Cleanup Procedure from SPE.**



The test tubes were rinsed 2 times with 3 mL methanol. Each of the cartridge rinses was collected in manifold. The eluate was concentrated to about 2.0 mL and final samples was made with methanol in 5 mL. This sample was directly injected to UFLC.

Flow Chart for SPE Cleanup Technique



The quantitative analysis of imidacloprid in soil and vegetable samples extracts was conducted by reverse phase Ultra Fast Liquid Chromatography (UFLC) instrument equipped with UV detector, kromosil C-18 column (250 mm length × 4.6 mm i.d. and 5 μm particle size) and LC-solution software was used.

Mobile phase A: 0.01% (v/v) acetic acid in water (60). Add 0.1 mL acetic acid and dilute to 1 litre with water. Mobile phase B: acetonitrile (40). The mobile phase was delivered to 1mL flow rate and detector set a 252 nm λmax was used for analysis. Imidacloprid standard showed sharp peak at 4.93 minute under the described HPLC conditions. Fig. 2 and 3 depict a typical chromatogram of the separation of imidacloprid reference standard and recovery in vegetable samples. The vegetable samples were analysed by LC-MS Ion Trap for imidacloprid peak confirmation and other matrix effects.

Instrumentation for LC-MS Ion Trap Instrument

The system was equipped with a binary solvent pump with autosampler. The imidacloprid MS spectrum of the m/z 256 parent ion is shown in **figure 4.** The MS system was constituted of a standard atmospheric pressure ionization source and positive mode configured as APCI. The LC system was fitted with C18 column and mobile phase A: 0.01% (v/v) formic acid in water (60) and Mobile phase B: acetonitrile (40) for samples analysis.

Results and Discussion

The linearity of the detector response was tested for imidacloprid, in solvent and in matrix (soil and vegetable extract) over the concentration range of 0.005 to 1.0 μg/mL. A very precise linear relation between the injected amount and the resulting peak area was observed over the entire concentration range with correlation coefficient value of 0.999 (**table 1 and figure 1)**. Ishii-Y, *et. al*. 1994 have also reported an HPLC method for determination of imidacloprid residue in 9 kinds of crops and soil. The method consisted of extraction with acetonitrile/water (80:20 v/v), pre-washing of the concentrated extracts with cyclohexane and alkaline solution, silica gel column chromatography, and finally reversed-phase HPLC. The recoveries of imidacloprid were 75-109%. The limits of detection were 0.005 and 0.01 μg/g for soil, cabbage and spinach, respectively.

We have validated the analytical method and extraction procedure for the determination of trace amounts of imidacloprid in/on soil, cabbage and spinach, using Ultra Fast

Liquid Chromatography (UFLC). The accuracy and precision of the method was evaluated on the basis of the recoveries obtained for fortified soil and cabbage and spinach samples. The limit of quantitation (LOQ) was found to be 0.01 µg/g in soil, cabbage and spinach. The limit of detection (LOD) was 0.005 µg/g. Recoveries for imidacloprid varied from 95.0 to 95.62, 96.82 to 99.52 and 90.34 to 97.02 for the used solid phase extraction procedure in soil, cabbage and spinach, respectively (**figure 2 & 3)**. Recoveries for imidacloprid varied from 95.0 to 95.62 and 89.39 to 94.41 used QuEChERS method procedure in cabbage and spinach, respectively. The accuracy (% recovery) data in soil, cabbage and spinach is depicted in Table 1. Ralf *et. al*. 2003 have reported a gradient HPLC – tandem MS method for imidacloprid recovery. In their study, repeatability of the method was determined for the analyte by running a set of five recoveries each at two different fortification levels for selected matrices. The resulting mean recovery rates ranged from 79 to104 % with relative standard deviations between 0.8 and 15.3%. Similar results were found by us in present method validation for soil and vegetable samples. The repeatability of the method in the present study was determined for each fortification level by running a set of five recoveries each at different fortification levels for selected matrices. The % RSD for the resulting mean recovery rates ranged from 94.66 to 95.27% in soil with relative standard deviations between 1.21 to 3.37 %. The % RSD for the resulting mean recovery rates ranged from 92.34 to 96.86% in water with relative standard deviations between 1.66 and 3.23%. . The vegetable samples were analysed by LC-MS Ion Trap for imidacloprid peak conformation and other matrix effect (**Figure 4**).

Conclusion

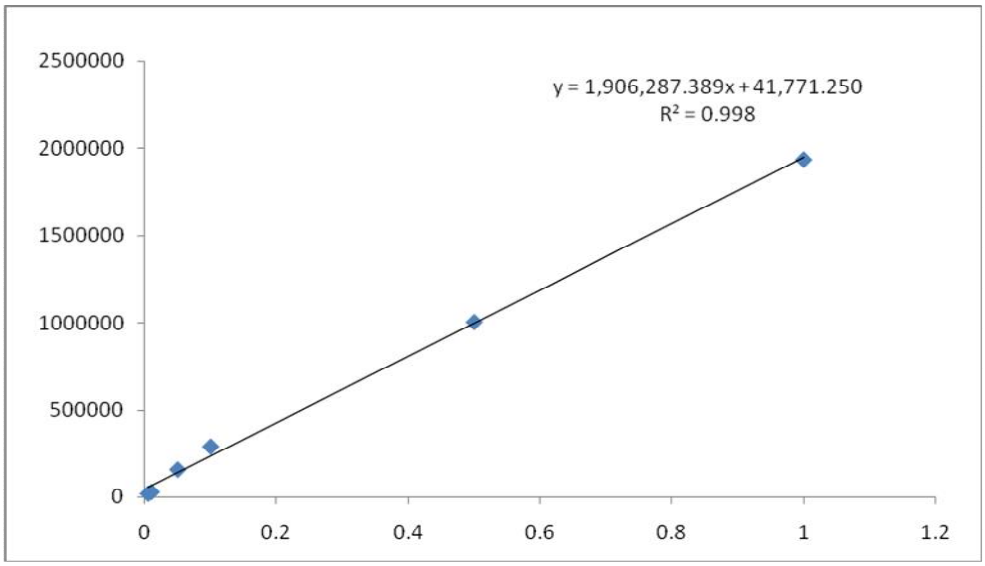
The present study reports trace level determination of imidacloprid in vegetables (cabbage and spinach) and soil by validating and using Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) extraction method and solid phase extraction followed by Ultra Fast Liquid Chromatography (UFLC). Comparison between (QuEChERS) extraction method and solid phase extraction for its efficiency and sensitivity has been carried out. A simple clean-up procedure using PSA was found to yield sufficiently clean samples. A quick, simple extraction procedure for the determination of imidacloprid in vegetables (cabbage and Spinach) and soil samples with good percentage recovery (89.39-99.52%) in all cases. The limit of detection was 0.005 µg/g. The satisfactory validation parameters such as linearity, recovery, precision and very

low limits were obtained and according to the SANCO guidelines. It has been proved that the proposed method and extraction procedure provides a good sensitivity; reproducibility and recovery (see Table 1). The results demonstrated that the proposed extraction technique is a viable alternative for determination of imidacloprid in vegetables, soil and fruit samples.

Table-1 Results summary of Imidacloprid

Parameters	Substrate				
	Cabbage		Spinach		Soil
	SPE	QuEChERS Method	SPE	QuEChERS Method	SPE
Specificity	**No interference**				
r^2	0.998	0.998	0.998	0.998	**0.999**
Limit of Detection	0.005 µg/g				
Limit of Quantitation	0.01 µg/g				
Precision (%RSD)					
LOQ LEVEL (0.01)	1.71	1.02	1.00	0.14	0.25
10 × LOQ LEVEL (0.10)	2.15	1.12	0.89	2.13	1.02
50 × LOQ (0.50) LEVEL	0.87	1.94	0.96	1.13	1.61
Accuracy (% Recovery)					
LOQ LEVEL (0.01)	96.82	95.00	90.34	89.39	95.00
10 × LOQ LEVEL (0.10)	96.39	94.00	93.13	94.41	94.00
50 × LOQ (0.50) LEVEL	99.52	95.62	97.02	90.4	95.62

(1) Linear Dynamic Range (LDR)



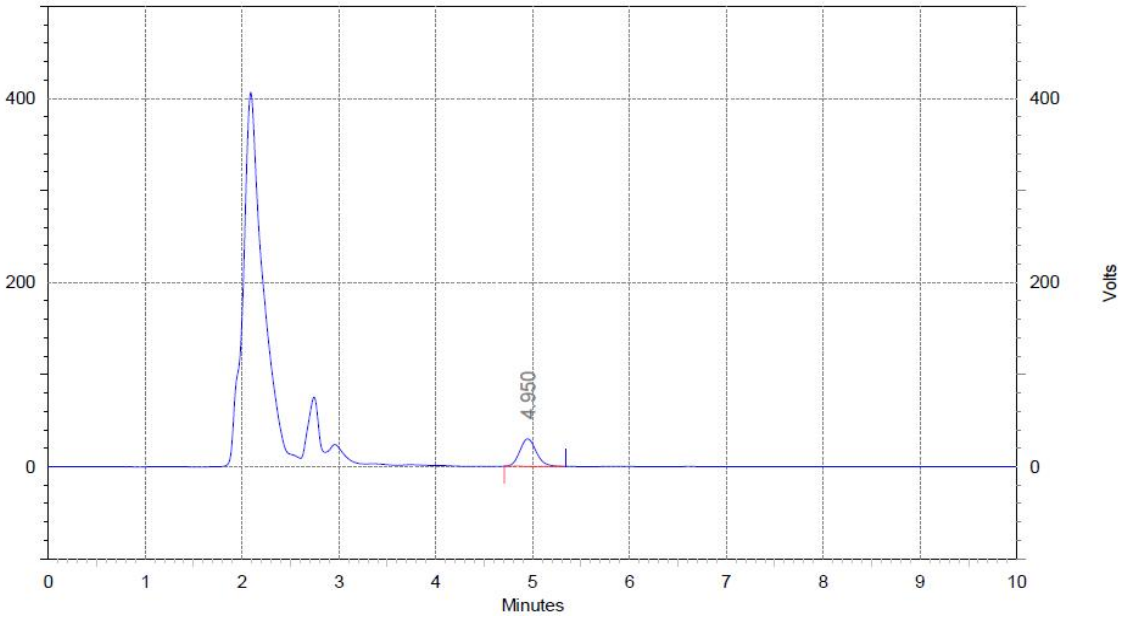


Figure (2) Chromatograms of imidacloprid spike at 10 × LOQ Level in vegetable samples (spinach)

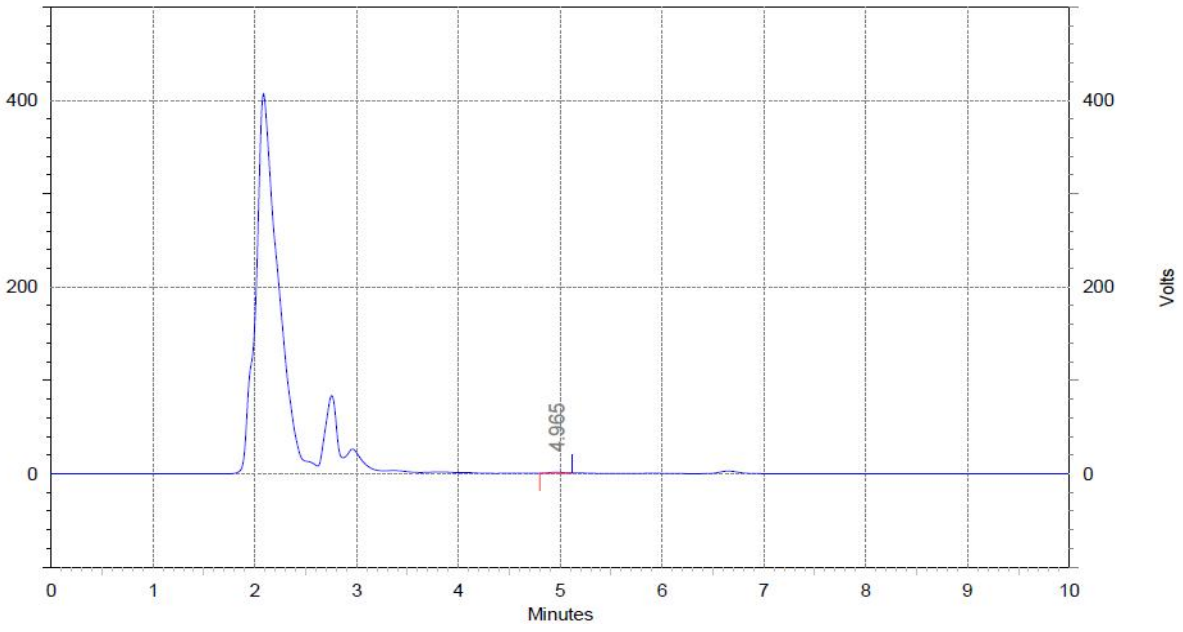


Figure (3) Chromatograms of imidacloprid spike at LOQ Level in vegetable samples (spinach)

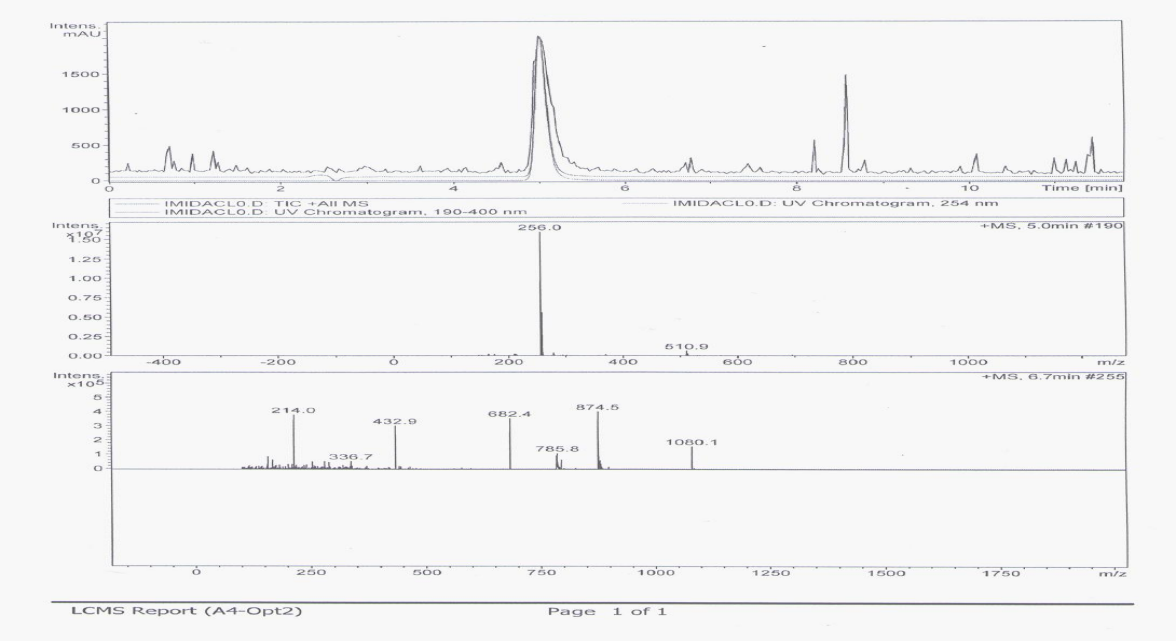


Figure (4) Mass Spectra for Extracted Vegetable Samples of Imidacloprid

References

Anastassiades, M., Lehotay, S. J., Stajnbaher, D.&Schenck, F. J., (2003), Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "Dispersive solid-phase extraction" For the determination of pesticide residues in produce, *J AOAC Int*, 86, (2), 412-431.

Brahma Reddy Gangadasu, Nagarjuna Reddy G., Dhanalakshmi K, (2015), Comparison of UPLC with UFLC: Liquid Chromatography,. Int. J. Pharm. Sci. Rev. Res., 31(1), 97-101.

Ishii-Y, Kobori-I, Araki-Y, Kurogochi-S, waya-K and Kagabu-S (1994) HPLC determination of the new insecticide imidacloprid and its behavior in rice and cucumber. Journal-of- Agricultural-and-Food-chemistry. 42, (12), 2917-2921.

Kapoor U, Srivastava MK, Srivastava AK, Patel DK, Garg V, Srivastava LP, (2013), Analysis of imidacloprid residues in fruits, vegetables, cereals, fruit juices, and baby foods, and daily intake estimation in and around Lucknow, India, Environ Toxicol Chem. 32, (3) 723-7.

Ralf Schoning, Richard Schmuck, (2003), Analytical determination of imidacloprid and relevant metaboliteresidues by LC MS/MS. Bulletin of Insectology. 56, (1) 41-50.

SANCO Guidelines., **(2009),** Method validation and quality control procedures for pesticide residues analysis in food and feed, Document NO. SANCO/10684/2009.

Srivastava Ashutosh K., Srivastava M.K., Patel D.K., Mudiam M.K.R. and Srivastava L.P (2012)“ Gas-ChromatographicDetermination of Imidacloprid in Water”Pesticide Toxicology Laboratory, Indian Institute of Toxicology Research, Lucknow (India) Journal of Environmental Research and Development, 7, (2), 643-651.

Sajjad Ahmad Baig , Niaz Ahmad Akhter, Muhammad Ashfaq, Muhammad Rafique Asi and Umair Ashfaq (2012), Imidacloprid residues in vegetables, soil and water in the southern Punjab, Pakistan Journal of Agricultural Technology, 8(3), 903-916.

Vilchez J.L., El-Khattabi R., Fernandez J.,Gonzalez-Casado and Navalon A., (1996). Determination of imidacloprid in water and soil sample by gas-chromatography-mass Spectrometry. *J. Chrom.* A., 746, (2), 289-294.

Watanabe, E., Eun, H., Baba, K., Arao, T., Ishii, Y., Endo, S., and Ueji, M. (2004). Rapid and simple screening analysis for residual imidacloprid in agricultural products with commercially available ELISA. Analytica Chimica Acta 521, 45-51.

Zhou, Q., Ding, Y., and Xiao, J. (2003). Sensitive determination of thiamethoxam, imidacloprid and acetamiprid in environmental water samples with solid-phase extraction packed with multiwalled carbon nanotubes prior to high-performance liquid chromatography. Analytical and Bioanalytical Chemistry 385, 1520-1525.