Chemical Contaminants in Rice, Spice and Vegetable Samples

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DECLARATION

Experimental work described in the thesis has been carried out by herself at the Laboratory of Organic Section, Department of Chemistry, University of Dhaka, Dhaka-1000, Bangladesh under our supervision. The work has not been and will not be presented for any other degree.

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DEDICATION

This thesis is dedicated to the Almighty Allah, my creator and provider

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3.4 RESULTS AND DISCUSSION

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LIST OF ACRONYMS AND ABBREVIATIONS

ACN	Acetonitrile
ADI	Acceptable Daily Intake
AFL	Aflatoxicol
AFB1	Aflatoxin B1
BDL	Below detection limit
BRRI	Bangladesh Rice Research Institute
EC	Emulsifiable concentrate
ECD	Electron capture detector
EI	Electron ionization
EPA	Environmental Protection Agency
EtOAc	Ethyl acetate
EU	European Union
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FID	Flame ionization detector
FLD	Fluorescence Detector
GC	Gas chromatography
GAP	Good agricultural practices
HCH	Hexachlorocyclohexane
HPLC	High performances liquid chromatography
i.d.	Internal diameter
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography with mass spectrometer
LOD	Limit of Detection
LOQ	Limit of Quantification
MRL	Maximum Residue Limit
NA	Not Applicable
ND	Not detected
OCPs	Organochlorine pesticides

OPPs	Organophosphorus pesticides
PCB	Polychlorinated biphenyls
PCDD	Polychlorinated dibenzo-*p*-dioxins
PCDF	Polychlorinated dibenzofurans
PDA	Photo Diode Array
pH	Degree of Acidity/Basicity
PHI	Pre Harvest Interval
pKa	negative base-10 logarithm of the acid dissociation constant
POPs	Persistent Organic Pollutants
PSA	Primary secondary amine
PTFE	Polytetrafluoroethylene
QA	Quality assurance
QC	Quality control
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
RSD	Relative Standard Deviation
RT	Retention time
SD	Standard Deviation
S/N	Signal to noise ratio
SIM	Selected ion monitoring
SP	Soluble powder
SPE	Solid-phase extraction
$t_{1/2}$	Half-life
tr	Trace concentration, LOD < trace < LOQ
UNEP	United Nations Environment Programme
USA	United States of America
US EPA	U.S. Environmental Protection Agency
UV	Ultraviolet
WCOT	Wall-Coated Open-Tubular
WHO	World Health Organization

CHEMICAL SYMBOLS

^{0}C	degree Celsius
cm	centimetre
g	gramme
g mol^{-1}	gramme per mole
kg	kilogramme
mg	milligramme
mg kg^{-1}	milligramme per kilogramme
mg L^{-1}	milligramme per litre
mm	millimetre
nm	nanometre
ng g^{-1}	nanogramme per gramme
ng mL^{-1}	nanogramme per millilitre
ng L^{-1}	nanogramme per litre
µg	microgramme
µg L^{-1}	microgramme per litre

ABSTRACT

The amount of food produced is very important as the human population increases. Over the last 40 years, food production has been increased 20-50 % where pesticides were played an important role. Proper use of pesticides can protect storage food and vegetable from damage without causing any obvious toxic effects, and final residues of pesticides in edible parts are under recommended maximum residue levels (MRLs). In developing countries, including Bangladesh, the cultivation of crops is mainly carried out by small farmers. To get more products, they use pesticides in overdose than needed in many cases. These are creating serious health problems in Bangladesh. As a part of PhD work, some survey about the present pesticides used in field and storage level have done. A total of 94 pesticides, with 299 trade names, of different groups and formulations, have been registered for use in agriculture. From the observation of the most recent government figures available, the total pesticides imported in Bangladesh are increases gradually. However, our field survey revealed that a large number of unregistered pesticides are being used for storage food and vegetable samples in the country. Therefore, the present research project has been undertaken to determine the residual pesticides/natural toxins in stored food and vegetable samples. In all the analyses certified standard reference samples (91-99 % purity) were used.

Rice is cultivated in three seasons in Bangladesh. As Bangladesh is a hot and humid country moisture content is increased even the crops dried properly. For this climate of Bangladesh, aflatoxins (natural mycotoxins that are produced by certain molds) can be grown in rice. The aim of the study was to assess the level of aflatoxins (if any) in some rice samples. Rice samples were collected from three districts of Bangladesh (Dhaka, Noakhali and Kurigram). The samples were extracted with aqueous methanol and the extract was purified by immunoaffinity column. The analytes were identified and quantified by reverse-phase high performance liquid chromatography where KOBRA Cell was attached after column for post-column bromo derivatisation (PCD) which gave fluorescence. Calibration curves were linear with coefficient of determinant $r^2 \geq 0.9998$, 09997, 0.9956 and 0.9969 for B1, B2, G1 and G2, respectively. The limit of detection (LOD) and quantification (LOQ) were 0.009 and 0.025 µg kg^{-1} for B1, 0.006 and 0.018 µg kg^{-1} for B2, 0.039 and 0.116 µg kg^{-1} for G1 and 0.025 and 0.075 µg kg^{-1} for G2,

respectively. The total aflatoxins (B1, B2, G1 and G2) in the rice samples were found to be in the range of trace to 3.54 µg kg^{-1}. Aflatoxin B1, B2, G1 and G2 were present in 70, 60, 40 and 10 % of rice samples, respectively. The results revealed that 18 out of 20 samples contained detectable amount of aflatoxins. Aflatoxin B_1 (in the range of 0.04 to 0.70 µg kg^{-1}), B2 (in the range of trace to 0.20 µg kg^{-1}), G1 (in the range of 0.22 to 1.82 µg kg^{-1}) and G2 (in the range of 0.12 to 1.56 µg kg^{-1}) were quantified in 17, 16, 6 and 4 samples, respectively. Recoveries (n = 4) were carried out at two different spiking concentrations (1.39 and 2.77 µg kg^{-1} for B1, 0.49 and 0.98 µg kg^{-1} for B2, 1.56 and 3.12 µg kg^{-1} for G1 and 0.51 and 1.01 µg kg^{-1} for G2) and were ranged from 56.71 ± 1.60 to 70.37 ± 5.59 % for B1, 57.71 ± 0.58 to 75.36 ± 6.77 % for B2, 65.53 ± 0.73 to 72.85 ± 5.93 % for G1 and 65.83 ± 2.92 to 99.20 ± 3.16 % for G2, respectively.

Commercial grade turmeric powder samples were analyzed for the presence of carbofuran residues by high performance liquid chromatography (HPLC) coupled with photodiode array (PDA) detector. A total 46 turmeric powder samples (37 were packet and 9 were loose samples) were extracted with ethyl acetate by following QuEChERS (quick, easy, cheap, effective, rugged and safe) method. The extract was cleaned up using an open column packed with mixture of florisil, alumina and charcoal (5:5:1 ratio). Calibration curves were linear with coefficient of determinant $r^2 \geq 0.9996$, 09973 and 0.9958. The limit of detection (LOD) and quantification (LOQ) were 0.01 and 0.03 mg kg^{-1} carbofuran, respectively. No residue was found when the sample was heated in a water bath for 30 min. The amount of carbofuran residues were found to be in the range of 2.5 ± 0.07 to 23.1 ± 0.30 and 2.06 ± 0.14 to 7.8 ± 0.32 mg kg^{--1} in the packet and loose samples, respectively. Recoveries (n = 7) were carried out at three different spiking concentrations (0.4, 0.8, 1.0 and 20 mg kg^{-1}) and were ranged from 92.52 ± 0.01 to 103.14 ± 2.41 %.

For the study of post-harvest intervals of diazinon and carbosulfan in cauliflower, bean, eggplant and tomato samples, the samples were collected from BARI (Bangladesh Agricultural Research Institute) experimental field. Three replicate treated samples of cauliflower, bean, eggplant and tomato and one control sample were collected from BARI at 0 (2 hours after spraying), 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 days after application of diazinon(2 mL L^{-1}) and carbosulfan (1.5 mL L^{-1}). Quantification of residue of diazinon was done on a gas chromatograph

(GC) with an electron capture detector (ECD). Nitrogen was used as carrier and make up gas. Separations were performed on Non-polar (HP-5 MS) capillary column of 30 m long x 250 µm i.d. x 0.25 µm film thicknesses from Agilent, USA. A QuEChERS method was used for extraction using ethyl acetate as an extraction solvent, and clean up was carried out using primary secondary amine. The linearity was excellent (r^2 > 0.9976, 0.9967, 0.9922 and 0.9905) in calibrations. The recoveries at three spiking levels were 99 to 105 % for tomato, 97 to 104 % for cauliflower, 89 to 108 % for bean and 93 to 104 % for eggplant with relative standard deviations in the range of 1.68 to10.64 %. The limit of quantification (LOQ) of this method was found to be 0.003 mg kg^{-1} whereas limit of detection (LOD) being 0.001 mg kg^{-1}. The results revealed that the dissipation pattern of diazinon was followed first-order kinetic. The residues of diazinon in tomato, bean, cauliflower and eggplant were found to be in the range of 0.02 ± 0.01 to 1.66 ± 0.24, 0.005 ± 0.001 to 0.152 ± 0.007, 0.03 ± 0.01 to 4.02 ± 0.37, and 0.02 ± 0.01 to 1.29 ± 0.09 mg kg^{-1}, respectively. The maximum residue limit (MRL) of diazinon on cauliflower, tomato, eggplant and bean has been fixed by CODEX is 0.5 mg kg^{-1}. The diazinon residues declined to a level below the maximum residue limits within 3, 3 and 10 days for eggplant, tomato and cauliflower, respectively. The residue of diazinon was below the maximum residue limit even at 0 day (two hours after spraying) for bean. The estimated dissipation half-life ($t_{1/2}$) of diazinon was found to be 2.63, 2.23, 1.12 and 0.90 days in cauliflower, tomato, bean and eggplant, respectively. The analysis of residue of carbofuran in tomato was done by using gas chromatography (GC) equipped with a flame ionization detector (FID). Nitrogen was used as carrier and makeup gas. Hydrogen and air were used for flame. Separations were performed on HP-5 (30 m long & 0.25 inner diameter) capillary WCOT quartz column. The tomato samples were extracted and cleaned up by QuEChERS method. The limit of detection (LOD) and limit of quantification (LOQ) were 0.1 and 0.3 mg kg^{-1}, respectively. Calibration curves were linear over the calibration ranges with coefficient of determinants 0.9978 and 0.9967 for carbosulfan. The half-life ($t_{1/2}$) of carbosulfan was found to be 5.25 days in tomato. According to Europion Union, the MRL value of carbosulfan in tomato is 0.05 mg kg^{-1}. The residue of carbosulfan in tomato was found to be above the MRL value (0.05 mg kg^{-1}) up to 14 days (9.43 ± 0.16 to 1.19 ± 0.06 mg kg^{-1}).

Forty five vegetable samples namely bean, eggplant, cauliflower and tomato were purchased from different markets of Dhaka city and Noakhali and Kurigram districts. Cypermethrin, chlorpyrifos, diazinon, fenvalerate and quinalphos were detected in some of them. Quantification of residues was done on a gas chromatograph (GC) with an electron capture detector (ECD). Calibration curves were linear with coefficient of determinant $r^2 \geq 0.9912$, 09962, 0.9929, 0.9947 and 0.9907 for chlorpyrifos, cypermethrin, diazinon, fenvalerate and quinalphos, respectively. The LOD was found to be determined 0.50 µg L^{-1} for chlorpyrifos, 2.50 µg L^{-1} for diazinon and quinalphos and 5.0 µg L^{-1} for cypermethrin and fenvalerate, respectively. LOQ was found to be determined 1.65 µg L^{-1} for chlorpyrifos, 8.25 µg L^{-1} for diazinon and quinalphos and 16.5 µg L^{-1} for cypermethrin and fenvalerate, respectively. Out of 10 bean samples, the residue of chlorpyriphos was detected in 3 samples (0.01 ± 0.01 mg kg^{-1}; MRL 0.01 mg kg^{-1}), cypermethrin was detected in 5 samples in the range of 0.05 ± 0.01 to 0.74 ± 0.09 mg kg^{-1} (MRL 0.05 mg kg^{-1}) and fenvalerate was detected in 3 samples in the range of 0.39 ± 0.05 to 0.55 ± 0.04 mg kg^{-1} (MRL 1.0 mg kg^{-1}). Among the 12 eggplant samples, the residue of chlorpyriphos was detected in 4 samples 0.02 ± 0.01 and 0.05 ± 0.01 mg kg^{-1} (MRL 0.5 mg kg^{-1}) and cypermethrin was detected in 2 samples in the range of 0.04 ± 0.01 to 0.13 ± 0.01 mg kg^{-1} (MRL 0.2 mg kg^{-1}). Out of 11 cauliflower samples, the residue of chlorpyriphos was detected in 10 samples in the range of 0.01 ± 0.01 to 0.79 ± 0.02 mg kg^{-1} (MRL 0.05 mg kg^{-1}), cypermethrin was detected in 3 samples in the range of 0.09 ± 0.01 to 0.74 ± 0.16 mg kg^{-1} (MRL 1.0 mg kg^{-1}) and quinalphos was detected in 4 samples in the range of 0.07 ± 0.01 to 0.49 ± 0.08 mg kg^{-1} (MRL 0.2 mg kg^{-1}). Among the 12 tomato samples, the residue of chlorpyriphos was detected in 9 samples in the range of 0.01 ± 0.01 to 0.33 ± 0.02 mg kg^{-1} (MRL 0.2 mg kg^{-1}) and cypermethrin was detected in 3 samples in the range of 0.05 ± 0.01 to 0.32 ± 0.04 mg kg^{-1} (MRL 0.5 mg kg^{-1}). The average recovery of chlorpyrifos in tomato (n = 5) was 98.48 ± 2.73% and in eggplant (n = 6) was 99.57 ± 6.98 % at spiking level of 0.05 mg kg^{-1}. In bean (n = 3), the average recovery of chlorpyrifos was 88.51 ± 2.64 % at spiking level of 0.15 mg kg^{-1}. The average recovery of cypermethrin in tomato (n = 5) was 79.65 ± 5.56 %, in eggplant (n = 6) was 86.29 ± 7.33 % and in bean (n = 3) was 97.43 ± 8.52 % at spiking level of 0.10 mg kg^{-1}. The average recovery of diazinon in tomato (n = 5) was 109.92 ± 2.33 % and in eggplant (n = 6) was 101.41 ± 4.72 % at spiking level of 0.10 mg kg^{-1}. In bean (n = 3), the average recovery of chlorpyrifos was 106.78 ± 3.55 % at spiking level of 0.15 mg kg^{-1}. For fenvelarate, the average recovery in tomato (n = 5) was 90.88 ± 2.15 %

and in eggplant (n = 6) was 84.10 ± 8.91 % at spiking level of 0.10 mg kg^{-1}. In bean (n = 3), the average recovery of fenvelarate was 90.04 ± 9.29 % at spiking level of 0.15 mg kg^{-1}. For quinalphos the average recovery in tomato (n = 5), eggplant (n = 6) and bean (n = 3) were 78.28 ± 4.85 , 85.15 ± 7.72 and 85.28 ± 2.32 % at the spiking level of 0.10,0.15 and 0.20 mg kg^{-1}, respectively.

1. INTRODUCTION

1.1 Background

Food is one of the basic needs in our life. In modern world humans are getting many challenging steps to make their life valuable and successful. Worldwide safe food consumption became a great challenge. The responsibility to ensure the safety of food is a combined duty of producers, industry, government, and consumers. Safe food should free from toxins, pesticides, chemical and physical contaminants, microbiological pathogens including bacteria, parasites, and viruses that can cause illness. In developed countries, many comprehensive and effective systems are followed to ensure safe food. To enhance consumer confidence the effectiveness of food systems in developed countries are improved by increasing the resilience of the source systems along the food chain, enhancing the scientific base for decisions, and providing organizational support for effective participation of all parties in the institutional debate. In developing countries, the extremely diverse food systems are suffering from a number of weaknesses and consumers are exposed to a wider range of potential food safety risks. In this context, awareness and scientific and technological advances are improving in developing countries. Bangladesh is a developing country and the rapid urbanization, globalization of food trade and lack of food safety knowledge and practice leads to consumption of unsafe food in many cases (Food Safety and Policy, www.iedcr.org). To solve this problem, the food safety component of World Health Organization (WHO) working in Bangladesh to build up the capacity of government institution, institute of public health to ensure safety of food from production to the point of consumption (World Health Organization, www.searo.who.int). In addition to this, the Government of Bangladesh made the Food Safety Act, 2013 on October 10, 2013 for establishment of an efficient activities relating to food production, import, processing, stockpiling, supplying, marketing and sales and to ensure the consumer's right to access the safe food (Bangladesh Food Safety Authority, www.bfsa.gov.bd).

1.2 Food Contaminants

Contaminants are substances added intentionally or unintentionally that makes food unfit for human consumption. These substances may be present in food as a result of the various stages of its production, packaging, transport or holding. They also might result from environmental contamination (Malin; 1995).

1.3 Analysis of Known Chemical Contaminants in Food

Our food may be contained chemical contaminants from various sources. The Codex Alimentarius Commission regulates the level of chemical contaminants internationally with national governments because contaminants typically pose a health concern. To ensure consumer safety and compliance with regulatory limits, the analysis of relevant chemical contaminants is an essential part of food safety testing programs. The known chemical contaminants in complex food matrices at very low concentration levels can be determined by modern analytical techniques. To discover and identify new or unexpected chemical contaminants, different techniques are being developed (Demenna et al.; 1995).

The chemical contaminants in foods are mainly the small organic molecules which are typically present in foods at low concentrations (parts per trillion to parts per million). In many cases their analyses in complex food matrices are often quite challenging. The analysis of the contaminants involve the basic analytical approach including an extraction using a suitable solvent, cleanup to remove interfering matrix components, a chromatographic separation and a selective detection.

As a detection technique Mass spectrometry (MS) has truly revolutionized the analysis of chemical contaminants in foods (Cairns et al.; 1995). MS can detect a wide range of compounds independent of their elemental composition and provide simultaneous quantitation and structural identification of detected analytes. Among the chromatographic separations MS also adds another degree of separation/selectivity on top and these unique features have made MS the number one choice for detection and identification/confirmation of trace-level organic chemical contaminants in food testing laboratories.

The analysis of volatile and semivolatile compounds, including many pesticide residues, PAHs, PCBs and other less-polar POPs, the combination of MS with gas chromatography (GC-MS) has become popular (Tsikas; 2010). The introduction of atmospheric ionization techniques, such as electrospray, for liquid chromatography-mass spectrometry (LC-MS) removes the difficulty to analyze polar and less volatile analytes. the direct analysis of many more polar contaminants, including modern, new-generation pesticides, and the majority of veterinary drugs and toxins, such as mycotoxins can be done by LC-MS. LC-MS has opened the door to analyze the emerging and recently identified contaminants, including acrylamide, melamine or Sudan dyes etc.

Thus, GC-MS and LC-MS are being utilizes by the modern food contaminant testing laboratories to cover the wide polarity range of possible organic chemical contaminants (http://www.scientific.org/tutorials/articles/gcms.html). The selectivity of LC-MS are increased by employing Tandem MS (MS/MS) that helps further distinguish target compounds from potential matrix interference.

1.4 Identification of Unknown Chemical Contaminants in Food

The analysis of unknown contaminants at low concentration levels is not an easy task. The information about the sample and potential sources of contamination are important in addition to expertise and a good analytical strategy. The clues, such as taste or texture, changes in smell, as well as a description of potential poisoning symptoms may be important in this respect. The analysis of control samples which are free from contaminants with the suspect samples is often essential to find differences and eliminate potential false positives.

To identify the suspected compound or a group of compounds, the targeted samples can be prepared by suitable analytical procedure and instrumental method(s) can be employed. Different extraction and separation techniques should be used to isolate the truly unknown compounds with a wide range of physicochemical properties (polarity, solubility, volatility, etc.). MS with full-spectra acquisition can be used for nontargeted analysis (Pang et al; 2009).

Statistical analysis of the acquired chromatographic and MS data of contaminated and noncontaminated samples are essential to identify differences and reduce the number of components that have to be examined. MS spectral libraries can be used to compare the acquired MS spectra of suspected contaminants. In LC-MS, time-of-flight (TOF) or orbitrap MS instruments should be used for added selectivity in high-resolution/accurate-mass measurements (MacMahona; 2012). To elucidate the structure of contaminants, tandem MS should be employed. Finally, strong knowledge and expertise in both analytical and food chemistry are typically required to succeed in this task.

The developments in analytical instrumentation are directly related to the current and future trends in the analysis of chemical contaminants. MS instruments enable analysis of many compounds in one analytical run. The advancements in high-resolution MS instruments and development of related software tools show great potential to bring this technology from the research environment into testing laboratories, where it could be employed for nontargeted testing of known and unknown chemical contaminants.

1.5 Sources of Contaminants in Food

Contaminants can be present in foods mainly as a result of the use of agrochemicals, such as residues of pesticides and veterinary drugs, contamination from environmental sources (water, air or soil pollution), cross-contamination or formation during food processing, migration from food packaging materials, presence or contamination by natural toxins or use of unapproved food additives and adulterants (Vazquez and Pico; 2012).

1.5.1 Pesticide Residues

The use of pesticides, such as insecticides, fungicides or herbicides, has become an integral part of modern agriculture to increase crop yields and quality by controlling various pests, diseases and weeds. Registration of new pesticides is a strictly regulated process that evaluates their toxicity and environmental fate, and sets maximum residue limits (tolerances) in raw and processed commodities. There are over 1,400 known pesticides. Some of them should no longer be used but may still be present in the environment. Older pesticides are being reevaluated based on currently available scientific data (Tomlin; 2006).

Approved uses of pesticides following Good Agricultural Practices should result in pesticide residues below maximum residue limits. However, global sourcing of raw commodities and global distribution of food products complicate the situation because pesticide registrations, uses and limits can be and are different in different countries. Consequently, an approved use in one country may result in an illegal pesticide residue in a food imported into another country, such as the recent case of the fungicide carbendazim in orange juice imported into the United States from Brazil (http://www.huffingtonpost.com/2012/01/11/orange-juice-imports-fda-fungicide- n 1200305.html). Furthermore, pesticides can be misused or present in food due to contamination during application (spray drift), storage or transportation or from environmental sources, such as contaminated water or soil.

1.5.1.1 Classification of Pesticides

Pesticides may be classified in a number of ways; these classifications can provide useful information about the pesticide chemistry, how they work, what they target, etc. Following are brief descriptions of some commonly used classification systems.

Classifications based on their origin are two types – chemical pesticides and bio pesticides.

1.5.1.1.1 Chemical Pesticides

Chemical pesticides are further divided into four types–

1.5.1.1.2 Organophosphate Pesticides

These are the chemical substances which are produced due to reaction between phosphoric acid and alcohols. This affects the nervous system by inhibiting the action of enzyme acetyl cholinesterase (AChE). This causes irreversible blockage leading to accumulation of the enzyme which results in overstimulation of muscles. These mainly include insecticides, nerve gases, herbicides, etc **(**Sharma et al., 2010**)**. Organophosphate pesticides are characterized by their multiple functions and the capacity of controlling a broad spectrum of pests. They are

nerve poisons that can be used not only as stomach poison but also as contact poison and fumigant. These pesticides are biodegradable, cause minimum environmental pollution and slow pest resistance. Temephos and F enitrothion are examples of organophosphate pesticides.

1.5.1.1.3 Carbamate Pesticides

These are esters of carbamic acids. The mode of action is inhibiting acetyl cholinesterase similar to that of the organophosphates but the bond formed for inhibition is less durable and thus reversible. These also include mainly of insecticides. They can be used as stomach and contact poisons as well as fumigant. Moreover, as their molecular structures are largely similar to that of natural organic substances, they can be degraded easily in a natural manner with minimum environmental pollution. Propoxur is an example of carbamate pesticides.

1.5.1.1.4 Organochlorine Pesticides

These are the derived from chlorinated hydrocarbons. These are endocrine disrupting agents which effect on the hormonal systems of the body, act as duplicates of the normal hormones and thus causing adverse health problems. They remain in environment for a long time by breaking down slowly and accumulating in the fat tissues of animals. A well-known example is DDT (dichloro diphenyl trichloroethane). Prolonged use in large quantities will easily lead to environmental pollution and accumulation in mammals, resulting in cumulative poisoning or damage. Organochlorine pesticides are therefore banned under general circumstances and gradually replaced by other pesticides.

1.5.1.1.5 Pyrethroid Pesticides

These are potent nuero poisons, endocrine disruptors and cause paralysis. Pyrethroids are synthetic version of pyrethrin a natural insecticide. They have similar chemical structure and similar mode of action as of pyrethrin which is obtained from chrysanthemum. These are derivatives of ketoalcoholic esters of chrysanthemic and pyrethroic acids and are more stable

in sunlight than pyrethrins. They are comparatively more stable with longer residual effects than natural pyrethrins. Synthetic-pyrethroid pesticides are highly toxic to insects but of only slight toxicity to mammals (Roberts and Hutson, 1999). These are most popular insecticides as they can easily pass through the exoskeleton of the insect. Few examples are- deltamethrin, cypermethrin, etc.

1.5.1.1.6 Biopesticides

Biopesticides are naturally occurring materials or derived naturally from living organisms or their metabolites, like bacteria, fungi, plants, etc. These are classified into three major groups-

1.5.1.1.7 Microbial Pesticides

This has microorganisms acting as pest controllers like bacteria, fungi or viruses. Each of it contains specific target. Widely used are strains of Bacillus Thuringenesis or Bt and its subspecies. The mode of action generally is producing a protein that binds to the larval gut receptor which starves the larvae.

1.5.1.1.8 Biochemical Pesticides

They are naturally occurring, nontoxic pest controllers. These include pheromones, natural plant and insect regulators, enzymes, bio repellents or attractants.

Insect growth regulators are compounds developed by copying insect juvenile hormone. The main functions are to interfere with the growth and hatching of larvae into adults, and to prevent the formation of exoskeleton so as to prohibit the growth of the insect. As its ability to live as a living organism is curtailed, the insect may die eventually as well as the whole insect population. Methoprene is an example of insect growth regulators.

1.5.1.1.9 Plant Incorporated Protectants (PIPs)

These substances are produced by plants naturally but the gene necessary for production of pesticide is introduced into the plant through genetic engineering. The substance produced by the plant and the genetic material introduced are together defined as plant incorporated protectants (PIPs).

1.5.1.2 Name and Structures of Some Common Pesticides

1.5.1.2.1 Carbofuran

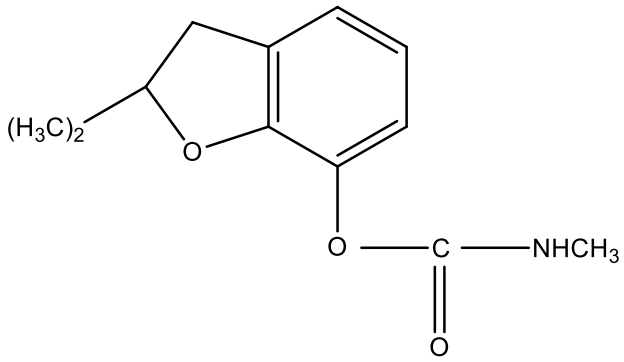


Figure 1: Carbofuran

Carbofuran (2, 2-dimethyl-3H-1-benzofuran-7-yl) N-methylcarbamate) is an N-methyl carbamate insecticide and nematicide (**Figure 1**) which has been registered to control pests in soil and on leaves in a variety of fruits and vegetable crops. It is marketed under the trade name 'Furadan' and characterized as having high acute toxicity to warm blooded animals. Since its toxic effect is due to the activity as a cholinesterase inhibitor it is considered as a neurotoxic pesticide. It is also a powerful endocrine disruptor that can cause transient alterations in the concentration of many hormones in animals and humans even at extremely low doses. These alterations may consequently lead to serious reproductive problems following repeated exposure (Goad *et al*.; 2004 & Lau et al.; 2007). In human, carbofuran is highly toxic by inhalation and ingestion and moderately toxic by dermal absorption that may cause burns to the skin or eyes (Hazardous Materials Advisory Committee; 1974, Hayes et

al.; 1982 & Kearney et al.; 1975). Exposure to carbofuran is risky for persons with asthma, diabetes, cardiovascular disease, mechanical obstruction of the gastrointestinal or urogenital tracts, or those in vagotropic states (Pesticide Properties Database, U.S.; 1990). Symptoms of carbofuran poisoning include: nausea, vomiting, abdominal cramps, sweating, diarrhea, excessive salivation, weakness, imbalance, blurring of vision, breathing difficulty, increased blood pressure or 'hypertension,' and lack of control of urine or feces release, referred to as 'incontinence.' Therefore, a respirator should be worn by farmers during application of carbofuran (Pesticide Properties Database, U.S.; 1990).

1.5.1.2.2 Carbosulfan

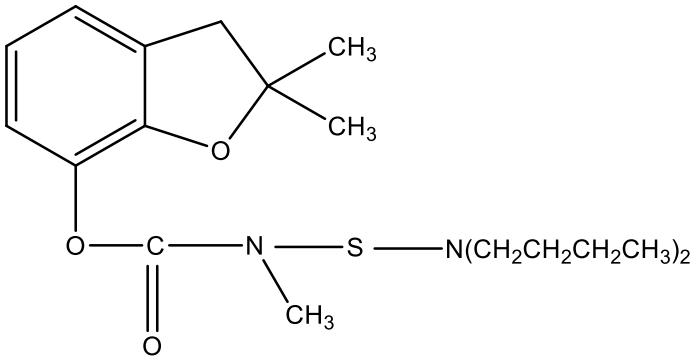


Figure 2: Carbosulfan

Carbosulfan [2, 3-dihydro-2 2-dimethylbenzofuran-7-yl (dibutylaminothio) methylcarbamate] is a broad spectrum carbamate pesticide (**Figure 2**) that acts by inhibiting the activity of acetylcholinesterase. It is used to control insects, mites and nematodes by soil, foliar and seed treatment applications, foliar pests may be controlled by soil applications via systemic action, and is said to be effective through direct contact or stomach ingestion (FAO/WHO, 1984).

1.5.1.2.3 Diazinon

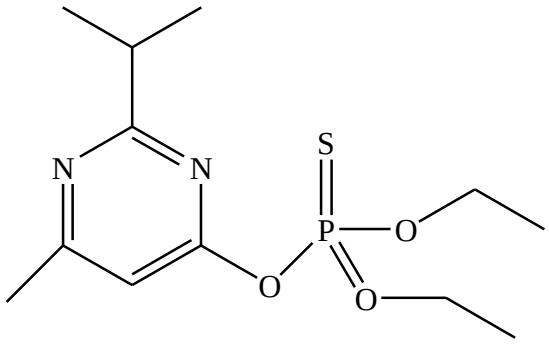


Figure 3: Diazinon

Diazinon (*O,O*-diethyl *O*-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate) is an organophosphate insecticide (**Figure 3**). Inside living things, diazinon is transformed into a molecule called diazoxon. Diazinon, and the more potent diazoxon (U.S. Dept. of Health and Human Services; 1996), kill insects by interfering with nervous system function, as do all members of the organophosphate chemical family. Normally, impulses are transmitted chemically from the end of one nerve cell to the beginning of another; one of the chemical transmitters used in animal nervous systems is called acetylcholine. After transmitting the nerve impulse, acetylcholine is destroyed by an enzyme called acetylcholinesterase (AChE) in order to clear the way for another transmission. Organophosphates attach to AChE and prevent it from destroying acetylcholine, causing overstimulation of the nerves (Ware; 2000). Symptoms of acute (short-term) diazinon poisoning in people are similar to the symptoms of any organophosphate insecticide poisoning: headache, nausea, dizziness, tearing, sweating, salivation (Reigart *et al*.; 1999), drowsiness, agitation, anxiety (U.S. EPA. Office of Prevention, Pesticides and Toxic Substances; 1997) and influenza- like symptoms (Murray *et al*.; 1992). Symptoms of higher exposure include an abnormal heart rate (Forbat *et al*.; 1992), muscle weakness, muscle twitching, pinpoint pupils (Reigartet al.; 1999), lung congestion (Rude *et al*.; 1984), cardiac arrest (Wecker *et al*.; 1985) and seizures (Halle *et al*.; 1987). Diazinon is fat soluble, there is potential for delayed toxicity if significant amounts of diazinon are stored in fatty tissues. Intermediate syndrome generally occurs within 24–96 hours after exposure. Intermediate syndrome in humans is characterized by difficulty breathing and muscular weakness, often in the face, neck and proximal limb muscles. Cranial nerve palsies and depressed tendon reflexes have also been reported.

1.5.1.2.4 Chlorpyrifos

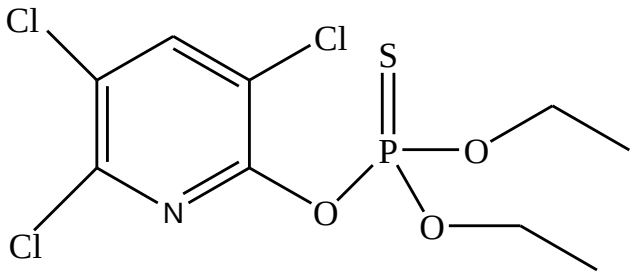


Figure 4: Chlorpyrifos

Chlorpyrifos (*O*,*O*-diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothioate) is a crystalline organophosphate insecticide, acaracide and miticide (**Figure 4)**. It is a broad-spectrum organophosphate insecticide. While originally used primarily to kill mosquitoes, it is no longer registered for this use. Chlorpyrifos is effective in controlling cutworms, corn rootworms, cockroaches, grubs, flea beetles, flies, termites, fire ants, and lice. It is used as an insecticide on grain, cotton, field, fruit, nut and vegetable crops, and well as on lawns and ornamental plants. Chlorpyrifos is moderately toxic to humans (U.S. Environmental Protection Agency; 1989). Poisoning from chlorpyrifos may affect the central nervous system, the cardiovascular system, and the respiratory system. It is also a skin and eye irritant (Gallo et al.; 1991). While some organophosphates are readily absorbed through the skin, studies in humans suggest that skin absorption of chlorpyrifos is limited (Gallo et al.; 1991).

1.5.1.2.5 Cypermethrin

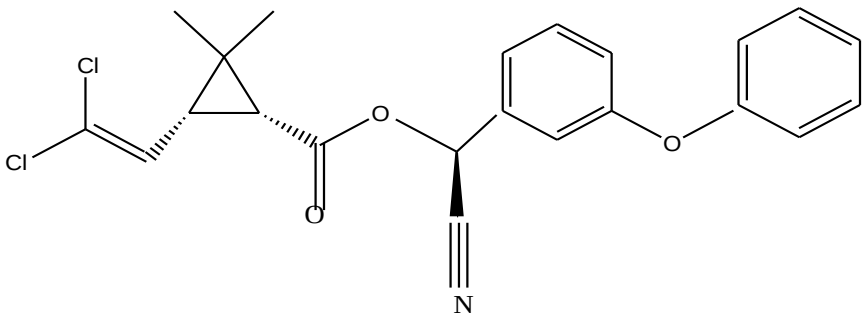


Figure 5: Cypermethrin

Cypermethrin (**Figure 5**) is a synthetic pyrethroid insecticide used to control many pests, including moth pests of cotton, fruit and vegetable crops (Meister; 1992). It is also used for crack, crevice and spot treatment for control of insect pests in stores, warehouses, industrial buildings, houses, apartment buildings, greenhouses, laboratories and on ships, railcars, buses, trucks and aircraft. EPA has classified cypermethrin as a possible human carcinogen (a chemical that causes cancer) because it causes lung tumors in female mice (U.S. EPA; 1995). Cypermethrin consists of eight isomers, four cis and four trans isomers, the cis isomers being the more biologically active. Depending on the manufacturing source, the cis:trans ratio varies from 40:60 to 80:20.

1.5.1.2.6 Fenvalerate

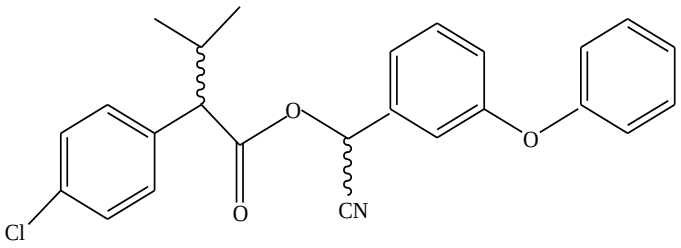


Figure 6: Fenvalerate

Fenvalerate [(RS)-α-cyano-3-phenoxybenzyl (RS)-2-(4-chlorophenyl)-3-methylbutyrate] is an insecticide (**Figure 6**). It is a mixture of four optical isomers which have different insecticidal activities. The 2-S *alpha* (or SS) configuration, known as esfenvalerate, is the most insecticidally active isomer. Fenvalerate consists of about 23% of this isomer.

1.5.1.3 Potential Adverse Consequences of Pesticides

Although pesticides are intended to harm only the target pest, if not used correctly, they can also harm people or the environment. The presence of a pesticide in the environment is not

necessarily a problem, but it may be a source of exposure (Antle and Pingali, 1994). As with all toxic substances, whether the exposure causes harm depends on the dose, how someone is exposed, how sensitive an individual may be to that toxin, and the toxicity of the pesticide involved.

1.5.1.4 Pesticide Residue in Food

The extent of pesticide contamination in the food stuffs, programs entitled 'Monitoring of Pesticide Residues in Products of Plant Origin in the European Union' started to be established in the European Union since 1996. In 1996, seven pesticides (acephate, chlopyriphos, chlopyriphos-methyl, methamidophos, iprodione, procymidone and chlorothalonil) and two groups of pesticides (benomyl group and maneb group, i.e. dithiocarbamates) were analysed in apples, tomatoes, lettuce, strawberries and grapes. An average of about 9 700 samples has been analysed for each pesticide or pesticide group. For each pesticide or pesticide group, 5.2% of the samples were found to contain residues and 0.31% had residues higher than the respective MRL for that specific pesticide. Lettuce was the crop with the highest number of positive results, with residue levels exceeding the MRLs more frequently than in any of the other crops investigated. The highest value found in 1996 was for a compound of the maneb group in lettuce which corresponded to a mancozeb residue of 118 mg/kg. In 1997, 13 pesticides (acephate, carbendazin, chlorothalonil, chlopyriphos, DDT, diazinon, endosulfan, methamidophos, iprodione, metalaxyl, methidathion, thiabendazole, triazophos) were assessed in five commodities (mandarins, pears, bananas,beans, and potatoes). Some 6 000 samples were analysed. Residues of chlorpyriphos exceeded MRLs most often (0.24%), followed by methamidophos (0.18%), and iprodione (0.13%). With regard to the commodities investigated, around 34% contained pesticide residues at or below the MRL, and 1% contained residues at levels above the MRL. In mandarins, pesticide residues were most frequently found at levels at or below the MRL (69%), followed by bananas (51%), pears (28%), beans (21%) and potatoes (9%). MRLs were exceeded most often in beans (1.9%), followed by mandarins (1.8%), pears (1.3%), and bananas and potatoes (0.5%). Estimation of the dietary intake of pesticide residues (based on the 90th percentile) from the above-mentioned commodities, where the highest residue levels of the respective pesticides were found, shows that there is no exceeding of the ADI with all

the pesticides and commodities studied (European Commission, 1999). In 1998, four commodities (oranges, peaches, carrots, spinach) were analysed for 20 pesticides (acephate, benomyl group, chlopyriphos, chlopyriphos-methyl, deltamethrin, maneb group, diazinon, endosulfan, methamidophos, iprodione, metalaxyl, methidathion, thiabendazole, triazophos, permethrin, vinclozolin, lambdacyalothrin, pirimiphos-methyl, mercabam). With regard to all four commodities investigated in 1998 (oranges, peaches, carrots, spinach), about 32% contained residues of pesticides at or below MRL, and 2% above the MRL (1.8% for EU-MRLs, 0.4% for national MRLs). Residues at or below the MRL were found most often in oranges (67%), followed by peaches (21%), carrots (11%) and spinach (5%). MRL values were exceeded most often in spinach (7.3%), followed by peaches (1.6%), carrots (1.2%) and oranges (0.7%). The intake of pesticide residues has not exceeded the ADI in any case. It was found to be below 10% of the ADI for all pesticides. The exposure ranges from 0.35% of the ADI for the benomyl group to 9.9% of the ADI for the methidathion group. In 1999, four commodities (cauliflower, peppers, wheat grains, and melon) were analysed for the same 20 pesticides as in the 1998 study (European Commission, 2001). Overall, around 4700 samples were analysed. Residues of methamidophos exceeded MRLs most often (8.7%), followed by the maneb group (1.1%), thiabendazole (0.57%), acephate (0.41%) and the benomyl group (0.35%). The MRL for methamidophos was exceeded most often in peppers and melons (18.7 and 3.7%, respectively). The residues of the maneb group exceeded the MRL most often in cauliflower (3.9%); residues of thiabendazole exceeded the MRL most often in melons (2.8% of the melon samples). With regard to all the commodities investigated, around 22% of samples contained residues of pesticides at or below the MRL and 8.7% above the MRL. Residues at or below MRL were found most often in melons (32%), followed by peppers (24%), wheat grains (21%) and cauliflower (17%). MRL values were exceeded most often in peppers (19%), followed by melons (6.1%), cauliflower (3%) and wheat grains (0.5%). The intake of pesticide residues did not exceed the ADI in any case. It was below 1.5% of the ADI for all pesticides. The exposure ranged between 0.43% of the ADI for methamidophos and 1.4% of the ADI for endosulfan. The intakes for the highest residue levels in a composite sample for chlorpyriphos, deltamethrin, endosulfan and methidathion were below the ARfD for adults. They range between 1.5% of the ARfD for deltamethrin and 67% of the ARfD for endosulfan (Nasreddine and Parent-Massin, 2002). In spite of food contamination, most pesticide deaths recorded in hospital surveys are the result of self-poisoning (Eddleston, 2000). The Global Burden of Disease Study 6 estimated that 798 000 people died from deliberate self-harm in 1990, over 75% of whom were from developing countries

(Murray and Lopez,1996). More recent WHO estimates showed that over 500 000 people died from self-harm in Southeast Asia and the Western Pacific during 2000 alone (WHO, 2001). Suicide is the commonest cause of death in young Chinese women and Sri Lankan men and women (Murray and Lopez, 1996; Sri Lankan Ministry of Health, 1995; WHO, 2001). In India the first report of poisoning due to pesticides was from Kerala in 1958, where over 100 people died after consuming wheat flour contaminated with parathion (Karunakaran, 1958). In a multi-centric study to assess the pesticide residues in selected food commodities collected from different states of India, DDT residues were found in about 82% of the 2205 samples of bovine milk collected from 12 states. About 37% of the samples contained DDT residues above the tolerance limit of 0.05 mg/kg (whole milk basis). The highest level of DDT residues found was 2.2 mg/kg. The proportion of the samples with residues above the tolerance limit was highest in Maharastra (74%), followed by Gujarat (70%), Andhra Pradesh (57%), Himachal Pradesh (56%), and Punjab (51%). In the remaining states, this proportion was less than 10%. Data on 186 samples of 20 commercial brands of infants formulae showed the presence of residues of DDT and HCH isomers in about 70 and 94% of the samples with their maximum level of 4.3 and 5.7 mg/kg (fat basis) respectively. Measurement of chemicals in the total diet provides the best estimates of human exposure and of the potential risk. The risk of consumers may then be evaluated by comparison with toxicologically acceptable intake levels. The average total DDT and BHC consumed by an adult were 19.24 mg/day and 77.15 mg/day respectively (Kashyap *et al*., 1994). Fatty food was the main source of these contaminants. In another study, the average daily intake of HCH and DDT by Indians was reported to be 115 and 48 mg per person respectively, which were higher than those observed in most of the developed countries (Kannan *et al*., 1992).

1.5.1.5 The Pesticide Market and Environmental Issues

Pesticides are useful to society because of their ability to exterminate disease-causing organisms and control insects, weeds and other pests. At the same time, most pesticides may be harmful to humans, animals and the environment because of their ecotoxicity, their potential bioaccumulating properties or their hormone disrupting effects. Pimentel and Greiner (1996) described and summarized the environmental and socio-economic costs of pesticide use as follows: pesticide use has an impact on national health and livestock, it results in less effective predators and higher costs due to pesticide resistance, it affects bees,

birds, fish and other aquatic organisms and it contaminates ground and surface water reserved for drinking water purposes. Pesticides are of concern for public authorities, as it seems that pesticide pollution is not a fleeting issue. Worldwide sales figures show that the agrochemical market has been relatively static for almost 20 years, increasing in line with inflation. In 2004, herbicides accounted for 45.4% of the agrochemical market, followed by insecticides 27.5%, fungicides 21.7% and other products 5.4%. The most noticeable trend is the increased share of herbicides and reduced share of insecticides (Dingham, 2005). When pesticides are applied on the field, only a certain percentage of the applied dose will reach the target crop. The remaining fraction will enter the soil, air, surface and groundwaters through different pathways. In the different compartments of the ecosystem, pesticides are then -to a smaller or larger extent- available for organisms. Depending on the exposure concentration and the mode of action, pesticides can be harmful to humans, animals and the ecosystem.

Therefore, public authorities and industry tried to minimize the negative consequences of pesticide use. The use of certain highly toxic pesticides has been banned and the use of triazine herbicides, organophophate and carbamate insecticide has been restricted. These groups of pesticides have been replaced by other classes of pesticides that have shorter half-lives or are applied in smaller amounts. Through monitoring and modelling, one can gain insight in the occurrence of pollutants in the different compartments of the ecosystem. Besides, models can be used as a decision tool in the selection of mitigation measures in order to reduce environmental contamination.

1.5.1.6 Global Demand of Pesticides

Worldwide, pesticides have helped achieve higher food productions, increased food security by reducing vulnerability of crops to plagues and pathogens, and lower morbidity and mortality rates for certain vector-borne diseases such as malaria. However, pesticides can have many adverse environmental impacts: persistence in soil can make once-rich soil unusable for farming, bioaccumulation can wipe out living creatures and sources of food, and runoff and groundwater infiltration can contaminate water, causing nutrient pollution (US EPA, 2005a). Additionally, pesticides have potential for various unintended negative consequences to human health ranging from respiratory issues, impairment to the central

nervous system, developmental issues in babies and children, to types of cancer such as lymphoma (Bus & Hammond, 2007). Many of these unintended negative consequences are not yet fully understood.

In 2007, five billion pounds of pesticides were sprayed worldwide (US EPA, 2013) and in 2014 world demand is expected to reach $52 billion (The Freedonia Group, Inc., 2010). In recent decades, the availability of pesticides has increased in developing countries, to the far-out reaches of the countryside where subsistence‖ agriculture is still the lifestyle. Previously it was assumed that the poorest of the poor could not afford pesticides, but they are especially cheap in countries where regulations are loose. Sometimes they are given free of charge by government agencies (Mokhele, 2011) or are sold by agriculture supply stores that they are ―pure medicine‖ for the crops. At times, pesticides that are banned or restricted for use in developed countries are brought to developing countries for crops that are then shipped back to the developed world for sale (Wright, 1986) or return by atmospheric transport (Mihelcic, 1999)—this is referred to as ―the circle of poison‖ (Wright, 1986).

Since 2000, pesticide sales in North America have only increased slightly and sales in Europe have increased by nearly $6 billion. Sales in the Middle East and Africa have remained steady probably due to the fact that the majority of farmers in sub-Saharan Africa remain too poor to use pesticides on a regular basis. Pesticide sales in Asia have also increased substantially as they are highly dependent on pesticide use, which remains a significant public health issue. For example, in Sri Lanka in the 1990s, death by pesticide poisonings exceeded death by infectious diseases (Eddleston *et al.*; 2002). In the last five years, Latin America has started to approach North America in pesticide sales. This is alarming because pesticide use and sales in Latin America remain generally unregulated.

By 2020, the developing world is projected to be responsible for one-third of the world's chemical production and consumption (including pesticides) (Rain, 2005). In the developing world, adverse environmental and health effects are typically greater due to laws that are non-existent or not enforced. Agricultural supply stores sell chemicals without restriction. Workers, who utilize agriculture chemicals, are typically untrained in chemical safety, do not wear personal protective equipment or know how to properly use or store chemicals, and sometimes are illiterate and unable to read the toxicity warning labels.

The World Health Organization's (WHO) International Program on Chemical Safety (IPCS), founded in 1980, rates hazardous pesticides as one of the top ten chemical or groups of chemicals of major public health concern worldwide (WHO, 2010). Around the same time as the founding of the IPCS, the Food and Agriculture Organization (FAO) of the UN released the International Code of Conduct on the Distribution and Use of Pesticides, intended to reduce negative impacts associated with pesticide use, specifically in developing countries. The proclamation is considered the globally accepted standard for pesticide management (Wesseling *et al*.; 2005) and includes standards for national governments to enforce regarding registration of pesticides. However, this document generally serves only as recommendation, as registration of pesticides and poisonings in Central America do not comply with the code (Wesseling *et al*.; 2005). Furthermore, most countries in Central America do not have their own legislation pertaining to pesticides but rather refer to the international legislation. In Central America, 98% of all pesticide poisonings go unreported (Science, 2013). El Salvador, Nicaragua, and Honduras have initiated efforts to restrict certain chemicals at the legislation level, while many countries lag behind (Wesseling et al., 2005).

1.5.1.7 Pesticide Dissipation

The behavior of a pesticide results from the interactions between the chemical and various components of the environment. Pesticides can undergo various routes of dissipation once applied in the environment. Major routes of pesticide dissipation include adsorption, transfer (i.e. volatilization, runoff, leaching, absorption) and degradation (i.e. photodegradation, chemical degradation, and microbial degradation). Adsorption occurs when the pesticide binds to soil particles. Soil sorption is the affinity a chemical has to adhere to soils. The extent to which a pesticide is adsorbed to soil depends on soil type, soil texture, soil pH, soil moisture and the pesticide itself (Parker and Doxtader, 1983; Wild, 1993; Gan *et al*., 1996; Dyson et al., 2002). Soil retention characteristics of pesticides, for example, vary with the number and location of polar functional groups of the pesticides. Cationic pesticides are strongly held to negatively charged soil by ionic bonds. Anionic pesticides are poorly held to negatively charged soil particles unless positively charged soil colloids are present; and nonionic pesticides are often weakly held at the soil surfaces through weak physical forces (McCarty *et al*., 2003). Clay or organic soils are more adsorptive than coarse, sandy soil due in part to their increased surface areas (Johnson et al., 2007). Soil pH can affect the

equilibrium between undissociated pesticide molecules and the anion molecules of the pesticide. Such an equilibrium shifts as soil pH changes in relation to pka value of the pesticide. The herbicide 2,4-dichlorophenoxyacetic acid, for example, has a pka of 2.8 (Wauchope *et al*., 1992). When soil pH goes above 2.8, 2,4-dichlorophenoxyacetic acid would exist primarily in its dissociated, negatively charged form. As soil pH increases, adsorption will decrease because the 2,4-D molecules are more repelled from the overall negative charges of soil colloids (McCarty *et al*., 2003). Pesticide volatilization occurs when the solid or liquid form of a pesticide is transformed into a gas. Volatilization can reduce effectiveness of a pesticide by reducing the amount of chemical that makes contact with the intended target. As temperature and ambient air movement increase, the potential for pesticide loss through volatilization increases (Yates *et al*., 2002; Haith *et al*., 2002). Henry's law summarizes the relationship between pesticide solubility and volatilization by stating that pesticide volatile loss is inversely proportional to pesticide solubility (Dearden & Gerrit; 2003). Pesticide loss in the air can also occur because of spray drift. Drift consists of droplets produced by nozzles of the spray equipment being suspended in air and carried away by air flows before reaching any surface. Pesticide runoff occurs when pesticides are carried away by surface water movement. Typically, if water addition to a field is faster than it can be absorbed into the soil, runoff occurs. Pesticide molecules can move either when dissolved in the water, or through attachment with soil particles or sediments (Bailey *et al*.; 1974). Factors that influence pesticide runoff include the physic-chemical properties of the pesticide, application method, soil property, hillside slope, timing, duration, intensity, climate, and agricultural practices (Zhang *et al*.; 1997; Lecomte *et al*.; 2001; Chaplot *et al*.; 2003). Losses from runoff can be greatest when it rains heavily immediately after a pesticide application (Smith and Bridges; 1996; Ma *et al*.; 1999). For example, Ma et al. (1999) simulated rainfall 1 d before and 1,2,4, and 8 d after field application of 2,4-D [(2,4-dichlorophenoxy) acetic acid], dicamba (3,6-dichloro-2- methylphenoxy-benzoic acid), and mecoprop [(±)-2-(4-chloro-2-methylphenoxy)- propanoic acid]. They found that both the mass and concentration of pesticide runoff decreased rapidly with each rainfall event, with the first posttreatment eventrunoff averaging 74.5, 71.7, and 73.0% of the total runoff of 2, 4-D, dicamba, and mecoprop, respectively. Leaching is the downward movement of chemicals in water through the soil. Pesticides that are easily leached have a high potential to reach groundwater. The characteristics of the soil and pesticide play an important role in influencing pesticide leaching. Sandy soils are more prone to leaching than clay textured soils because macropore flow in sandy soil is more extensive than in clay textured soil. When macropore flower is less

extensive, adsorption is stronger and leaching potential is lower (Roulier & Jarvis; 2003). Pesticides having low water solubility, high soil adsorption, and low persistence are less likely to leach than pesticides that are highly soluble, less adsorptive to soil, and more persistent (Webb *et al*.; 2008; Magri & Haith; 2009). Absorption is the movement of chemicals from the surface to the interior of the plant. Plant absorption can occur either through leaves or roots of the plant. Systemic pesticides, for example, move inside a plant following absorption of the plant. Degradation is the process of pesticide breakdown after application and it is a very important route of pesticide dissipation. As pesticides are broken down, the possibility of the pesticide chemicals reaching ground or surface water and thus creating environmental or health related concerns is generally minimized. Pesticides can be broken down by microbes, chemical reactions, and light; these processes are known as biodegradation, chemical degradation, and photodegradation, respectively (Wheeler; 2002). Microbial degradation is the breakdown of pesticides by fungi, bacteria, and other microorganisms that use pesticides as an energy source. The aerobic and anaerobic oxidation and reduction of pesticides by microbial populations produce energy for the microbes (Magri & Haith; 2009). In soil-enriched environments, soil conditions such as moisture, temperature, pH, and the amount of organic matter affect the rate of microbial degradation due to their direct or indirect influence on microbial growth and microbial activity (Wise & Trantolo, 1994; Magdoff & Weil; 2004). Chemical degradation is the breakdown of pesticides by processes where living organisms are not involved. Major chemical reactions such as hydrolysis, oxidation, and reduction, without the influence of microbial activity, are processes involved in chemical degradation. Photodegradation is the breakdown of pesticide by sunlight. The rate of breakdown is influenced by the intensity and spectrum of sunlight, length of exposure, and the properties of the chemical. Photodegradation can occur by direct or indirect absorption of light (Zepp & Cline; 1977).In direct photolysis, for example, the substance absorbs UV-visible light energy and undergoes transformation (Konstantinou *et al*.; 2001).

1.5.1.8 Use of Pesticides and their Risk in Bangladesh

As a part of PhD work, some survey about the present pesticides used in field and storage level have done. The climate of Bangladesh is suitable to cultivate a wide variety of crops-

rice, wheat, jute, potato, oilseeds, pulses, maize, tobacco, cotton, sugarcane, fruits, and vegetables. Rice is our staple food and grows in three crop growing seasons. In early times (up to 1956), traditional methods have done to control pest but after introduction of synthetic pesticides, the use of pesticides became very popular among the farmers for pest control of rice and other crops. A total of 94 pesticides, with 299 trade names, of different groups and formulations, have been registered for use in agriculture (List of Registered Agricultural and Public Health Pesticides in Bangladesh; 2008). From the observation of the recent government figures available (**Figure 7**), the total pesticides imported in Bangladesh is increases gradually.

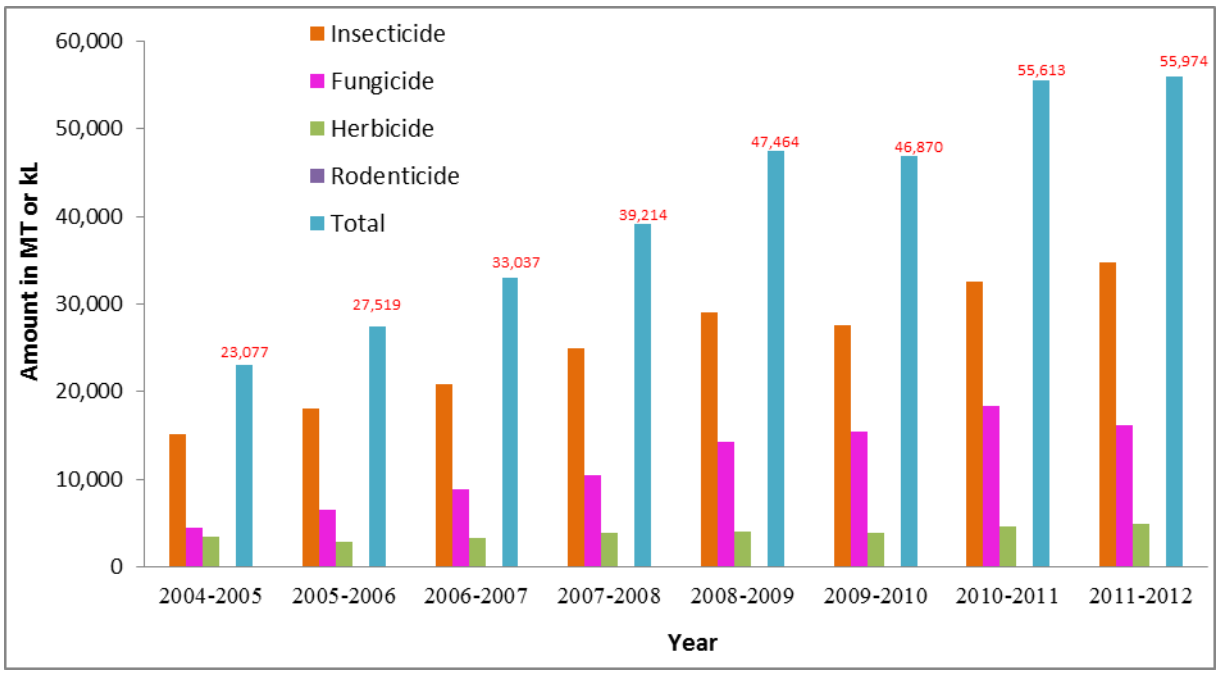


Figure 7: Imported pesticides (MT/kL) in the year 2004 to 2012

The farmers of Bangladesh do not have enough knowledge about the side effects of pesticides. To get more products, they use overdose than needed. In field level, the farmers do not take any precaution during pesticide using (**Figure 8**). Not only that, the empty container after uses attract them and they use these as household purpose even as food container. These are creating serious health problems in Bangladesh.

Figure 8: The farmer is spraying pesticide in a vegetable field

Survey was done in Mymensingh Sadar (**Table 1**) and Lalmonirhat District (**Table 2**). During survey in Patgram Thana (Lalmonirhat District) which is adjacent to Burimary border, it was surprising that a lot of banded pesticides are available in farmer level with relatively low cost though they sale these in hidden way. Some of these are very popular to farmers due to those pesticides are more profitable and prompt re-active than the other registered pesticides. The actual fact is that when they use heptachlor or endrin they need not to use other herbicides or insecticides for long time. This indiscriminate uses of risky pesticides are creating dangerous environmental problems.

Table 1: Pesticides Uses in Mymensingh Sadar

Name of places: 1. Jublighat, Shotobazar, Ketoali Thana, 2. Heshbulla Market, Tankpatri and 3. Balashpur, KewatKhali

Active compounds	Trade name	Half-life DT_{50}	Physical state
Chlorpyrifos	Morter 48EC	10-120 d	Liquid
Cypermethrin	Buster , Ustad, Mazic and Ripcord(10 EC)	60 d	Liquid
Carbofuran	Marshal 6G	30-60 d	Liquid
Diazinone	Basudin, Diazinon(10 G)	11-21 d	Liquid
Malathion	Malaphos(57 EC)	3-4 d	Liquid
Carbaryl	Sevin(85 SP)	7-28 d	Powder
Permethrin	Finis	<38 d	Powder
Aluminium Phosphide	Alumphos 56%	-	Tablet

Table 2: Pesticides Uses in Lalmonirhat District

Name of places: 1. Thanzara, Baura, Hatibandha Thana and 2. Rasulganj, Patgram Thana

Active compounds	Trade name	Half-life DT_{50}	Physical state
Chlorpyrifos	Relothrin, Polon, Darsban, Nitro	10-120	Liquid
Hexachonazol	Contaf	-	White crystalline solid
Pretilachlor	Comit	200	Liquid
Cypermethrin	Nitro, Polon	60	Liquid
Cabrio Top	Cabrio Top 60% WG	11-17	Powder
Dolomite	Dolomite mineral powder	-	Powder
Mancozeb	Indofil ,Jineb	6-15	Powder
Carbendazim	Bavistin	8-32	Crystalline Powder
Carbofuran	Carbotaf, Autotaf 3G	30-60	Powder
Fipronil	Regent	122-128	Powder
Diazinon	Basudin, Diazinon (10 G)	11-21	Liquid
Malathion	Malaphos (57 EC)	3-4	Liquid
Carbaryl	Sevin (85 SP)	7-28	Powder
Permethrin	Finis	<38	Powder
Aluminium Phosphide	Alumphos 56%	-	Powder
Pretilachor	Longfit	>200	Liquid
Naphthalene	Naphthalene	71 hours (Surface water)	Crystalline solid
Endosulfan	Thiodin	9 months-6 years	Solid
Heptachlor	Aahepta	9-10 months	Crystalline solid
Endrin	Endrex	14 years	Solid
Aldrin	Aldrex	5 years	Solid

Rice is cultivated in three seasons in Bangladesh. This decreases the demand of storage in farm level. The moisture content is control traditionally by drying under sunlight. Disasters including floods, cyclones (and tidal surges), river bank erosion, tornadoes, earthquakes, etc are common phenomenon in Bangladesh agriculture. To manage these emergency situation rice and other agricultural products are stored by public and private sectors. There are about 750 godowns to preserve nearly 0.6 million metric tons of food grain in Bangladesh. Commercially rice parboiling includes both soaking and steaming in rice mill. The maximum rice in market are very good polished and glazy. To improve the gauziness the paddy are half boiled. Though rice is stored for short time in godawn or shop but due to this fact the chance of attacking fungus and pest are increased.

It was very difficult to collect information about pesticide uses in storage level. The storage businessman was enough literate about the side effect of pesticides, but for more profit they also uses hazardous chemical pesticides. From labors it was surveyed that they mixes finish powder, spraying cypermethrine, chlorpyrifos and gas tablet. In big rice and pulse shops, the labors sleep adjacent place, they said during using gas tablet they feel very uneasy for bad smelling.

1.5.2 Veterinary Drug Residues

Similar to pesticides, veterinary drugs are agrochemicals that undergo a thorough registration process, resulting in setting of their maximum residue limits/tolerances in animal-derived foods. The major classes of veterinary drugs include antibiotics, anthelmintics, coccidiostats, nonsteroidal anti-inflammatory drugs, sedatives, corticosteroids, beta-agonists and anabolic hormones. These drugs, which are administered to live animals, can remain as residues in animal tissues. Liver and kidney are highly susceptible to residues given their biological function (Baert et al.; 2003).

Certain antibiotics, such as penicillin, can cause severe allergic reactions in sensitive individuals, which is an important reason for enforcing their residue limits in foods of animal origin. Another important justification for limiting antibiotic usage in food-producing animals is to reduce the risk of pathogenic microorganisms becoming resistant to antibiotics. Most veterinary drugs are not of acute toxicological concern, but some substances, such as nitrofurans, chloramphenicol, clenbuterol and diethylstilbestrol, have been banned in most countries due to their carcinogenicity. Concern about endocrine-disrupting effects has become another reason for regulation of certain veterinary drugs, such as beta-agonists and hormones.

1.5.3 Environmental Contaminants

Environmental contaminants can be man-made or naturally occurring substances present in air, water or soil (Chen et al.; 2000). They can enter into the food chain and even bio-accumulate. Some can pose an acute health risk if present at higher concentrations, but the

major concern related to the presence of environmental contaminants in foods is for their potential endocrine disruption, developmental, carcinogenic and other chronic effects.

Examples of environmental contaminants that enter into the food chain include heavy metals, polychlorinated biphenyls (PCBs), "dioxins" (polychlorinated dibenzodioxins and dibenzofurans), persistent chlorinated pesticides (e.g., DDT, aldrin, dieldrin, heptachlor, mirex, chlordane), brominated flame retardants (mainly polybrominated diphenyl ethers), polyfluorinated compounds, polycyclic aromatic hydrocarbons (PAHs), perchlorate, pharmaceutical and personal care products or haloacetic acids and other water disinfection byproducts (Karagas et al.; 2012).

The manufacture and use of PCBs and other persistent organic pollutants (POPs) have been banned for years, but they remain in the environment due to their high stability. PAHs can be found in the environment as a result of industrial pollution or can originate from oil spills; thus, they were of concern in seafood after the oil spill accident in the Gulf of Mexico in 2010.

Environmental contaminants are chemicals that accidentally or deliberately enter the environment, often, but not always, as a result of human activities. Some of these contaminants may have been manufactured for industrial use and because they are very stable, they do not break down easily. If released to the environment, these contaminants may enter the food chain. Other environmental contaminants are naturally-occurring chemicals, but industrial activity may increase their mobility or increase the amount available to circulate in the environment, allowing them to enter the food chain at higher levels than would otherwise occur.

A wide variety of environmental contaminants including lead, arsenic, mercury, bromate, brominated flame retardants, chlorinated naphthalenes, dioxins, furans, PCBs, perchorate and perfluorinated chemicals etc have been detected in foods.

1.5.4 Food Processing Contaminants

Certain toxic or undesirable compounds can be formed in foods during their processing (Cristina et al.; 2016), such as during heating, baking, roasting, grilling, canning, hydrolysis

or fermentation. Precursors of these contaminants can occur naturally in the food matrix, such as in the case of acrylamide being formed during the Maillard reaction between the amino acid asparagine and a reducing sugar (especially in potato- and cereal-based, heat-treated products). Alternatively, certain processing contaminants, such as nitrosamines, can be formed by interaction of natural food components with food additives. Carcinogenic and genotoxic chlorpropanols, such as 3-monochloropropane-1, 2 diol (3-MCPD), are formed during the acid hydrolysis of wheat, soya and other vegetable protein products.

Examples of other processing contaminants include PAHs (in grilled and smoked products), ethyl carbamate (in yeast-fermented alcoholic beverages and other products) or furan (in a variety of heat-treated foods, especially coffee and canned/jarred food). Food processing may also be a source of cross-contamination, such as contamination of nonallergenic foods with known food allergens.

Undesirable chemicals can be formed in certain foods during processing as a result of reactions between compounds that are natural components of the food. In some cases an undesirable chemical may be formed as a result of a food additive being intentionally added to food and reacting with another compound in the food. When foods are heat-processed (baked, deep-fried, etc.), there are reactions that occur between components of the food, resulting in the desired flavour, appearance and texture of the food. However, some of these reactions can lead to the production of undesirable compounds. Similarly, certain storage or processing conditions may allow reactions to occur that otherwise would not. These reactions could generate potentially harmful compounds. Such chemicals can be collectively referred to as food-processing-induced chemicals. Some of these chemical reactions involve naturally-occurring components in the food, while other reactions may involve food additives, ingredients, or food packaging materials that were intentionally used.

In many cases, the presence of processing-induced chemicals (acrylamide, benzene, chloropropanols, ethyl carbamate, furan, heterocyclic aromatic hydrocarbons, nitrosamines, polycyclic aromatic hydrocarbons and semicarbazide) in food cannot be avoided; however, understanding the processes by which these products are formed can allow us to optimize or adjust food preparation methods, formulae or processes, thereby reducing or eliminating the formation of such chemicals.

1.5.5 Migrants from Packaging Materials

Direct contact of foods with packaging materials can result in chemical contamination caused by migration of certain substances into foods (Arvanitoyannis and Bosnea; 2004). Examples of migrants of health concern may include bisphenol A or phthalates from plastic materials, 4-methylbenzophenone and 2-isopropylthioxanthone from inks, mineral oil from recycled fibers or semicarbazide from a foaming agent in the plastic gaskets that are used to seal metal lids to glass packaging.

1.5.6 Unapproved Food Additives and Adulterants

Food adulteration can happen accidentally when unapproved additives are introduced to the food, or the wrong additive is introduced through formulation error. This results in mislabeled food. Perhaps a larger health issue is when foods are adulterated intentionally for economic reasons to sell a low-value food or material for more or to mask food spoilage. Some adulteration may just mislead or cheat consumers, such as adding high fructose corn syrup to honey, but some may be harmful to them. The most notorious example from recent years is the addition of melamine to whey and other protein concentrates to increase their apparent protein content analyzed as total nitrogen (Liu et al.; 2010). Other examples include the use of toxic Sudan dyes in adulterated chili powders or adulteration of virgin olive oil with hazelnut oil, which can cause unexpected allergic reactions in sensitive individuals.

1.5.7 Toxins

Toxins are naturally occurring substances that are produced by various organisms, with mycotoxins and marine biotoxins typically representing the major concerns in foods. Other examples of toxins in foods may include bacterial toxins (e.g., staphylococcal toxins) or certain plant toxins, such as pyrrolizidine alkaloids that can be found in honey, milk or eggs. While the bacterial/fungal contamination can be eliminated with heat treatment, the toxins can remain in the food product as contaminants.

Mycotoxins are toxic secondary metabolites produced by fungi (molds) that can colonize

various crops. They are of concern mainly in cereals, nuts, infant formula, milk, dried fruit, baby food, coffee, fruit juice and wine. There are many mycotoxins, but only a few are currently regulated, with the European Union having a more comprehensive list than most other countries, which includes aflatoxins, ochratoxin A, patulin, deoxynivalenol, zearalenone, fumonisins and T-2/HT-2 toxins. Different mycotoxins are prevalent in different climates and in various growing and storage conditions.

Marine biotoxins, such as saxitoxin, domoic acid, okadaic acid or ciguatoxin, are highly toxic compounds produced by phytoplankton. During so-called harmful algal bloom events, they can accumulate in fish or shellfish, such as clams, mussels, scallops or oysters, to levels that can pose serious health risks or even be lethal to humans.

Under certain conditions, some plants have the capacity to naturally produce compounds that are toxic to humans when ingested. For example, under certain conditions, microscopic algae (tiny plants) in the ocean can produce compounds that are toxic to humans but not to shellfish that eat this algae. When people eat shellfish that contain these toxins, illness can quickly follow (Quilliam et al.; 1989).Certain climatic conditions may favour the growth of toxin-producing fungi on food crops (toxins produced by fungi are called "mycotoxins"). Shellfish may contain toxins as a result of filter-feeding on microscopic algae. In such a case, the algal toxin does not harm the shellfish but can be harmful to humans. Natural toxins are are not harmful to the organisms themselves but they may be toxic to other creatures, including humans, when eaten. Mycotoxins are another group of natural toxins. The word mycotoxin is derived from the Greek word for fungus 'mykes' and the Latin word 'toxicum' meaning poison. Mycotoxins are toxic chemical products formed by fungi that can grow on crops in the field or after harvest. The foods that can be affected include cereals, nuts, fruit and dried fruit, coffee, cocoa, spices, oilseeds and milk. There are now more than 300 known mycotoxins of widely different chemical structures and differing modes of action - some target the kidney, liver, or immune system and some are carcinogenic. Common mycotoxins include aflatoxins, ochratoxin A, ergot alkaloids, fumonisins, patulin, trichothecenes (such as deoxynivalenol which is also known as vomitoxin) and zearalenone.

1.6 Aflatoxins

Aflatoxins are secondary metabolitis, produced by *A. flavus* link *A. parasiticus* (Cleveland, *et al*.;1992 & Cotty ; 1997). They are secondary metabolites which considered as one of the threatening factors of food and feed consumer health. These fungi survive in a wide range of environments and can be found in soil, plant and grains and their products (Pitt; 2000). Those fungi are responsible for spoilage of stirred grains and their products around the world (Reddy *et al.* 2008). *A. flavus* is the main fungus that causes preharvest aflatoxin contamination field crops. The food and agriculture organization of the united nations (FAO) estimated that at least 25% of the worlds cereal grains are contaminated by mycotoxins including aflatoxins (Bhatnagar *et al.*; 2004 & Dai; 1997). Because of the toxic and potent carcinogenic of aflatoxins, many developed countries have established very stringent regulations limiting the maximum allowable amount of aflatoxins in food and feed (Massey *et al*.; 1995 & Hussein *et al*.:2001).

1.7 Types of Aflatoxins

At least 18 different aflatoxins are produced in nature (*Boutrif; 1998*). Aflatoxin B_1 is considered the most toxic. Aflatoxin M_1 is present in the fermentation broth of *Aspergillus parasiticus*, but it and aflatoxin M_2 are also produced when an infected liver metabolizes aflatoxin B_1 and B_2. The four major aflatoxins (**Figure 9**) are called B_1, B_2, G_1, and G_2 based on their fluorescence under UV light (blue or green) and the aflatoxin metabolic byproducts are M_1 and M_2.

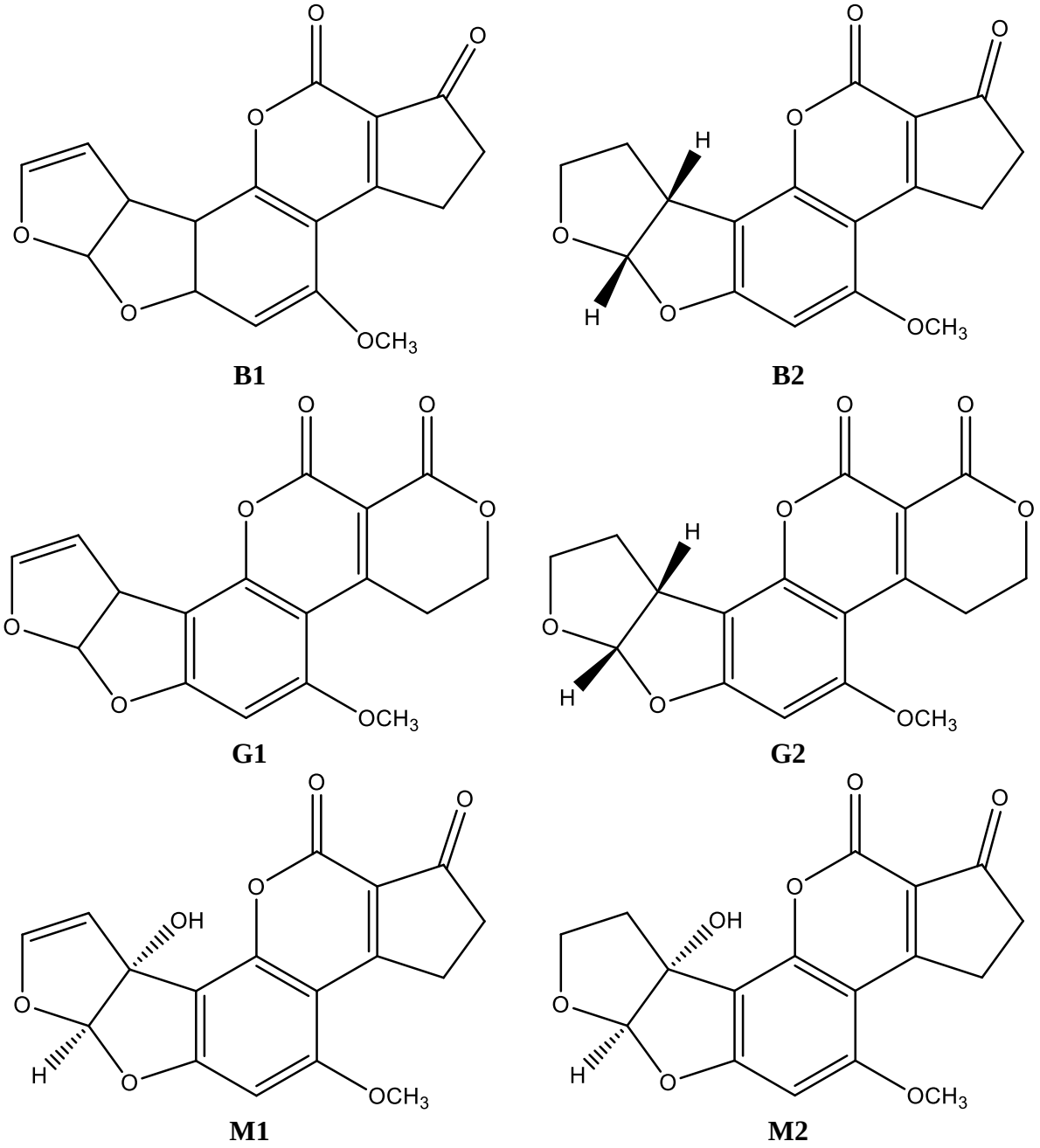


Figure 9: Structure of six aflatoxins (B1, B2, G1, G2, M1 and M2)

Aflatoxin B_1 and B_2, produced by *A. flavus* and *A. parasiticus.* Aflatoxin G_1 and G_2, produced by some Group II *A. flavus* and *Aspergillus parasiticus* (*Geiser; 2000*). Aflatoxin M_1, metabolite of aflatoxin B_1 in humans and animals (exposure in ng levels may come from a mother's milk). Aflatoxin M_2, metabolite of aflatoxin B_2 in milk of cattle fed on contaminated foods. Aflatoxin Q_1, major metabolite of B_1 in *in vitro* liver preparations of other higher vertebrates (*Smith; 1991*).

Aflatoxins are normally refers to the group of difuranocoumarins and classified in two broad groups according to their chemical structure:

1. Difurocoumarocyclopentenone series: (AFB1, AFB2, AFB2A, AFM1, AFM2, AFM2A and aflatoxicol).
2. Difurocoumarolactone series: (AFG1, AFG2, AFG2A, AFGM1, AFGM2, AFGM2A and AFB3).

The aflatoxins display potency of toxicity, carcinogenicity, mutagenicity in the order of

AFB1 > AFG1 > AFB2 > AFG2

The aflatoxins fluoresce strongly in ultraviolet light (ca. 365 nm):

B1 and B2 produce a blue fluorescence where as G1 and G2 produce green fluorescence.

The most important AFs are aflatoxin B1 (AFB1)**,** B**2** (AFB2)**,** G1 (AFG1) and G**2** (AFG2). However, AFB1 is the most frequently occurring among all of AFs. Moreover, the presence of AFB1 owing to its extreme toxicity is often associated with severe hepatotoxigenicity and hepatocarcinogenicity. Biotransformation of AFB1 occurs mainly in the liver through cytochrome P450 enzymes that can act in detoxification (Wang *et al*., 1998). The optimal temperature for AFs production ranged between 20–35°C. Elevation of temperature upto 40°C or decline upto 100C could result in reduced toxins production. The high temperature within the optimal range favours the production of aflatoxin B (AFB). On the other hand, low temperature favours the production of aflatoxin G (AFG) (Schroeder *et al*.; 1967).

1.8 Toxicity of Aflatoxins

Aflatoxins (AFs) are a group of fairly distributed mycotoxins. AFs are reported as hepatotoxic, mutagenic, immunosuppressive and neoplastic. According to the quantity ingested, frequency of intake and the age of individual, result could be cirrhosis, necrosis of the liver, encephalopathy and increased susceptibility to hepatitis B (Lereau et al., 2012).

Aflatoxins adversely affect the human health. Aflatoxicosis is a disease caused by the aflatoxins. Sudden death occurs as a result of acute aflatoxicosis and in chronic aflatoxicosis the prolonged pathological changes like cancer and immune suppression happens (Magnussen *et al*.; 2013). Aflatoxin is well known agent for its hepatocarcinogenic properties. The risk of liver cancer is almost 30 times higher in the subjects exposed to aflatoxin than unexposed ones (Liu *et al*.; 2010).

Aflatoxicosis causes acute liver damage, liver cirrhosis, induction of tumors, impaired central nervous, skin disorders and immune defects. The overall toxicity of aflatoxin in an animal appears to be determined by the rate of formation of the reactive intermediate binding to the largest macromolecules (DNA and RNA) (Makun *et al*.; 2007 & Speijers *et al*. 2004). Then for both feed safety and economic reasons, aflatoxin contamination is therefore a serious concern throughout the world.

1.9 Aflatoxins in Food

Aflatoxins may be present in a wide range of food commodities, particularly cereals, oilseeds, spices and tree nuts. Maize, groundnuts (peanuts), pistachios, brazils, chillies, black pepper, dried fruit and figs are all known to be high risk foods for aflatoxin contamination, but the toxins have also been detected in many other commodities. Milk, cheese and other dairy products are at risk of contamination by aflatoxin M. The highest levels are usually found in commodities from warmer regions of the world where there is a great deal of climatic variation.

It is important to recognise that, although it is primary food commodities that usually become contaminated with aflatoxins by mould growth, these toxins are very stable and may pass through quite severe processes. For this reason they can be a problem in processed foods, such as peanut butter.

Fungal proliferation depends upon environmental favorable conditions like, high humidity and temperature (Choudhary *et al*.; 2010). The permitted limits in cereal grains set by European Union Commission Regulation (EC) No. 1881/2006 for total aflatoxins are 4.0 μg/kg and for aflatoxin B1 is 2.0 μg/kg (*Journal of the European Union*; 2006).

1.10 Evidence of Exposure to Aflatoxins

The main route of exposure to aflatoxin is through the direct consumption of contaminated food. For humans, aflatoxin is predominantly perceived as an agent promoting liver cancers, although lung cancer is also a risk among workers handling contaminated grain (Kelly *et al*.; 1997). The increased risk of hepatomas is caused by deletion mutations in the P53 tumor-suppressing gene and by activation of dominant oncogenes (Dragan & Pitot; 1993). The risk of cancers due to exposure to the various forms of aflatoxin is well established (Gorelick *et al*.; 1993) and is based on the cumulative lifetime dose. The International Cancer Research Institute identifies aflatoxin as a Class 1 carcinogen, resulting in the regulation of this toxin to very low concentrations in traded commodities [20 ppb in grains and 0.5 ppb in milk in the United States; 4 ppb in foods in some European countries (Henry *et al*.; 1999)].

However, in many developing countries, epidemics of hepatitis B virus (HBV) and hepatitis C virus (HCV) affect ≤20% of the population. A strong synergy is observed between aflatoxin and these biological agents for liver cancer. In hepatitis B surface antigen-positive subjects, aflatoxin is ≈30 times more potent than in persons without the virus (Henry *et al*. 2002), and the relative risk of cancer for HBV patients increases from ≈5 with only HBV infection to ≈60 when HBV infection and aflatoxin exposure are combined (Groopman; 1993). In some areas where aflatoxin contamination and HBV occur together, hepatomas are the predominant cancer (64% of cancers; Wang *et al*.; 2001), and they may be a predominant cause of death: ≈10% of males in Gambia die of liver cancer (CP Wild, 1999), and in Qidong, China, 10% of all adult deaths were due to this cancer (Wang *et al*; 1996). Thus, to minimize the risk of liver cancer, it is critically important that exposure of HBV- and HCV-infected persons to aflatoxin is minimized.

A factor in this greater potency of aflatoxin in HBV-positive people is the finding that HBV positivitity reduces the person's ability to detoxify aflatoxin (Allen *et al*; 1992). Whereas this synergy is recognized as an important factor for cancer, it is also of great potential importance for immunologic and nutritional toxicities, because it increases the level of biological exposure.

1.11 Objective of the Work

Bangladesh is one of the major rice growing countries of the world and rice is the staple food of the people. In order to increase the production of rice, vegetable and other crops many registered pesticides are being used. Early seventies, Bangladesh had taken a step of "green revolution" to produce sufficient amount of agricultural crops to have food security. Large amount of fertilizer, pesticides were supplied and irrigation system has been improved to produce high yielding varieties rice, vegetable and other crops like pulses, lentil, grains etc. During production of bumper crops, food crops are stored by public and private sectors to meet the emergency when food loss occur due to tornado, cyclone, flood etc or by the businessman to sell in off season for more profit. The Government of Bangladesh collects food grains from the farmer and reserve food for urgent needs. For proper storage, moisture content of the food crops must be written 8% (eight percent) to inhibit microbial attack. As Bangladesh is hot and humid country moisture content is increased even the crops dried properly. For this reason, aflatoxins (natural mycotoxins that are produced by certain molds) can be grown in rice. Rice is the primary source of carbohydrate, protein, fibre, some vitamins like the B complex & E and minerals. The 75% of the weight of rice grain comprises of starch, the major carbohydrate (Vaughan and Geissler ; 2009). Although the nutritional value of rice is very good but despite all this the problem is the toxin production due to fungal attack on crops or during storage. Rice is not a favourable commodity for growth of Aspergillus and aflatoxin contamination under normal conditions, but high humidity and heavy rains could enhance the capacity of rice grains for risk to aflatoxin contamination (Siruguri *et al*. 2012). Aflatoxins are produced as by product during the growth of fungi, *A. flavus* and *A. parasiticus*. Aflatoxins adversely affect the human health. Aflatoxicosis is a disease caused by the aflatoxins. Sudden death occurs as a result of acute aflatoxicosis and in chronic aflatoxicosis the prolonged pathological changes like cancer and immune suppression happens (Magnussen and Parsi. 2013). Aflatoxin is well known agent for its hepatocarcinogenic properties. The risk of liver cancer is almost 30 times higher in the subjects exposed to aflatoxin than unexposed ones (Liu and Wu. 2010). In order to control aflatoxins in rice, there is a need to determine and quantify aflatoxin levels for making a comparison with the permissible levels set by the food regulatory authorities in a pursuit to ensure safe food supply. The present study was therefore planned to determine the aflatoxins levels in different types of rice consumed in different regions of Bangladesh.

Presence of pesticide residues in vegetables and fruits is an indicative change in use pattern of pesticides in Bangladesh, where shift has taken place from Organochlorines to the easily degradable groups of pesticides over the last few years.

Pesticide and aflatoxin residue above maximum residue level (MRL) / maximum tolerance limit (Codex Alimentarius Commission, 2010) is a risk factor for human health and food safety is reduced.

Proper management of store grain and ensuring safe food is still a problem in this country. Therefore, the present research project has been undertaken to determine the residual pesticides/aflatoxin in rice, spice and vegetable samples. The behavior of pesticides in the agricultural product (Omirou et al., 2009) is of great importance since the disappearance, persistence and partial transformations of these compounds determine their usefulness or potential effects on our health and in the environment. Any inappropriate use of insecticides and pre harvest could cause residual problems. Pesticide residue is becoming a major food safety concern of consumers. Most of the farmers of Bangladesh do not have adequate knowledge about actual dose and pre-harvest interval of pesticides on crops, and toxic & harmful residual effects on human health and in the environment (Philp; 2003).

Turmeric is one of the common spices of curry in Indian sub-continent (Tilak et al.; 2004). Traditionally, turmeric paste was made from boiled and dried rhizome and was used in curry. With the change of life style and more urbanization, turmeric powder became popular and is available in every corner of the country. Although boiled and dried turmeric is hard, powder is susceptible to pest infestation.

Therefore, the objectives of the present study are:

i) To assess the level of aflatoxins (if any) in some rice samples.

ii) To modify and validate the reported methods of extraction, clean-up and analysis of pesticide/aflatoxin residues in rice, spice and vegetable samples

iii) To the multi-residue analysis of pesticides in spice (commercial turmeric powder) samples

iv) To study the dissipation pattern of organophosphorus and carbamate groups of pesticides in some vegetable samples

v) To analyse multiple pesticide residues in market vegetable samples

2. EXPERIMENTAL

2.1 General

2.1.1 Materials and methods

2.1.1.1 Chemicals

Potassium chloride, potassium dihydrogen phosphate, anhydrous disodium hydrogen phosphate, anhydrous magnesium sulphate, anhydrous sodium sulphate, sodium chloride, neutral alumina and nitric acid were purchased from Merck, Germany. Primary Secondary Amine (PSA) and Florisil from Supelco USA and activated Charcoal Powder from Unichem, China were purchased. Kit of Afla Toxins for derivative preparation *i.e.* Immuno-affinity column (AflaCLEAN™, LCTech) and Potassium bromide from Scharlau, Spain were purchased.

2.1.1.2 Solvents

HPLC grade acetonitrile (ACN) & methanol, analytical grade n-hexane, acetone and ethyl acetate were purchased from Merck, Germany. HPLC grade water (Milli-Q water) which is free from cations, anions and hydrocarbons was used to carry out the study.

2.1.1.3 Certified Standards

The certified standard of aflatoxins, a mixture of B1 (99.00 % purity), B2 (98.00 % purity), G1 (99.70 % purity) and G2 (99.00 % purity) in benzene: acetonitrile (98:2) were purchased from Supelco, Sigma Aldrich, USA.

Certified standards of pesticides; carbofuran (98.50 %) , carbosulfan (95.00 % purity), diazinon (97.50 % purity), chlorpyrifos (99.50 % purity), cypermethrin (91.00 % purity),

fenvalerate (98.50 % purity) and quinalphos (99.20 % purity) were purchased from Dr. Ehrenstorfer, Germany.

2.1.1.4 Glass and Plastic Lab ware

Solid phase extraction unit (Alltech Vac Master, Brazil), calibrated volumetric flasks and pipettes by BSTI (Bangladesh Standard Testing Institute), glass 100µL trace syringe micro injector, mobile phase vacuum filtration apparatus set (1000 mL), glass column, glass desiccator, vial, round bottom flask, pear shaped flask, graduated test tube, funnel, glass syringe, micro pipette, teflon tube and plastic syringe were used in the present studies.

2.1.1.5 Equipment

Minor Equipment

Four digit balance and standard weight used for balance were calibrated by BSTI (Bangladesh Standard Testing Institute) and gave certificate for each. The equipment including, pH meter, rotary evaporators ((Büchi R-210, Switzerland) and Heidolph (Model No.517-61000-00-0, Germany), vortex machine, ultrasonic bath, oven (Salvis, Inventory No. G-1020), water purification system (Boeco, Germany) and furnace (GSM 11/8 Hope valley, S336RB, England) were used to carry out research. The samples were centrifuged by SIGMA 2-16 Bench-top, 10000 rpm and Cowbell brand centrifuge machines.

Major Equipment

Two Gas Chromatographic systems; Shimadzu 2010 (GC-ECD) & 2025 (GC-FID); high performance liquid chromatographic system; Shimadzu UFLC Prominence (LC-PDA/FLD) and Tandem Mass Spectrometric system (LC-MS/MS; 8050) were calibrated by the technical persons of local agent of the Company (AQC) and gave certificate for each of the equipment. Picture of the major equipment used to carry out the research are given in **Figure 10**.

(a)

(b)

(c)

(d)

Figure 10: Major equipment; Shimadzu Prominence Ultra-Fast Liquid Chromatograph (a), Shimadzu LCMS-8050 (b), Shimadzu GC-2010 (c) and Shimadzu GC-2025 (d)

2.1.1.6. Methods

All evaporations were carried out by rotary vacuum evaporator at water bath temperature not exceeding 40^0C. The residual solvent of the dried mass was removed by a freeze dryer.

All glass wares used for the analysis were cleaned by detergent, rinsed with LC grade water followed by acetone and the cleaned glass wares were dried in an oven at 105 °C.

Alumina, florisil, anhydrous sodium and magnesium sulphates were heated at 300ºC in a furnace for 4 hours and the moisture freed materials were kept in a vacuum desiccator until use.

Activated charcoal powder was washed with distilled water then with methanol followed by acetone. The cleaned powder was dried at 300 ºC in a furnace and the dried powder was kept in vacuum desiccator until further use.

2.1.2 Instrument

2.1.2.1 Homogenizer

Vegetable samples were homogenized by using Kitchen blender and rice samples were powdered by a grinding machine (Model no. M400C).

2.1.2.2 Liquid Chromatograph

Shimadzu Prominence Ultra-Fast Liquid Chromatograph (Prominence Degassing Unit DGU-$20A_{5R}$; Column Oven CTO-$10AS_{VP}$; Solvent Delivery Unit LC-20AD) having Photo Diode Array Detector (Shimadzu SPD-M20A) & Fluorescence Detector (Shimadzu RF-20A) was used in the studies (**Figure 10a**) Separations were performed on a Phenomenex Luna C_{18} column (250 x 4.6 mm i.d.; particle size 5 µm ; theoretical plates ≮ 4500) for carbofuran and Shimadzu C18 (Shim-Pack VP-ODS; 150 mm x 4.6 mm i.d.; 5.0 µm; theoretical plate ≮2560) column for aflatoxins analysis. The injector was manual and the injector loop size was 20 µl. Methanol, acetonitrile, Nano particle water and buffer solution were used as mobile phases with different proportion. The total run time was kept within 15 min by changing the proportion of mobile phases and sharp peak was chosen by applying different wavelengths at a time. The flow rate was kept at 1mL/ min. The column oven temperature was fixed at 30 and 40^0 C for analysis of carbofuran and aflatoxins.

2.1.2.3 Gas Chromatograph

Two gas chromatographs (one with electron capture detector and one with flame ionization detector) were used for analysis of pesticides. Gas chromatograph with electron capture detector was Shimadzu-2010 (**Figure 10c**) and Gas chromatograph with a flame ionization detector (**Figure 10d**) was Shimadzu-2025. Gas chromatographs were attached with auto injectors.

2.1.2.4 Liquid Chromatography-Mass Spectrometry

Liquid chromatography-mass spectrometry analyses were carried out using Shimadzu LCMS-8050 with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), a triple quadrupole LC-MS/MS. Shimadzu Prominence Ultra-Fast Liquid Chromatograph (Communications Bus Module CBM-20A; Degassing Unit DGU-$20A_{3R}$; Column Oven CTO-10AC ; Solvent Delivery Unit LC-20AD; Auto Sampler SIL-$20AC_{HT}$) was used in the studies (**Figure 10b**). Nebulizing and collision gas was N_2. Separations were performed on a Shim-pack GISS C_{18} column (250 x 4.6 mm i.d.; particle size 5 µm). The carrier gas pipe was 5 m.

2.1.3 Preparation of Standard Solutions

Primary standard solution of 11 certified standards (aflatoxins B1, B2, G1 and G2, carbofuran, carbosulfan, chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos) were made. Among them 5 were analysed by LC and their solution were prepared with MeOH (aflatoxins B1, B2, G1 and G2) and ACN (carbofuran). Other 6 (carbosulfan, chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos) were analysed by GC and solution were made with using n-hexane.

2.1.4 Statistical Analysis

Analysis of Variance (ANOVA) is a particular form of statistical hypothesis testing used in the analysis of experimental data. It was used for comparing (testing) three or more means (variables) for statistical significance. The standard deviation (SD) and relative standard deviation (RSD) was calculated by using this statistical model.

The dissipation kinetic of the pesticide in vegetable was determined by plotting residue concentration against time and the maximum squares of correlation coefficients found were used to determine the equations of best fit curves. For all the samples studied, exponential relationships were found to apply, corresponding to first order rate equation. Confirmation of the first order kinetics was further made graphically from the linearity of the plots of Log Concentration against time. The rate equation was calculated from the first order rate equation:

$$C_t = C_0 e^{-kt}$$

Where, C_t represents the concentration of the pesticide residue at time t, C_0 represents the initial concentration and k is the rate constant.

The half-life ($t_{1/2}$) was determined from the *k* value for each experiment, being as calculate by Wang et al. (2007) and Liang et al. (2011).

$$T_{1/2} = \ln (2) / k$$

PART-A

Analysis of Aflatoxins; B1, B2, G1 and G2 in Rice Samples

2.2 Experimental

2.2.1 Sample Collection

Twenty different rice samples (six were good and fourteen were of inferior quality) were purchased from different markets of Dhaka city, Kurigram and Noakhali districts of Bangladesh during May to August 2016. The collected samples were labelled as R1 to R20; such as Swarna (R1), Nazir shail (R2 & R18), Paijam (R3), Minikit (R4, R9 & R17), Brri 28 (R5, R6, R10 & R11), Brri 29 (R7, R8, R12, R13 & R14), Heera (R15 & R16), Chinigura (R19 & R20). Among them R1 to R18 were from the grocery shop which are being sold in unpacket form (**Figure 11**). Two rice samples; Chinigura (Rupchanda brand, Batch No. OPPT160714320) and Chinigura of Square Company (Batch No. 16071484) were purchased from Mina Bazar supermarket at Lalbag, Dhaka. The good quality rice samples were R1, R4, R17, R18, R19 and R20. All the collected rice samples were ground into powder separately by a grinding machine, kept in twenty different zip locked plastic bags, labelled and stored in a freezer at -20^0 C until extraction was carried out. Name of the rice samples with Code No., place and date of collections are presented in **Table 3**.

Figure 11: Picture of some rice samples

Table 3: Rice Samples from Three Different Parts of Bangladesh

Sample Code	Name of the Rice Sample	Place of Collection	Date of Sampling
R1	Swarna	Ananda Bazar, Dhaka	10/05/16
R2	Nazir shail	New Market, Dhaka	19//05/16
R3	Paijam	New Market, Dhaka	19//05/16
R4	Minikit	Polashi Bazar, Dhaka	19//05/16
R5	Brri 28	Chowmani bazar, Noakhali	05/07/16
R6	Brri 28	Chomer Munshirhat, Noakhali	08/07/16
R7	Brri 29	Jamidarhat, Noakhali	08//07/16
R8	Brri 29	Shenbag bazar, Noakhali	10/07/16
R9	Minikit	Mirpur Kazipara, Dhaka	10/07/16
R10	Brri 28	Pkhirhat bazar, Kurigram	02/07/16
R11	Brri 28	Hazirhat bazar, Kurigram	03/07/16
R12	Brri 29	Neoyashi bazar, Kurigram	06/07/16
R13	Brri 29	Kharibari bazar, Kurigram	08/07/16
R14	Brri 29	Kurigram town Bazar	15/06/16
R15	Heera	Mirpur-1, Dhaka	01/07/16
R16	Heera	Mirpur-10, Dhaka	20/07/16
R17	Minikit	Ananda Bazar, Dhaka	13/08/16
R18	Nazir shail	Ananda Bazar, Dhaka	13/08/16
R19	Chinigura, Rupchanda company	Mina Bazar, Lalbag, Dhaka	19/08/16
R20	Chinigura, Square company	Mina Bazar, Lalbag, Dhaka	19/08/16

2.2.2 Preparation of Phosphate Buffer Saline (PBS)

Potassium chloride (0.20 g), potassium dihydrogen phosphate (0.20 g), anhydrous disodium hydrogen phosphate (1.16 g) and sodium chloride (8 g) were added to 900 mL LC grade water for preparation of phosphate buffer saline **(**PBS). The solution was made to 1.0 L with LC grade water. The pH of the solution was measured and found to be 7.4.

2.2.3 Preparation of Mobile Phase

The acidic mobile phase A was prepared by dissolving 216.4 mg of KBr and 159.1 µL of concentrated HNO_3 in LC grade water by ultra-sonication and the volume was made up to 1L. LC grade methanol (Merck, Germany) was used as another mobile phase B. Water phase C and acetonitrile phase D were used for conditioning the LC system. Four mobile phases (water, acetonitrile, buffer and methanol) were filtered through LC solvent filter (pore size 0.22 µm) and degassed by vacuum suction.

2.2.4 Conditioning of Immune-Affinity Solid Phase Extraction Column (SPE)

Afla Immune-Affinity Solid Phase Extraction Column (length 1.5 x 1.0 cm; solid phase area and 6 x 1.2 open space filled with buffer) was taken out from the refrigerator and kept outside until come to room temperature. The column was attached to vacuum solvent filtration system (Vac Master), then it was opened and allowed to pass off the buffer at gravitational flow. The open space of the column was filled with 10 mL LC grade water and the water was passed through under slow vacuum. The column was ready to load the sample extract for clean up.

2.2.5 Post Column Derivatization Device (Kobra cell)

Post Column Derivatization Device (Kobra cell; obtained from FAO as gift) was to be fitted between the LC column and the fluorescence detector. Kobra cell is an electrochemical cell

which consists of a platinum working electrode and a stainless steel auxiliary electrode separated by an ion exchange membrane. It is used to make bromo-derivative of the aflatoxins separated from LC column.

2.2.6 Analytical Conditions of Liquid Chromatograph-Fluorescence Detector (LC-FLD)

A C18 (Shim-Pack VP-ODS; 150 mm x 4.6 mm ID; 5.0 µm; theoretical plate 2560) column was used to carry out the separation. Elution was done by isocratic system using aqueous phase as A (1 L contained 216.4 mg of KBr and 159.1 µL of concentrated HNO_3) and methanol as B. An Isocratic system was used to elute the samples from column (mobile phase A:B:: 55:45) was used. The column oven temperature was fixed at 40°C, loop size was 20 µL, Excitation and Emission wavelengths of the detector (FLD) were set at 360 and 425 nm, respectively. The column flow rate was set at 1.0 mL min^{-1}.

2.2.7 Preparation of Primary Standard Solution of Certified Aflatoxins (B1, B2, G1 and G2)

Certified aflatoxins mixture; B1, B2, G1 and G2 was in 1 mL benzene: acetonitrile; 98:2 solution was in an amber color ampule. Concentration of the aflatoxins B1, B2, G1 and G2 were 0.88, 0.31, 099 and 0.32 µL mL^{-1}, respectively. The ampule was broken and the mixture was transferred into an amber color 2 mL sample vial. From that standard solution 10 µL was taken out in another 2 mL amber color vial then the solution was evaporated by gentle flow of N_2 gas. The dry mass was dissolved in 1 mL methanol. The concentration of primary standard solution of aflatoxins B1, B2, G1 and G2 were 8.8, 3.1, 9.9 and 3.2 ng mL^{-1}, respectively.

The primary standard solution of aflatoxins mixture (8.8, 3.1, 9.9 and 3.2 ng mL^{-1} of B1, B2, G1 and G2, respectively) was serially diluted to made ten different working standard solutions. The concentrations of the diluted solutions are given in **Table 4**.

Table 4: Concentration of Dilute Standard Solutions of Aflatoxins (B1, B2, G1 and G2)

Solution No.	Concentration of aflatoxin ng mL^{-1}			
	B_1	B_2	G_1	G_2
1	8.80	3.10	9.90	3.20
2	4.40	1.55	4.95	1.60
3	2.20	0.775	2.475	0.80
4	1.10	0.3875	1.2375	0.40
5	0.55	0.1938	0.61875	0.20
6	0.275	0.0969	0.3094	0.10
7	0.1375	0.0485	0.1546	0.05
8	0.0687	0.0242	0.0773	0.025
9	0.0343	0.0121	0.0386	0.0125
10	0.0171	0.006	0.0193	0.0062
11	0.0085	0.003	0.0096	0.0031
12	Solvent blank (methanol)			

2.2.8 Preparation of Calibration Curves

The LC system was conditioned by passing water and acetonitrile (mobile phase C and D) in different proportions until a smooth base line was obtained (**Figure 12a**). The Kobra cell was connected between column and FLD detector. Then the system was conditioned with mobile phase A and B for half an hour. The mobile phase B, methanol was injected as solvent blank and the retention time of the solvent blank was at 2 min (**Figure 12a**). The mixture of eleven working standard solutions of aflatoxins (B1 were 8.8 , 4.4, 2.2, 1.1, 0.55, 0.275, 0.1375, 0.0687, 0.0343, 0.0171 and 0.0085 ng mL^{-1}, aflatoxin B2 were 3.1, 1.55 0.775, 0.3875, 0.1938, 0.0969, 0.0485, 0.0242, 0.0121 0.006 and 0.003 ng mL^{-1}, aflatoxin G1 were 9.9 , 4.95 , 2.475, 1.2375, 0.61875, 0.3094, 0.1546, 0.0773 ,0.0386 , 0.0193 and 0.0096 ng mL^{-1} and aflatoxin G2 were 3.20 , 1.60 , 0.80, 0.40, 0.20, 0.10, 0.05 ,0.025, 0.0125, 0.0062 and 0.0031 ng mL^{-1}) were injected gradually into LC-FLD from the lowest to the highest concentration level. Then gradually the eleven mixtures of working aflatoxins solutions were injected, retention times of B1, B2, G1 and G2 were found at 5.11, 6.12, 7.48 and 9.16 min, respectively. Two representative chromatograms of standard aflatoxins mixture are given in **Figure 12b & 12c**. The limit of detection (LOD) and quantification (LOQ) were found to be 0.009 & 0.025, 0.006 & 0.018, 0.039 & 0.116 and 0.025& 0.075 ng mL^{-1} for B1, B2, G1 and G2, respectively (**Table 5**).

From the twelve chromatograms of mixture of aflatoxins (one for solvent blank and others for eleven concentration levels of aflatoxins mixture) calibration curve at different concentration levels was made using MS Excel software by plotting area of the eluted standard vs concentration. A few calibration curves are presented in (**Figure 13, 14, 15 and 16**) for aflatoxin B1, B2, G1 and G2, respectively. Linear range of elution and correlation coefficient are given in **Table 5**.

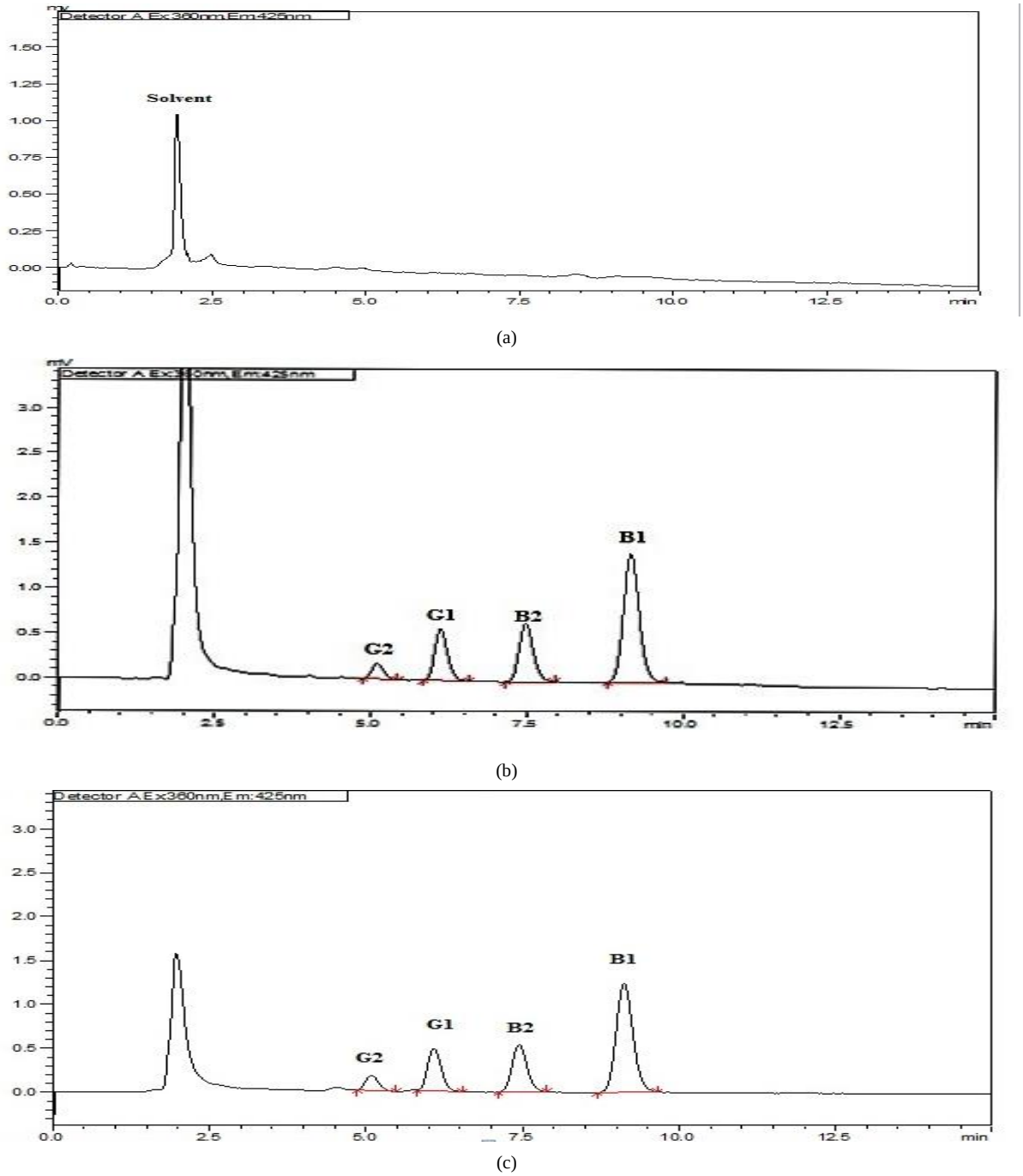
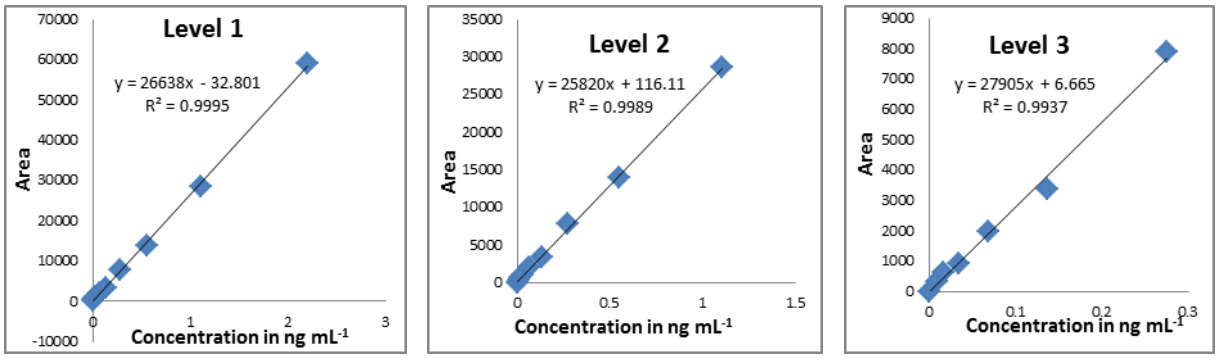


Figure 12: Chromatograms of solvent blank (a), certified standard aflatoxins in lower (b) and higher (c) concentration levels

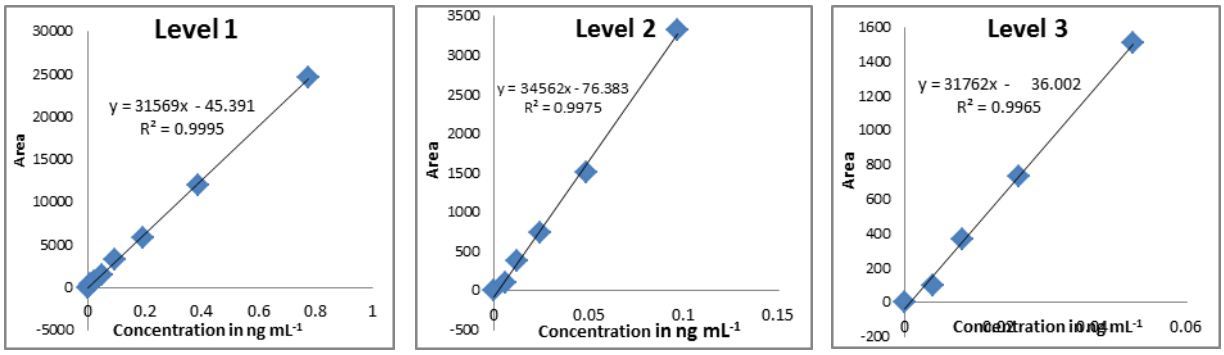
The three calibration curves for aflatoxin B1 are given in **Figure 13**. The correlations of coefficients were 0.9995, 0.9989 and 0.9937 for higher, medium and lower concentrations, respectively.



(a) (b) (c)

Figure 13: Calibration curves of B1 in higher (a), medium (b) and lower (c) concentration levels

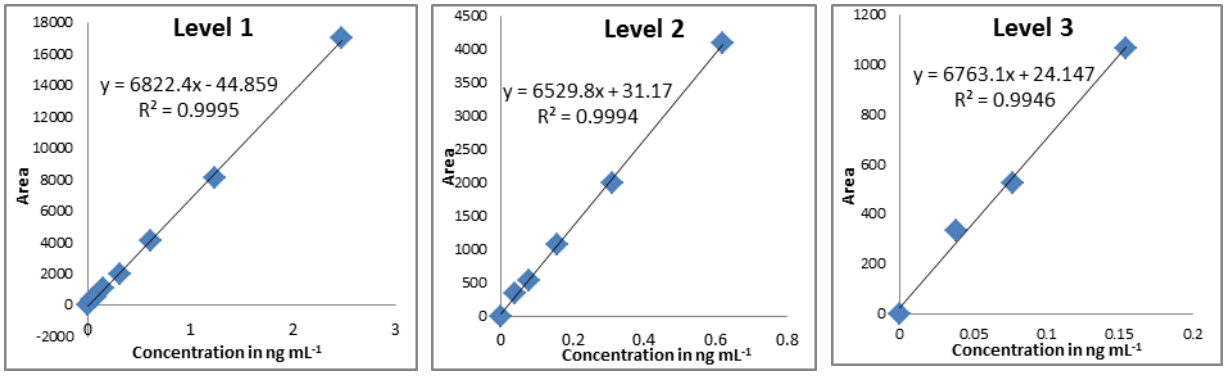
The three calibration curves for aflatoxin B2 are given in **Figure 14**. The correlations of coefficients were 0.9995, 0.9975 and 0.9965 for higher, medium and lower concentrations, respectively.



(a) **(b)** **(c)**

Figure 14: Calibration curves of B2 in higher (a), medium (b) and lower (c) concentration levels

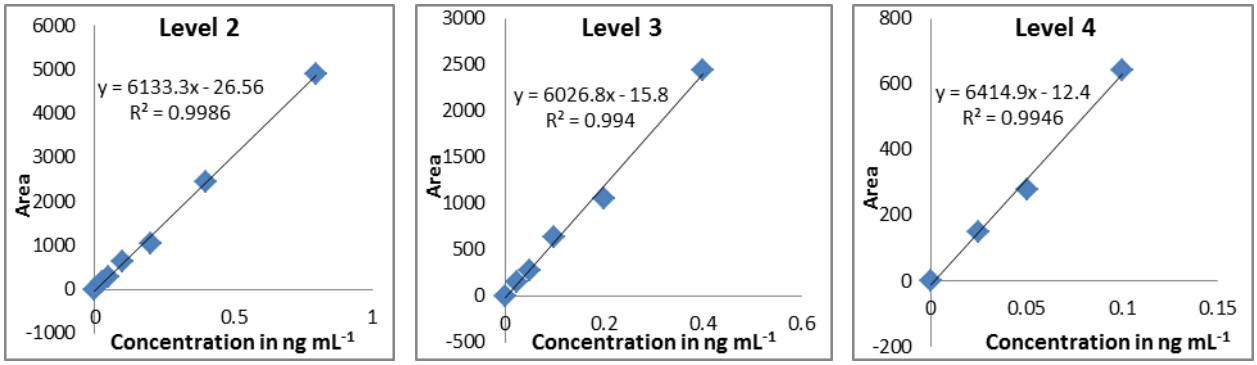
The three calibration curves for aflatoxin G1 are given in **Figure 15**. The correlations of coefficients were 0.9995, 0.9994 and 0.9946 for higher, medium and lower concentrations, respectively.



(a) **(c)** **(d)**

Figure 15: Calibration curves of G1 in higher (a), medium (b) and lower (c) concentration levels

The three calibration curves for aflatoxin G2 are given in **Figure 16**. The correlations of coefficients were 0.9986, 0.9940 and 0.9946 for higher, medium and lower concentrations, respectively.



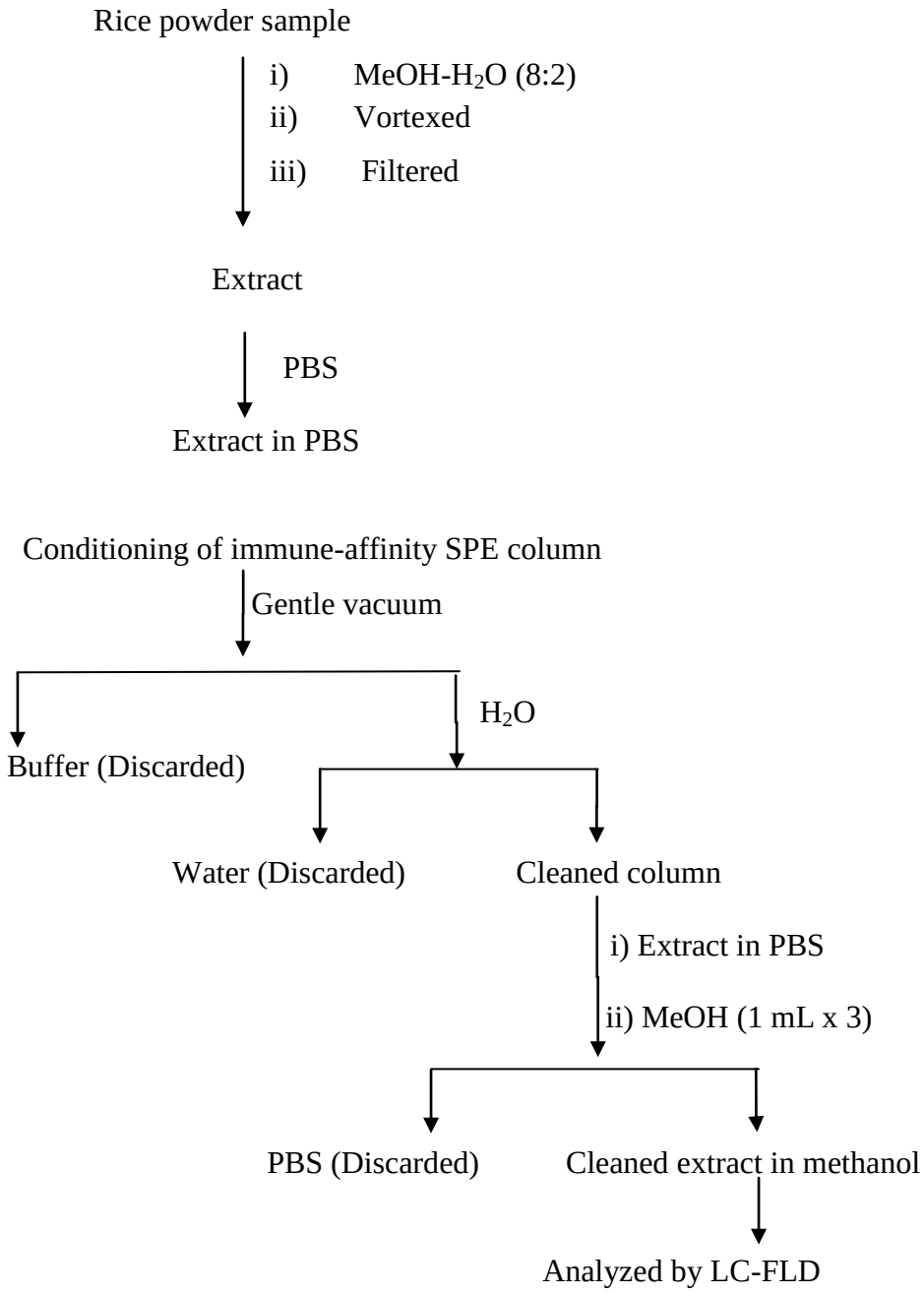
(a) **(b)** **(c)**

Figure 16: Calibration curves of G2 in higher (a), medium (b) and lower (c) concentration levels

2.2.9 Extraction and Clean-up of Rice Samples

Rice powder sample R1 (6.25g; amount according to ISO 16050 method) was taken into a 50 mL teflon tube and a mixture of methanol-LC grade water in 8:2 ratio (32 mL) was added to the sample. The mixture was vortexed for 5 min and filtered. From that extract 3.5 mL was taken in a graduated screw cap test tube, mixed with 21.5 mL phosphate buffer saline (PBS), total volume was 25 mL. From that diluted extract in PBS, 20 mL was taken and loaded into the pre-conditioned immune-affinity SPE column. The PBS was removed by slow vacuum. The cleaned sample was eluted with methanol (1 mL x 3). All the collected rice samples (R2-R20) were extracted and cleaned up by following the same method.

The cleaned extract of rice samples (R1- R20) were analysed by LC-FLD (**Scheme 1**).



Scheme 1: Extraction and clean-up procedure of rice samples

2.2.10 Analysis of Aflatoxins (B1, B2, G1 and G2)

Twenty cleaned extracts of twenty different rice samples were analysed by Liquid Chromatograph-Fluorescence Detector (LC-FLD). Solvent was injected before each injection from the twenty different chromatograms amount of four different aflatoxins were calculated using external calibration curve (**Figure 13, 14, 15 and 16**). Results are given in **Table 6**.

Aflatoxin derivatization system including Kobra cell were cleaned with water for more than ½ hour. Kobra cell was disconnected and LC system was washed with water-acetonitrile in different compositions before quite the instrument.

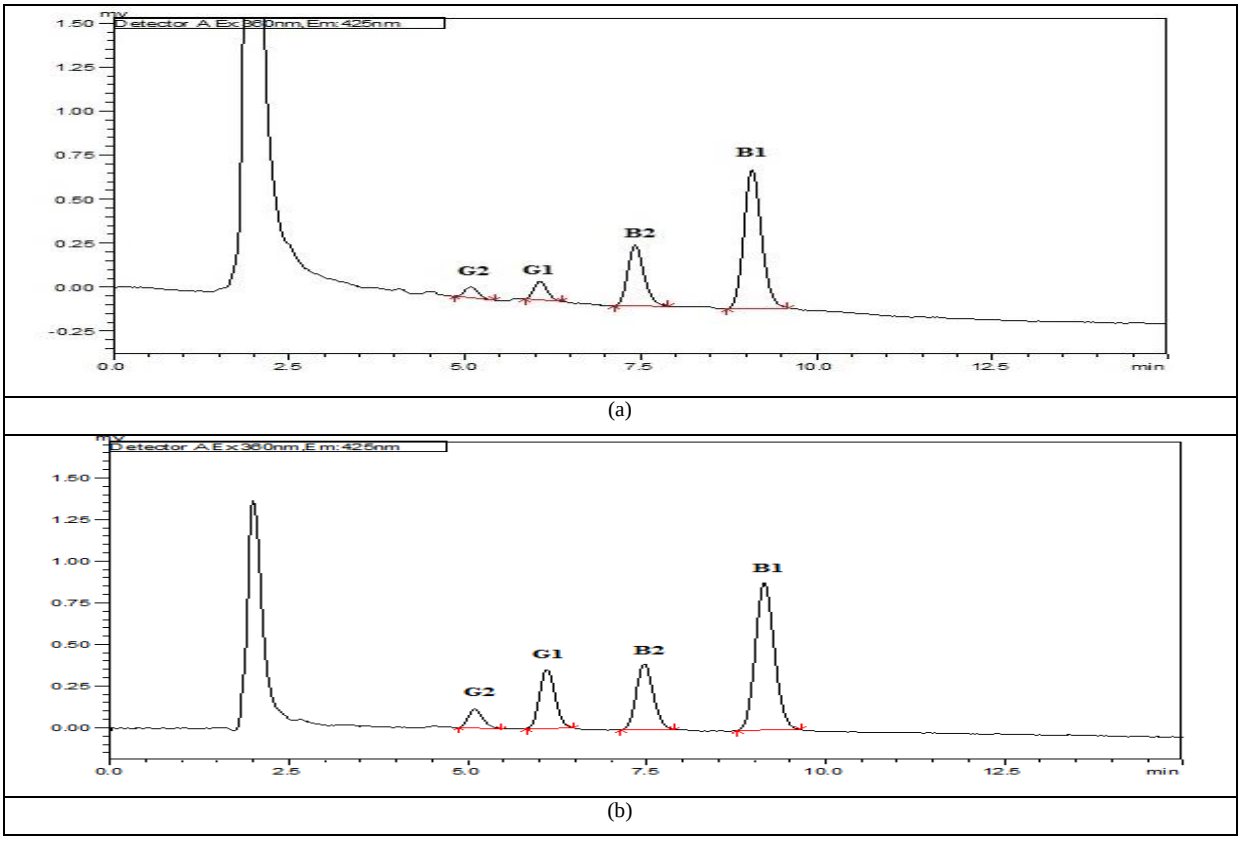


Figure 17: Chromatograms of Rice Sample (a) and Spiked Rice Sample (b)

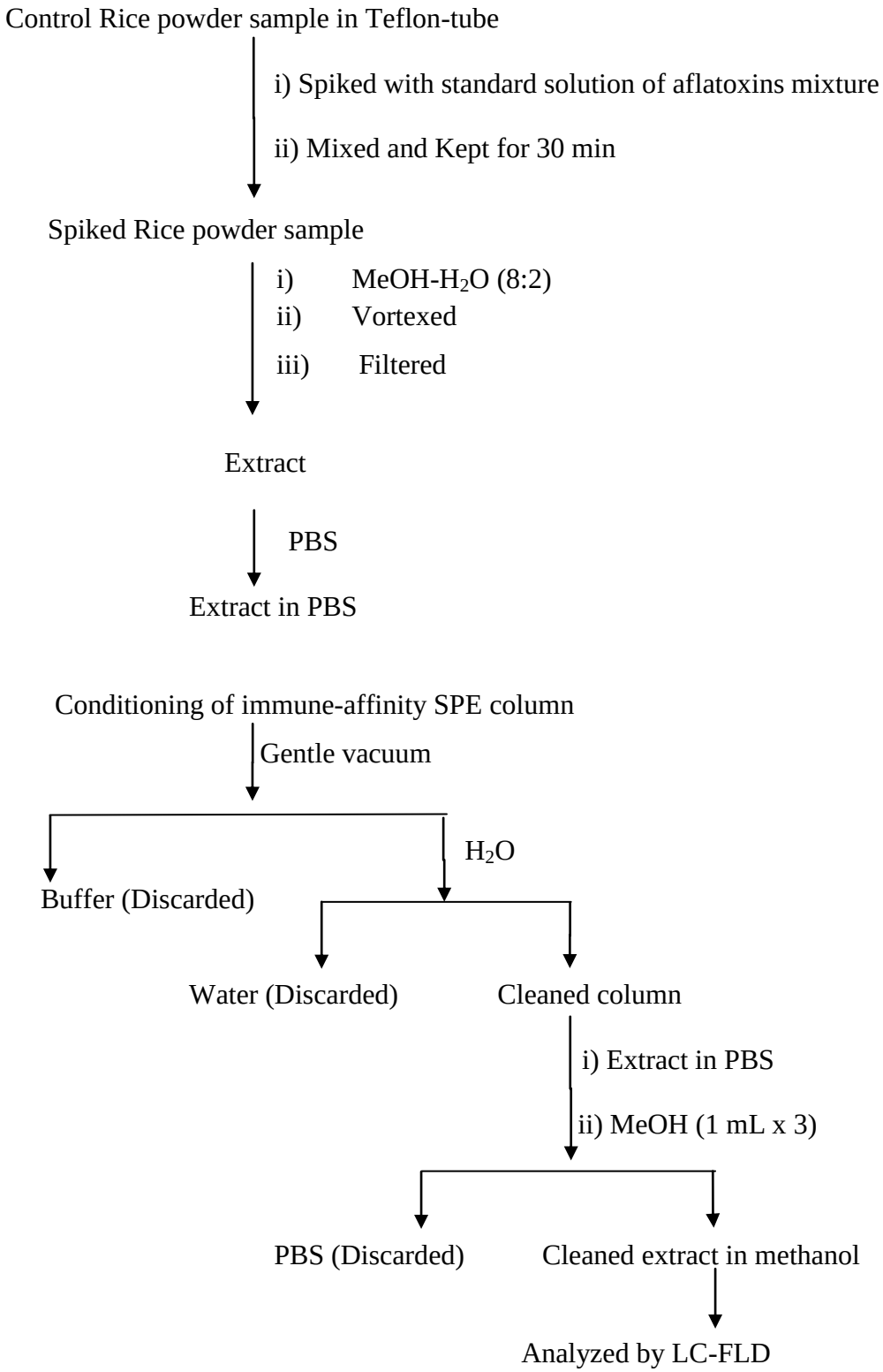
2.2.11 Control Sample

Good quality swarna (R1) rice sample was taken as control (sample blank).

2.2.12 Recovery Experiment

The control rice powder sample (6.25 g) was taken into a 50 mL teflon tube and 10 µL solution of aflatoxins mixture containing 8.8, 3.1, 9.9 and 3.2 ng mL^{-1} concentration of B1, B2, G1 and G2 was added. The spiked rice sample was shaken manually for 0.5 min and allowed to stay for 30 min at room temperature. Then the aflatoxins mixture was extracted and cleaned up by the same procedure as was done for experimental rice sample powder

(**Scheme 2**). The cleaned extract was passed through LC column and made into their respective bromo derivatives in Kobra cell then was detected by fluorescence detector (**Figure 17**). Four recovery experiments were done in same day (intra-day recovery) at two different spiking levels of each standard. The recovery of four different aflatoxins was calculated by a formula given below (**Section 2.2.14**). The results are given in **Table 7**. Standard deviation and relative standard deviation were calculated. Similarly four recovery experiments were done on another day (Inter-day) at two spiking levels by spiking same amount of standard aflatoxins mixture, extracted and cleaned following exactly same procedure and analysis were done under same condition. Percent recovery was calculated and the results are given in **Table 7**.



Scheme 2: Recovery procedure of aflatoxins in control rice powder sample

2.2.13 Calculation of Aflatoxins

A linear regression was obtained by plotting the peak versus concentration. Aflatoxins were quantitatively determined from the linear regression equation: y = mx ± c.

$$Concentration\ of\ Aflatoxin\ B1, B2, G1, G2\ (mg/L) = (Peak\ area - intercept\ )/slope$$

$$w_{Toxins}\ (\mu g/kg) = \frac{p_{smp}\ x\ V_s\ x\ V_E\ x\ V_D}{m_0\ x\ V_{AE}\ x\ V_{A/Ac}}$$

Where:

P_{smp}: Concentration of aflatoxin calculated from linear regression [ng/mL]

V_s: Solvent taken for extraction [mL]

V_E: Final volume achieved after elution from immunoaffinity column [mL]

V_D: Volume achieved after dilution with phosphate buffer saline, PBS [mL]

m_o: Sample material taken for analysis [g]

V_{AE}: Aliquot taken from extract [mL]

$V_{A/AC}$: Aliquot taken from immunoafinity clean up [mL]

W_{Toxins}: Amount of aflatoxin in sample material [µg kg^{-1}]

3.1 Results and Discussion

3.1.1 Result of the Rice Samples

Bangladesh is a country which produces rice 3 times in a year in the same field. The harvested paddies are dried by sunlight. If rain comes during harvesting period paddy has to be kept until rain gets over. Due to hot and humid weather there is high risk of fungal growth. The low quality rice is much cheaper than high quality and low quality rice are mostly consumed by low income family members. There is a potential risk for the consumers of rice for aflatoxins.

Aflatoxins produced by fungal attack on maize, wheat, rice etc. Rice is the staple food of Bangladesh. High grade and inferior quality rice samples were chosen for identification of aflatoxins if any present in the rice samples. Food safety demand rice sample should be free from any kind of chemical and biological contaminants. The present study was aimed to evaluate the growth of natural toxins in different kinds of rice (if any) from different parts of Bangladesh.

Twenty rice samples were purchased from different markets of Dhaka city, Kurigram and Noakhali districts**.** The rice powder samples were stored in freezer to prevent growth of natural toxins (if any). Calibrated balance and volumetric flask and certified standard and micropipettes were used for accurate result and amber color vial and bottle were used to prevent possible degradation of aflatoxins in certified standard and test samples. Prominence LC-FLD was calibrated by local agent of Shimadzu. Kobra cell, certified standard aflatoxins and immune-affinity SPE column were supplied by FAO local office of Bangladesh under a laboratory network upgrading program. The determination was carried out by LC–FLD with Kobra cell. The Kobra cell is basically an electrochemical cell, fitted between the HPLC column, the detector which consists of a platinum working electrode and a stainless steel auxiliary electrode, separated by an ion exchange membrane. The principle of the technique is to deliver to the Kobra cell, the mobile phase containing the derivatization agent precursor, a potassium bromide salt, and aflatoxins. Bromine is then generated electrochemically by applying a constant potential at the working electrode. The resulting electrochemical reaction enables aflatoxins B1, B2, G1 and G2 to be derivatisized in-situ to their brominated

derivatives, which gives an enhanced fluorescence response. Significantly increases the fluorescent signals of aflatoxins allowing detection of the four individual toxin in very low concentration level. The post column derivatisized compound produced in situ and gives more enhancing signal for aflatoxin gives very low level detection and the result accurate and low level.

Extraction and clean-up were done following the officially recognized ISO 16050 method. The method was validated in the laboratory following the standard procedures required. The rice samples were extracted as described in the **Section 2.2.9** and analysed by LC-FLD (**Section 2.2.10**).

The method for analysis of aflatoxins in rice sample was tested and validated in terms of selectivity, linearity, sensitivity and recovery.

The selectivity of the method was evaluated by injecting extracted blank samples. The absence of signals above a signal-to-noise ratio of at the retention times of the target compounds showed that the method is free of interferences.

The analysis of aflatoxins in the spiked and market samples were done by using calibration curves. Calibration curves were prepared (**Figure 13, 14, 15 and 16**) with different concentration levels and the linearity was excellent with correlation coefficients of $r^2 \geq$ 0.9998, 09997, 0.9956 and 0.9969 for B1, B2, G1 and G2, respectively (**Table 5)**.

Table 5: Correlation Coefficients (r^2), LODs (Limit of Detection) and LOQs (Limit of Quantification) of Aflatoxins

Aflatoxins	Linear range (ng mL^{-1})	r^2	LOD (ng mL^{-1})	LOQ (ng mL^{-1})
B1	0.009-4.40	0.9998	0.009	0.025
B2	0.006-1.55	0.9997	0.006	0.018
G1	0.005-0.25	0.9956	0.039	0.116
G2	0.005-0.25	0.9969	0.025	0.075

The limit of detection (LOD) and limit of quantification (LOQ) were determined by serially diluting the standard solutions of aflatoxins B1, B2, G1 and G2. The dilute standard solutions were injected one by one giving interval of solvent blank until the peak heights of the standards were same to the noise level. The limit of detection (LOD) and quantification (LOQ) were 0.009 and 0.025 ng mL^{-1} for B1, 0.006 and 0.018 ng mL^{-1} for B2, 0.039 and 0.116 ng mL^{-1} for G1 and 0.025 and 0.075 ng mL^{-1} for G2, respectively (**Table 5**). The lowest limit of detection was found for B2 than other (**Figure 18**).

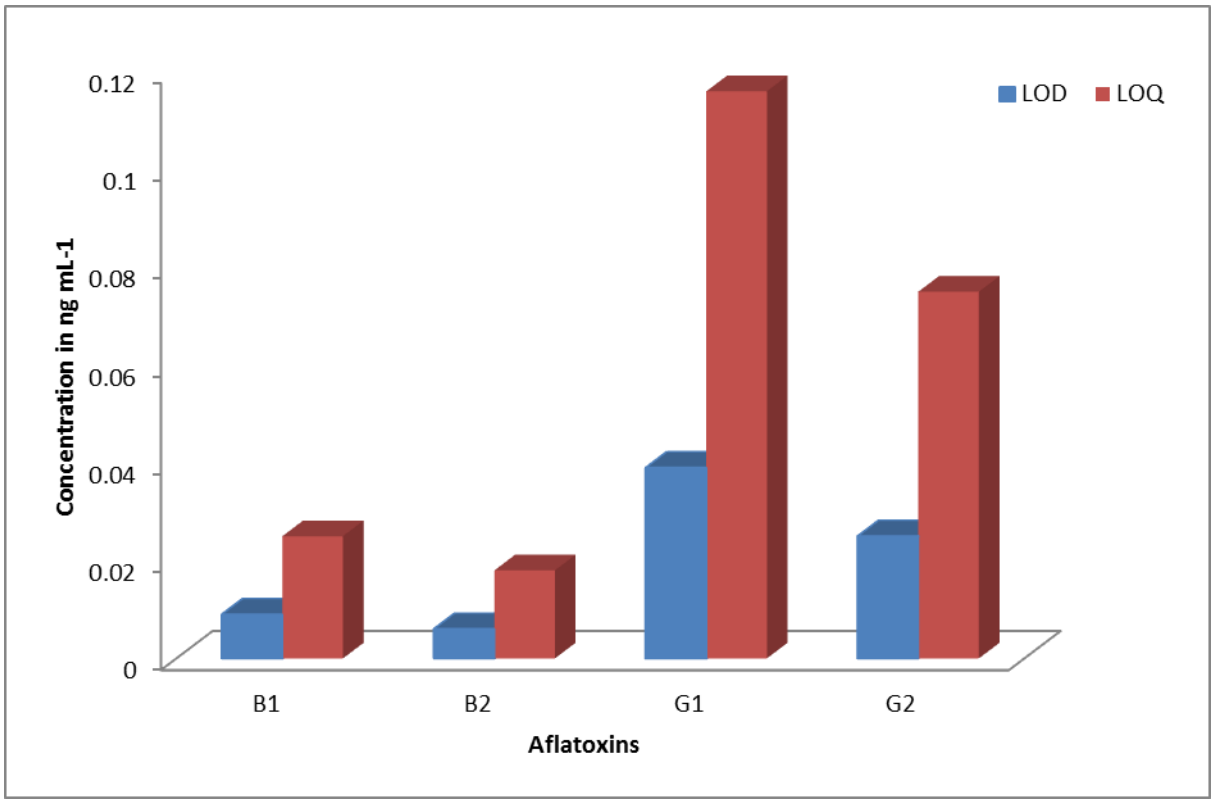


Figure 18: LOD and LOQ of Aflatoxins B1, B2, G1 and G2

The extraction efficiency of the analytical procedure was evaluated via recovery experiment. Recovery experiment was carried out by spiking control rice sample with known concentrations of aflatoxin standard working solutions. The rice sample R1 was used as control sample where no targeted compound was present. The control sample was extracted three replicates followed by respective extraction and clean-up procedure (**Scheme 1**) to determine the matrix effect under analysis condition (**Figure 12a**). Solvent blank methanol was injected to confirm the smooth base line in LC-FLD (**Section 2.2.7**). The control rice powder sample was spiked with 10 µL of aflatoxins standard solution of 8.8, 3.1, 9.9 and 3.2

ng mL^{-1} concentrations of B1, B2, G1 and G2, respectively. The spiked rice sample was shaken manually and was allowed to stand at room temperature for 30 min to be adsorbed the standard aflatoxins into the sample prior to extraction. The extraction and cleaned up method was same as test samples of rice powder (**Scheme 2**). The spiked concentration of aflatoxins were 0.77, 0.27, 0.86 and 0.28 µg kg^{-1} for B1, B2, G1 and G2, respectively for spiking 10 µL mixture of aflatoxins. Validation was done by intra-day and inter-day recoveries. For intra-day, recovery experiments were conducted in four replicates in same day at 10 µL spiking concentration level. Similarly, the same recovery experiments were conducted in four replicates at 10 µL spiking concentration level for inter- day recovery. Another intra-day and inter-day recovery experiments were conducted by spiking 20 µL of aflatoxins standard solution of 8.8, 3.1, 9.9 and 3.2 ng mL^{-1}concentration of B1, B2, G1 and G2, respectively. The spiked concentrations of aflatoxins were 1.54, 0.54, 1.73 and 0.56 µg kg^{-1} for B1, B2, G1 and G2, respectively for spiking 20 µL mixture of aflatoxins. Four replications were also done for the 20 µL spiking concentration level of mixture of aflatoxins at the same day for intra-day recovery. The same recovery experiment (spiked with 20 µL) with four replications was done in another day for inter-day recovery. The standard deviation (SD) was calculated by considering the results of replicate analysis and the relative standard deviation (RSD) was calculated from standard deviation and average value of the replicate studies. The % recovery was calculated by considering the spiked and recovered concentrations of aflatoxins B1, B2, G1 and G2. For spiking 10 µL mixture of aflatoxins the intra-day recovery was 70.37 ± 5.59 % for B1, 75.36 ± 6.77 % for B2, 72.85 ± 5.93 % for G1 and 99.20 ± 3.16 % for G2 and inter-day recovery was 56.71 ± 1.60 % for B1, 57.71 ± 0.58 % for B2, 65.53 ± 0.73 % for G1 and 76.34 ± 4.03 % for G2 (**Table 7**). For spiking 20 µL mixture of aflatoxins the intra-day recovery was 65.08 ± 2.21 % for B1, 63.39 ± 2.36 % for B2, 71.85 ± 1.90 % for G1 and 65.83 ± 2.92 % for G2 and inter-day recovery was 63.40 ± 3.55 % for B1, 61.39 ± 3.42 % for B2, 69.57 ± 5.19 % for G1 and 79.18 ± 7.10 % for G2 (**Table 7**).

Table 6: Amount of Aflatoxins (µg kg^{-1}) in Rice Samples

Sample Code	B1 (µg kg^{-1})	B2 (µg kg^{-1})	G1 (µg kg^{-1})	G2 (µg kg^{-1})	Total Aflatoxins (µg kg^{-1})
R1	ND*	ND	ND	ND	ND
R2	ND	ND	ND	ND	ND
R3	0.04	ND	ND	ND	0.04
R4	ND	tr**	ND	ND	tr
R5	0.70	0.06	0.63	ND	1.39
R6	0.45	0.14	1.04	ND	1.63
R7	0.21	0.10	ND	ND	0.31
R8	0.16	ND	1.82	1.56	3.54
R9	0.09	0.11	ND	ND	0.20
R10	0.29	0.10	0.93	ND	1.32
R11	0.40	0.20	ND	0.46	1.06
R12	0.15	0.08	ND	ND	0.23
R13	0.28	0.18	ND	0.34	0.80
R14	0.33	0.11	0.23	0.12	0.79
R15	0.25	0.11	0.22	ND	0.58
R16	0.24	0.10	ND	ND	0.34
R17	0.12	0.08	ND	ND	0.20
R18	0.04	0.05	ND	ND	0.09
R19	0.06	0.04	ND	ND	0.10
R20	0.06	0.07	ND	ND	0.13
Mean	0.23	0.10	0.81	0.62	0.75
Median	0.21	0.10	0.78	0.40	0.34
Maximum	0.70	0.20	1.82	1.56	3.54

*ND – Not Detected

tr**- Trace

The results (**Table 6**) revealed that 18 out of 20 samples contained detectable amount of aflatoxins. Aflatoxin B_1 (in the range of 0.04 to 0.70 µg kg^{-1}), B2 (in the range of trace to 0.20 µg kg^{-1}), G1 (in the range of 0.22 to 1.82 µg kg^{-1}) and G2 (in the range of 0.12 to 1.56 µg kg^{-1}) were quantified in 17, 16, 6 and 4 samples, respectively (**Figure 19**).

Table 7: Intra-day and Inter-day Recovery of Aflatoxins (B1, B2, G1 and G2) in rice sample

Day	Aflatoxins	Spiking level (µg kg^{-1})	Average recovery ± SD (%)	(RSD %)
Intra-day (n= 4)	B1	0.77	70.37 ± 3.59	7.94
		1.54	65.08 ± 2.21	3.40
	B2	0.27	75.36 ± 2.77	8.99
		0.54	63.39 ± 2.36	3.73
	G1	0.86	72.85 ± 2.93	8.14
		1.73	71.85 ± 1.90	2.64
	G2	0.28	99.20 ± 3.16	3.19
		0.56	65.83 ± 2.92	4.43
Inter-day (n = 4)	B1	0.77	56.71 ± 1.60	2.83
		1.54	63.40 ± 3.55	5.61
	B2	0.27	57.71 ± 0.58	1.01
		0.54	61.39 ± 3.42	5.57
	G1	0.86	65.53 ± 0.73	1.12
		1.73	69.57 ± 3.19	7.47
	G2	0.28	76.34 ± 4.03	5.28
		0.56	79.18 ± 3.10	8.97

*SD = Standard deviation and **RSD = Relative standard deviation

3.1.2 Discussion

Aflatoxins are naturally produced toxins by fungal growth of *Aspergillus* species. These mycotoxins can contaminate a variety of agricultural and food commodities including maize, nut, wheat, rice, corn, spice, dairy products etc. In the present study rice was chosen for analysis of aflatoxins because it is our staple food. Due to hot and humid condition aflatoxins can be unavoidable contaminants of foods in Bangladesh. Various surveys conducted in different parts of the world indicated considerable levels of aflatoxins in rice (Gummert et al.; 2009 & Tanaka et al.; 2007). It was found that there is a list of different countries where rice

samples were analyzed for checking the presence of aflatoxins but no such data are available for Bangladesh. But heavy rains or high humidity could become susceptible to aflatoxin contamination in the staple food (rice) of Bangladesh.

The analysis of aflatoxins in rice samples was done by following the established method (ISO 16050; 2003). The immune affinity column (3 mL; polypropylene) containing a gel material loaded with monoclonal antibodies against aflatoxins B1, B2, G1 and G2 were used in the present study for sample clean up purposes. The total aflatoxins (B1, B2, G1 and G2) in the rice samples were found to be in the range of trace to 3.54 μg kg^{-1} (**Table 6**). Aflatoxin B1, B2, G1 and G2 were present in 70, 60, 40 and 10 % of rice samples, respectively (**Figure 19**). The aflatoxins tolerance level is different for different country. European Union (EU) Maximum Tolerated Level is 4 μg kg^{-1} (Fredlunda et al., 2009; Ghali et al., 2010; Ruadrew et al., 2013; Sales & Yoshizawa, 2005; Suarez- Bonnet et al., 2013), AFs contamination more than MTL of 20 μg kg^{-1} as assigned by food authorities of USA (FDA and FAO). The WHO has set 30 mg kg^{-1} for aflatoxin in food, and this high limit has been used in several countries for aflatoxin in rice without any regards to the daily intake of rice (Nguyen et al., 2007). A comparable regulation have been reported in Malaysia of 35 mg kg^{-1} g (Reddy et al., 2011) and in Indian permissible limit of 30 mg kg^{-1} for total aflatoxin (Toteja et al., 2006).These limits have been set for food in general, but it may not be suitable for a global staple food like rice. From literature survey, it was found that the level of aflatoxin in rice (**Table 8**) was different for different countries.

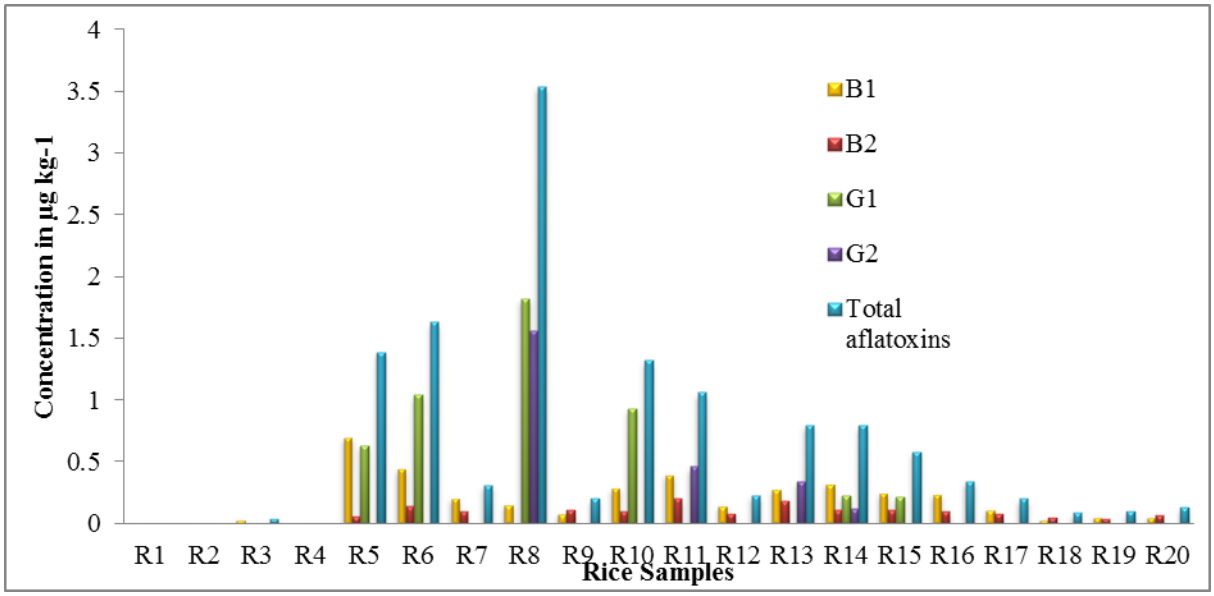


Figure 19: Amount of Aflatoxins (µg kg^{-1}) in rice samples

Table 8: **The Level of Total AFs in Rice in Different Countries**

Country	**Toxin types**	**Number of positive samples (%)**	**Range (µg kg^{-1})**	**Year of survey**	**Authors**
Sweden	B1	58	0.1-46.2		Fredlund *et al.* (2009)
	Total AFs	58	0.1-50.7		
Canada	B1	56	1.44-7.14	2008	Bansal *et al.* (2011)
	B1	43	1.45-3.48	2009	
India	B1	67.8	0.1-308.0	2008	Reddy *et al.* (2009)
Pakistan	B1	49	0.1-16.65	2010	Firdous *et al.* (2012)
Iran	B1	68.9	0.20-4.3	2010	Feizy *et al.* (2010)
United Arab Emirates	B1	-	1.2-16.5	1994	Osman *et al.* (1999)
South Korea	B1	5.6	1.8-7.3	2005	Park *et al.* (2005)
Japan	B1	3	0.3-3.6		Tabata *et al.* (1993)
Philippines	B1	94.8	0.03-11.0		Sales and Yoshizawa (2005)
Columbia	B1	36.3	1.0-52.8		Cespedez and Diaz (1997)
Tunisia	B1	0	0		Ghali *et al.* (2008)
	Total AFs	12.5	2.0-7.5		

* AFs = Aflatoxins (B1, B2, G1 and G2)

The two samples (R1 and R2) were found to be free from aflatoxins (**Table 6**) were collected from Dhaka city. The level of aflatoxins in rice differs from one place to another. This is due to various factors like temperature, relative humidity and agricultural practices. In general, hot and humid conditions are supposed to be favorable for the growth of toxigenic fungi and mycotoxin production in agricultural products (Reddy *et al.*, 2008). The results of present study revealed that the aflatoxins (AFs) level in Bangladeshi rice does not concurrently present a potential risk to the human health. The effect attributed to aflatoxin in rice in Bangladesh is still unclear and the low level does not absolutely mean safer rice food regarding to its high consumption.

3.1.2.1 Method Validation for Rice Samples

The method was validated following the procedure described in **Section 2.2.13**. Specificity was evaluated by comparing the chromatograms of the standard, blank and spiked sample (**Figure 12 & 17)**. No interference peak at the retention times of B1, B2, G1 and G2 was observed in the blank rice sample.

The LOD and LOQ were 0.009 and 0.025 µg kg^{-1} for B1, 0.006 and 0.018 µg $kg^{-1}$for B2, 0.039 and 0.116 µgkg^{-1} for G1 and 0.025 and 0.075 µg kg^{-1} for G2, respectively (**Table 5**). The LOD and LOQ were different for four different aflatoxins B1, B2, G1 and G2 due to their different sensitivity in LC-FLD with Kobra cell system.

The extraction efficiency of the ISO 16050 method in rice sample was evaluated by recovery experiments at two spiking concentrations (0.77 and 1.54 µg kg^{-1} for B1, 0.27 and 0.54 µg kg^{-1} for B2, 0.86 and 1.73 µg kg^{-1} for G1 and 0.28 and 0.56 µg kg^{-1} for G2) in 4 (n=4) replicates analyses. The spiking concentration levels were chosen according to the results of test rice samples. Both intra-day and inter-day recoveries were carried out to evaluate the efficiency of the method. The results for the intra-day recovery was 70.37 ± 5.59 % for B1, 75.36 ± 6.77 % for B2, 72.85 ± 5.93 % for G1 and 99.20 ± 3.16 % for G2 and inter-day recovery was 56.71 ± 1.60 % for B1, 57.71 ± 0.58 % for B2, 65.53 ± 0.73 % for G1 and 76.34 ± 4.03 % for G2 for spiking 10 µL mixture of aflatoxins (**Table 7**). For spiking 20 µL mixture of aflatoxins the intra-day recovery was 65.08 ± 2.21 % for B1, 63.39 ± 2.36 % for B2, 71.85 ± 1.90 % for G1 and 65.83 ± 2.92 % for G2 and inter-day recovery was 63.40 ±

3.55 % for B1, 61.39 ± 3.42 % for B2, 69.57 ± 5.19 % for G1 and 79.18 ± 7.10 % for G2 (**Table 7**). The relative standard deviation (RSD) value of the recovery experiments were in the range of 1.01 to 8.99 % which showed the excellent performance of the method. The recovery was within 70 to 99 % for seven recovery experiments and 56 to 69 % for nine recovery experiments. The percent recovery below 70 may be due to the low spiking concentration. Since the detected maximum concentration of aflatoxins in the test rice samples were 0.70 μg kg^{-1} for B1, 0.20 μg kg^{-1} for B2, 1.83 μg kg^{-1} for G1 and 1.56 μg kg^{-1} for G2 , the spiking concentrations chosen for recovery experiment were also low. From the previous report (Kirstin *et al.*;2014) it was found that the acceptable recovery ranges for aflatoxins B1, B2, G1 and G2 is 50-120 , 70-110 and 80-110 % for spiking concentration <1.0 , 1-10 and >10 μg kg^{-1}, respectively. The recovery results of the present study can be compared with Biljana *et.al* study (Biljana *et al*.; 2013) for determination of aflatoxins (B1, B2, G1 and G2) in peanuts and peanut based products. For B_1 the recovery was 92.33% and the recovery for G_1 was 84.37%. There were some minor deviations concerning the values of B_2 (127.86%) and G_2 (48.31%). But according to the Commission Regulation 466/2001, 2174/2003 and 1881/2006 those deviations are not likely to affect the result significantly. So, the recovery result of the present study was satisfactory. The average percent recovery can be increased by increasing the concentration of spiking level.

Due to the delay in drying and moisture content, post-harvest contamination of aflatoxins can (Asghar *et al*. 2013) be done. There are six seasons of Bangladesh but the rice samples in the present study were collected in rainy season which can regulates fungal grows and mycotoxin expression. More study is needed for investigation of aflatoxin in rice samples in other seasons.

The International Agency for Research on Cancer (IARC) classified aflatoxicol (AFL) and aflatoxin B1 (AFB1) as class I agents (International Agency for Research on Cancer; IARC, 1993).). Previous study showed that aflatoxins are relatively stable during the cooking process, suggesting that a major reduction in the exposure to these mycotoxins cannot be expected to occur by cooking rice. Regarding the fate of aflatoxicol (AFL) using naturally contaminated rice, Park *et al*. (Park *et al*., 2005 &2006) reported that 31%–36% of AFB1 was lost during cooking at 160 °C for 20 min. In another study (Hisako *et al.*, 2013), it was observed that only a 7% reduction of aflatoxin B1 (AFB1). The reason for this difference was

thought to be due to the differences in the cooking temperatures. Mohamadi *et al*. (Mohamadi *et al*., 2012) examined the reduction of the total AFL (AFB1, AFB2, AFG1, AFG2) using a steaming-based rice cooker (Toshiba Corp., Tokyo, Japan) and reported that the average total AFL reduction was 24.8%. The reduction of the total AFL in the Hisako et al study was almost the same as the result reported by Mohamadi *et al*. Hussain *et al*. (Hussain *et al*., 2009) studied the reduction of AFB1 in polished basmati rice by boiling with water and microwave oven cooking using artificially contaminated rice. They reported that boiling and microwave oven cooking showed a reduction up to 84.0%–87.5% and 72.5% of initial contents, respectively. These results demonstrated that the total AFL content was retained at 82.3% of the original level under the ordinary cooking conditions used in Japan.

Since people consume a larger amount of staple foods compared to other foods the regulation of B1 for staple foods should be more restricted than for other foods. Based on the results of the present study, if the toxicant was contained in a staple food, such as rice, there was a notable increase in the cancer risk due to the high consumption of that food. The study recommends that for staple foods, the maximum residue level should be considered and that the permissible level for staple foods should be more restricted than for other foods.

PART-B

Analysis of Spice (Turmeric Powder)

2.3 Experimental

2.3.1 Sample Collection

Forty six Turmeric powder samples were purchased from different shops of Dhaka city (**Figure 20**). Thirty seven samples were in packet form of different local companies of Bangladesh and nine samples were not in packet. The packet turmeric powder samples were coded as P1 to P37 and loose samples were coded as U1-U9.

The packet samples were from eight different companies; Pran (P1-P17), Radhuni (P18-P24), Rani (P25-P27), Rupchanda (P28-P31), Arku (P32-P34), BD (P35), Fresh (P36) and Pure (P37). The turmeric powder samples were kept in forty six different zip locked plastic bags, labelled and store in a freezer at -20^{0}C until extraction was carried out. The code No. of samples with Batch No., name of the companies, name of market, date of production, expiry and purchase are given in **Table 9**.

Figure 20: Packet and loose turmeric powder samples

Table 9: Sampling of Packet and Loose Turmeric Powder Samples

Sample Code	Place of Collection	Date of Collection	Batch No.	Company Name	Date of Production	Date of Expired
P 1	Kazipara Bazar, Mirpur	13/05/13	13/03/432	Pran	02/03/13/ A	01/09/14
P 2	Polashi Bazar	15/05/13	13/03/431	,,	02/03/13	01/09/14
P 3	Green Road	17/06/13	13/03/543	,,	30/03/13/ A	29/09/14
P 4	Central Road	20/06/13	12/12/107	,,	30/12/12/ CHA	29/06/14
P 5	New market	25/06/13	13/04/602	,,	13/04/13/ A	12/10/14
P 6	New market	25/06/13	12/09/103	,,	24/09/12/ A	23/03/14
P 7	Agora, Dhanmondi	09-07-13	12/12/064	,,	26/12/12/ B	25/06/14
P 8	Polashi	09-07-13	12/07/151	,,	21/07/12/ B	20/01/14
P 9	Nazira Bazar	19/07/13	13/01/291	,,	30/01/13/ A	29/07/14
P 10	Nazira Bazar	10/07/13	13/05/907	,,	27/05/13/ A	26/11/14
P 11	Kazipara,Mirpur	10/07/13	13/03/431	,,	02/03/13/ A	01/09/14
P 12	Bashabo	10/07/13	12/03/178	,,	20/03/12/ B	19/09/13
P 13	Kazipara,Mirpur	12/07/13	12/03/204	,,	23/03/12/ A	22/09/13
P 14	Sheorapara, Mirpur	12/07/13	12/04/090	,,	09/04/12/ B	08/10/13
P 15	Kochukhet Bazar	12/07/13	11/11/032	,,	05/11/11/ A	04/05/13
P 16	Farmgate	06/10/13	13/03/432	,,	02/03/13/ A	01/09/14
P 17	Fular Road	20/08/13	13/03/012	,,	10/03/13/ A	09/09/14
P 18	Fular Road	20/08/13	13050978	Radhuni	May,13	Nov,14
P 19	Kazipara,Mirpur	22/08/13	13070780	,,	July,13	Jan,15
P 20	Kazipara,Mirpur	22/08/13	13031465	,,	March,13	Sep,14
P 21	Alu Bazar,Old Dhaka	04/09/13	13060091	,,	June,13	Dec.,14
P 22	Nazira Bazar,Old Dhaka	04/09/13	13041384	,,	April,13	Oct.,14
P 23	Farmgate	06/10/13		,,	April,13	October,14
P 24	Polashi Bazar	10/02/14	13090235	,,	Sep, 13	March, 15

P 25	Kazipara,Mirpur	26/08/13	296	Rani	July,12	Jan,14
P 26	Kamrangir Chor	16/09/13	389	,,	Dec,12	June,14
P 27	Kamrangir Chor	16/09/13	421	,,	Feb,10	Aug,14
P 28	Kazipara,Mirpur	26/08/13	Ha 225 Kha	Rupchanda	July,13	Jan,15
P 29	Kamrangir Chor	16/09/13	Bha 217 Kha	,,	Sep,12	March,14
P 30	Nazira Bazar	02/10/13	Ha 225 Kha	,,	July,13	Jan,15
P 31	New Market	07/11/13	Bha 217 Kha	,,	Sep, 12	March,14
P 32	Kazipara,Mirpur	26/08/13	S047	Arku	11/03/13	10/03/14
P 33	Kamrangir Chor	22/09/13	121106	,,	07/11/12	06/11/13
P 34	Dhanmondi 6	07/01/14	6894	,,	14/09/13	13/09/14
P 35	Nazira Bazar	02/10/13	01281	BD	28/06/13	27/06/14
P 36	Kamrangir Chor	22/09/13	1307445	Fresh	27/07/13	26/07/13
P 37	Kazipara,Mirpur	26/08/13	T28 A	Pure	Jan,13	July,14
U 1	Alu Bazar,Old Dhaka	04/09/13	N/A*	N/A	N/A	N/A
U 2	Nazira Bazar,Old Dhaka	04/09/13	N/A	N/A	N/A	N/A
U 3	Nawa Bazar,Old Dhaka	16/09/13	N/A	N/A	N/A	N/A
U 4	Kamrangir Chor	22/09/13	N/A	N/A	N/A	N/A
U 5	Kamrangir Chor	22/09/13	N/A	N/A	N/A	N/A
U 6	Panthopath Bazar	24/09/13	N/A	N/A	N/A	N/A
U 7	Section Bazar	24/09/13	N/A	N/A	N/A	N/A
U 8	Polashi Bazar	24/09/13	N/A	N/A	N/A	N/A
U 9	Nazira Bazar	02/10/13	N/A	N/A	N/A	N/A

*N/A= Not applicable

2.3.2 Control Sample

Fresh turmeric rhizomes were purchased from Dhaka city market. The rhizomes were washed and boiled with water (1 hour) and dried in sunlight for 2 weeks. Dried turmeric was made into powder by grinding machine (Mesh size 200) and used as control.

2.3.3 Mobile Phase

LC grade acetonitrile from Merck, Germany and LC grade water produced at the laboratory were used as mobile phases. The mobile phases were filtered (0.25 µm solvent filter) and degassed prior to use.

2.3.4 Analytical Condition of Liquid Chromatograph with Photo Diode Array Detector (LC-PDA)

Liquid Chromatograph (Shimadzu Prominence UFLC) with Photo Diode Array Detector (PDA) was used in the present study. Separations were performed on a C_{18} column (250 x 4.6 mm; loop size 20.0 µl). The flow rate was 1 mL min^{-1}. Isocratic condition mobile phase acetonitrile-water (1:1 ratio) was used to elute the compounds. The column oven temperature was fixed at 30^{o} C and the wave length was set at 280 nm. LC was conditioned with mobile phase until smooth base line was observed.

2.3.5 Preparation of Standard Solutions of Carbofuran

Preparation of primary standard solution

The primary stock solution of carbofuran was made by dissolving 10 mg of certified standard in 100 mL acetonitrile to obtain 100 mg L^{-1} solution.

The prepared solution in 100 mL amber bottle was labelled indicating name of the standard, concentration and the date of preparation. The meniscuses of the solution was marked with permanent black ink and stored in the freezer (-20 °C) away from the sample storing area until further use.

Preparation of middle and working standard solutions

The primary standard solution of carbofuran (100 mg L^{-1}) was diluted with acetonitrile to obtain a medium stock solution (50 mg L^{-1}) and the medium standard was diluted with the

same solvent to obtain the working standard solutions (5.00, 4.00, 2.00, 1.00, 0.50, 0.20, 0.10, 0.05, 0.04, 0.02 and 0.01 mg L^{-1}).

2.3.6 Preparation of Calibration Curves

The LC system was conditioned with mobile phase A (water) and B (acetonitrile) in different proportions until a smooth base line was obtained. The mobile phase B, acetonitrile was injected as solvent blank and the retention time of the solvent blank was at 2.4 min (**Figure 21 a**). The working standard solutions of 5.0, 4.0, 2.0, 1.0, 0.5, 0.2, 0.1, 0.05, 0.04, 0.02 and 0.01 mg L^{-1} of carbofuran were injected gradually into LC-PDA from the lowest to the highest concentration level. The peak of carbofuran was obtained at 7.25 min. Two representative chromatograms of standard carbofuran are given in **Figure 21b & 21c**. From the twelve chromatograms of carbofuran solution (one for solvent bank and eleven concentration levels of carbofuran); limit of detection (LOD) and limit of quantification (LOQ) were found out to be 0.01 and 0.03 mg L^{-1}, respectively (**Table 10**). The calibration curve at different concentration levels was made using MS Excel software by plotting area of the eluted standard vs concentration. Three calibration curves; higher (0.2-5.0 mg L^{-1}), medium (0.1-2.0 mg L^{-1}) and lower 0.05-0.01 mg L^{-1}) are presented in (**Figure 22a, 22b & 22c**) for carbofuran. Linear range of elution and correlation coefficient are given in **Table 10**.

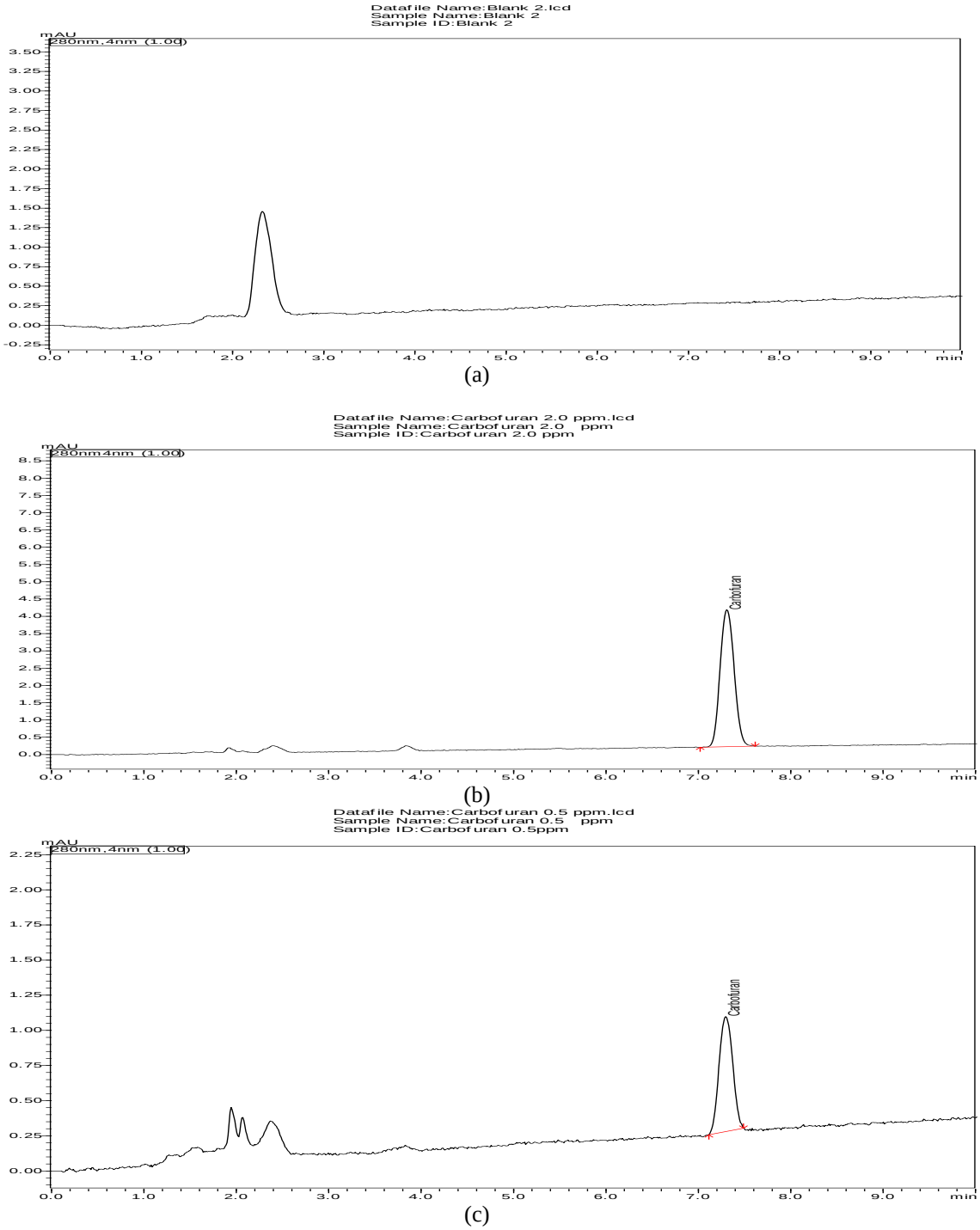
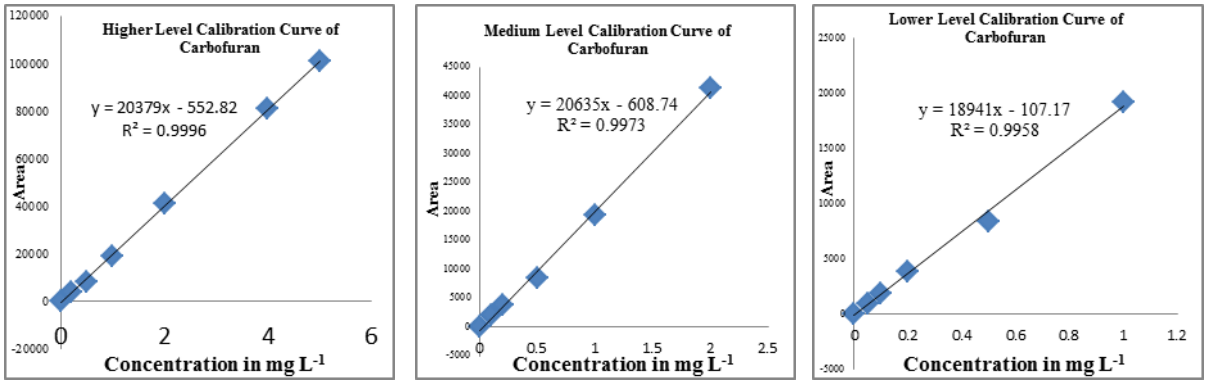


Figure 21: LC chromatogram of blank solvent (a) and certified standard carbofuran at higher (b) & lower (c) concentration levels



(a) **(b)** **(c)**

Figure 22: Calibration curves of standard carbofuran in higher (a), medium (b) and lower (c) concentration levels

2.3.7 Preparation of Turmeric Samples

Extraction

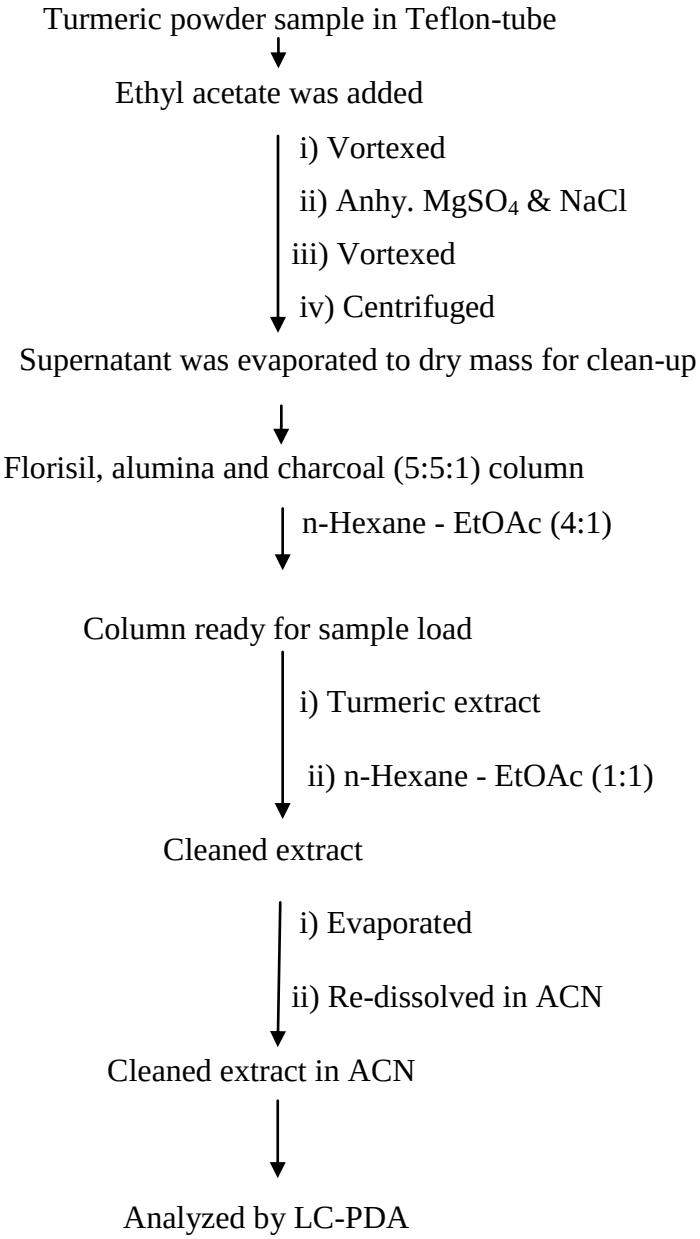
The samples were extracted by following QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method (Anastassiades *et al*., 2003) with minor modification. Turmeric powder sample (5 g each) was taken in a screw cap teflon tube (50 mL), ethyl acetate (15 mL) was added and vortexed (1 min). Anhydr. $MgSO_4$ (6 g) and NaCl (1.5 g) were added to it and the mixture was vortexed (1 min) and then centrifuged (4000 rpm) for 10 min. The supernatant (5 mL) was taken into a round bottom flask (100 mL) and evaporated to dry mass for clean-up.

Clean-up Process

Preparation and conditioning of column

A glass column (40 cm long & 12 mm internal diameter) was packed with a mixture of florisil (5 g), alumina (5 g) and charcoal (1.0 g) with a small amount of sodium sulphate added on the top using a mixture of n hexane-ethyl acetate (4:2 ratio). The column was equilibrated by passing 2 column volumes of mobile phase. The extract was applied into the column and eluted with n-hexane and EtOAc mixture (100 mL) in 1:1 ratio. The eluent was

evaporated and reconstituted with 2 mL acetonitrile. The sample was filtered by using 0.25 µm solvent filter and analyzed by LC-PDA (**Scheme 3**). Results are given in **Table 11**.



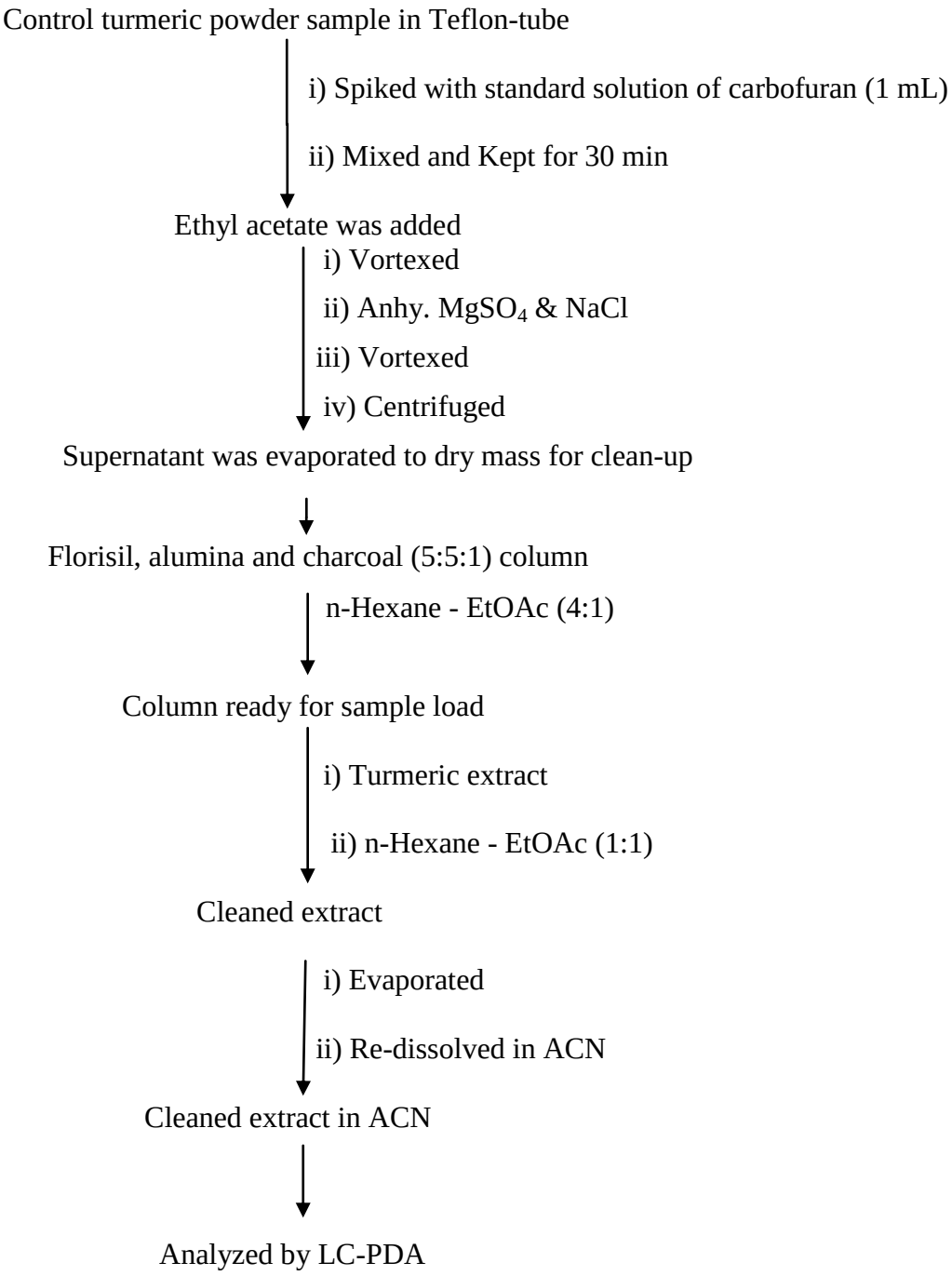
Scheme 3: Extraction and clean-up procedure of turmeric powder samples

2.3.8 Analysis of Turmeric Powder Sample

Forty six cleaned extracts of forty six different turmeric powder samples were analysed by Liquid Chromatograph-Photo Diode Array Detector (LC-PDA). Solvent was injected before each injection. From the forty six different chromatograms amount of carbofuran was calculated using external calibration curves (**Figure 22a, b and c**). Results are given in **Table 11**. LC system was washed with water-acetonitrile in different compositions before quite the instrument.

2.3.9 Recovery Experiment

The control turmeric powder sample (5 g) was taken into a 50 mL teflon tube and 1 mL solution of 2 mg L^{-1} standard carbofuran was added. The spiked turmeric sample was shaken manually for 0.5 min and allowed to stay for 30 min at room temperature. Then the sample was extracted and cleaned up by the same procedure as was done for experimental turmeric sample powder (**Scheme 3**). The cleaned extract was passed through LC column and was detected by photo diode array detector. Four recovery experiments with seven replications were done at four different spiking concentration levels (0.4, 0.8, 1.0 and 20 mg kg^{-1}). The recovery of carbofuran was calculated by a formula given below. The results are given in **Table 10**. Standard deviation and relative standard deviation were calculated. Similarly second, third and fourth recovery experiments with seven replications were done by spiking 1 mL of 4, 5 and 100 mg L^{-1} standard carbofuran, respectively , extracted and cleaned following exactly same procedure and analysis were done under same condition. The chromatograms of test turmeric sample and spiked sample are given in **Figure 23**. Percent recovery was calculated and the results are given in **Table 10**.



Scheme 4: Recovery procedure of carbofuran in control turmeric powder samples

The recovery of carbofuran was calculated according to:

$$R = \frac{A_m \times C_{st}}{A_{st} \times C_m} \times \frac{100}{M_{st}}$$

Where R is the recovery (%), A_m is the peak area of the analyte in the matrix, A_{st} is the peak area of the analyte in the standard, C_m is the concentration of the analyte in the matrix, C_{sr} is the concentration of the analyte in the standard, and M_{st} is the mass of the analyte in the standard.

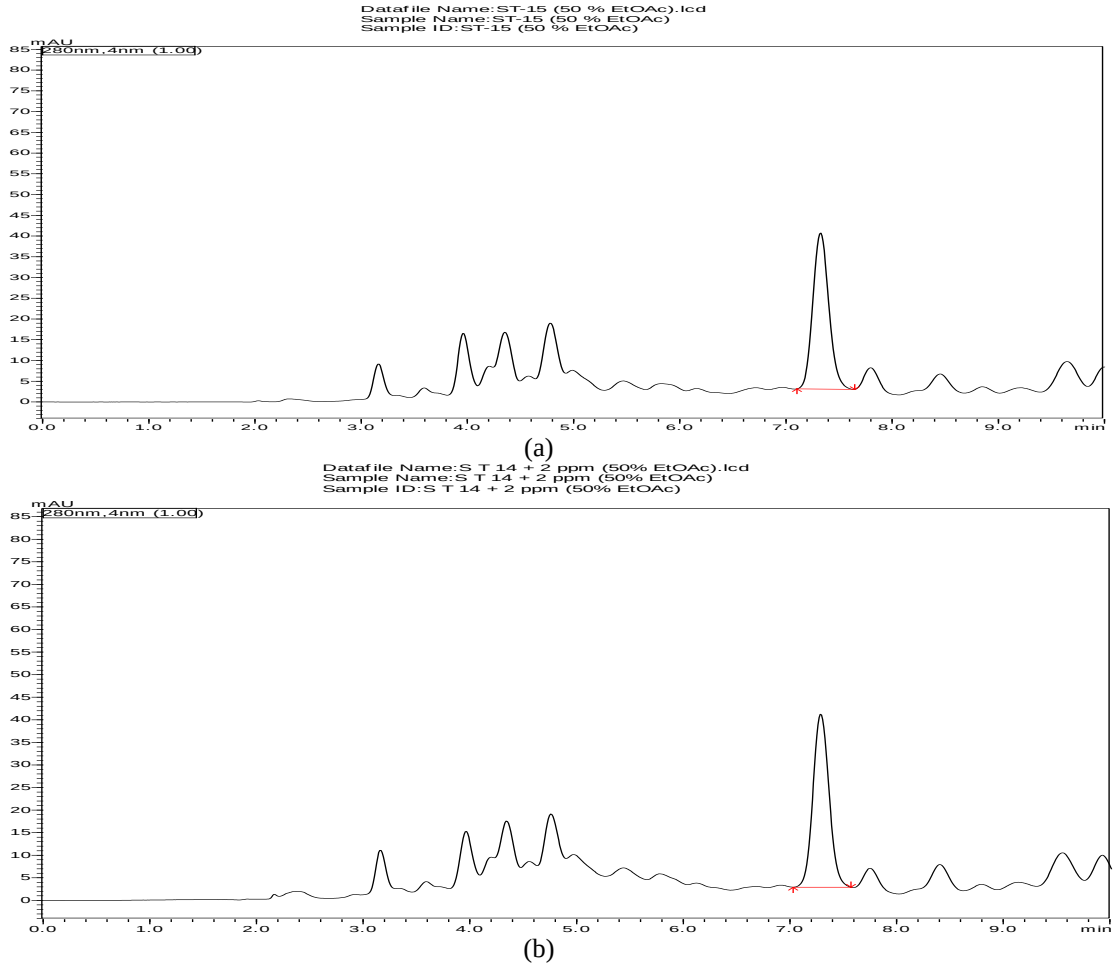


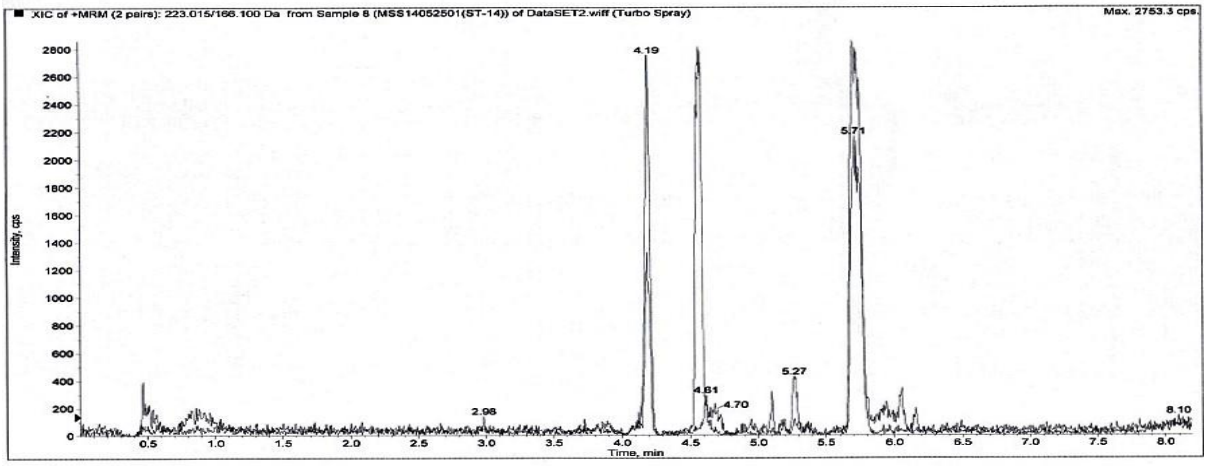
Figure 23: LC chromatogram of turmeric sample (a) and spiked turmeric sample (b)

2.3.10 Heating of Turmeric Powder

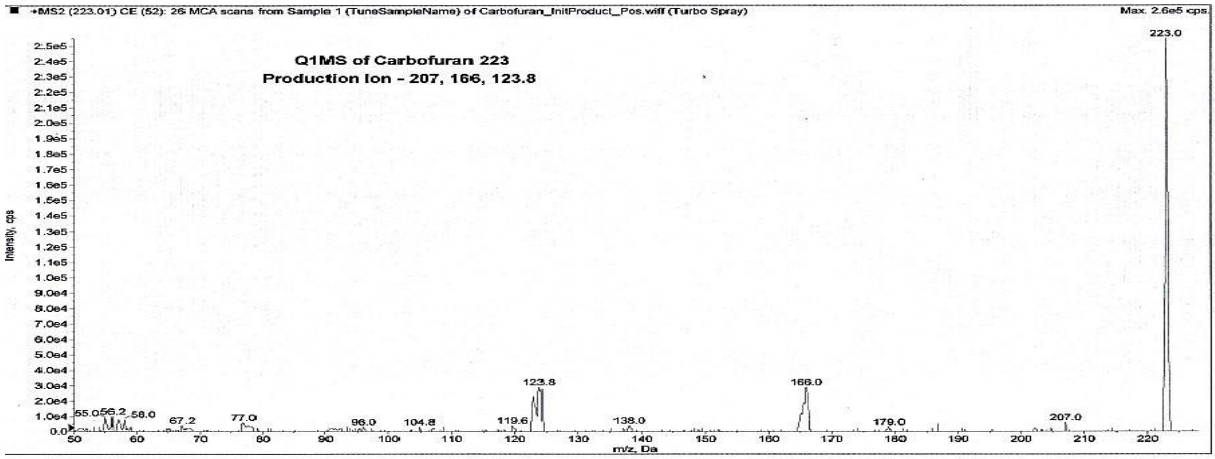
The samples (5 g) were taken in a 100 mL round bottom flask and 15 mL LC grade water was added to it, heated for 30 min in a boiling water bath, evaporated to dry mass by rota-vapor followed by a freeze dryer. The dried sample was extracted, cleaned up and analyzed following the same procedure as described in **Section 2.3.7** and **Scheme 3**.

2.3.11 Liquid Chromatography-Mass Spectrometry

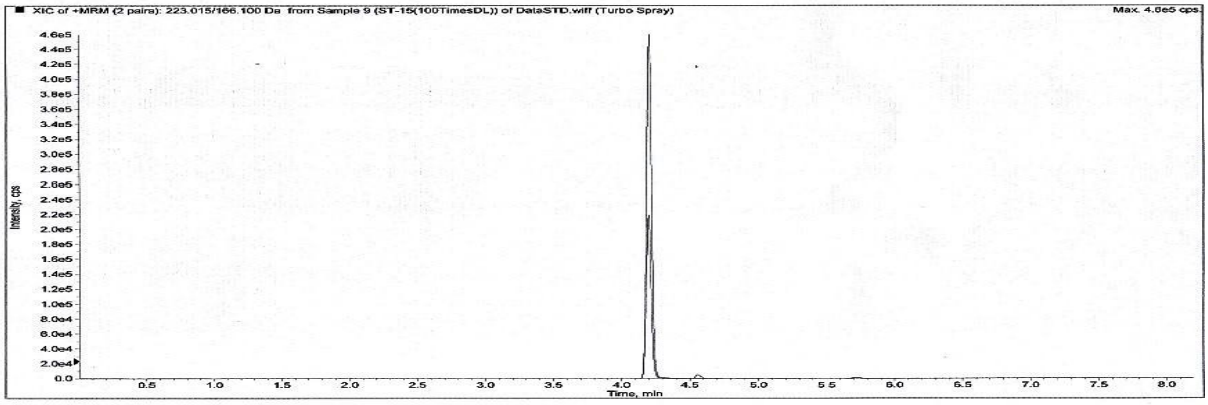
The turmeric powder extract was analysed by liquid chromatography-mass spectrometry using Shimadzu LCMS-8050 with electrospray ionization (ESI), a triple quadrupole LC-MS/MS. Nebulizing and collision gas was N_2. Separations were performed on a Shim-pack GISS C_{18} column (250 x 4.6 mm i.d.; particle size 5 µm). The carrier gas pipe was 5 m. The molecular mass of carbofuran is 221.26 g mol^{-1}. Multiple–reaction monitoring (MRM) measurement was conducted by ESI using positive switching. The carbofuran peak was found at 4.19 min (**Figure 24a**). The m/z ratio for carbofuran was at 223 → 166 and the major fragment ions were observed at m/z 207 and 166 and 123.8, respectively. Q1MS of the peak and standard carbofuran are given in **Figure 24 b& c**.



(a)



(b)



(c)

Figure 24: LC-MS/MS chromatogram of turmeric extract (a), Q1 MS of carbofuran peak (b) and standard carbofuran (c)

3.2 Results and Discussion

3.2.1.1 Results of the Turmeric Powder Sample

Commercial turmeric powder samples of different companies and different batch No. of the same companies were purchased for analysis of pesticides residue. The packet powder samples were in different sizes (15, 50, 100 and 200 g). The open or loose powder samples were purchased from different markets of Dhaka city. The control sample for blank experiment was prepared by following the traditional method of Bangladesh. Fresh, raw and whole turmeric of five kilogram was collected from market. After removing extra dry shell and root, the samples were washed with water and boiled for 1 hour. Then the sample was dried under sunlight. About 10-14 days were needed for drying and ground into powder with grinding machine. For the analysis of pesticide residue, the samples were extracted as described in the **Section 2.3.7** and were analysed by LC-PDA (**Section 2.3.8**).

Table 10: Linear Ranges, Correlation Coefficients (r^2), Limit of Detection (LOD), Limit of Quantification (LOQ) and Recovery Experiments of Carbofuran in Turmeric Powder

Linear range (mg L^{-1})	Linearity (r^2)	LOD (mg L^{-1})	LOQ (mg L^{-1})	Spiking level (mg kg^{-1}; n=7)	Mean recovery ± SD* (%)	RSD** (%)
0.05 - 1.00	0.9958	0.01	0.03	0.40	92.52 ± 0.01	3.33
0.10 - 2.00	0.9973			0.80	97.97 ± 0.02	2.35
0.20 - 5.00	0.9996			1.00	99.95 ± 0.05	4.82
				20.00	103.14 ± 2.41	11.70

*SD = Standard deviation and **RSD = Relative standard deviation

The calibration curves of carbofuran (**Figure 22**) were made by injecting the working standard solutions of certified standard carbofuran to LC-PDA. The linearity of the calibration curves was excellent with correlation coefficients $r^2 \geq 0.9996$, 0.9973 and 0.9958. Limit of detection (LOD) and limit of quantification (LOQ) were determined by serially diluting the working standard solutions. The diluted standard solutions were injected one by

one giving interval of solvent blank until the peak heights of the standards were same to the noise level. The area of the target peak was three times than the noise was for limit of detection (S/N; 3:1) and the area of the target peak was ten times than noise for limit of quantification (S/N ratio, 10:1). The limit of detection (LOD) and quantification (LOQ) were 0.01 and 0.03 mg L^{-1}, respectively (**Table 10**).

Recovery experiment was carried out by spiking control turmeric powder sample with known concentrations of carbofuran standard working solutions. The control sample was extracted three replicates followed by respective extraction and clean-up procedure (**Scheme 3**) to determine the matrix effect under analysis condition. Solvent blank acetonitrile was injected to confirm the smooth base line in LC-PDA. The control turmeric powder sample was spiked with 1 mL of carbofuran standard solution of 2, 4, 5 and 100 mg L^{-1} concentration of carbofuran standard solutions, respectively. The spiked turmeric powder sample was shaken manually and was allowed to stand at room temperature for 30 min to be adsorbed the standard carbofuran into the sample. The extraction and cleaned up method was same as test samples of turmeric powder (**Scheme 4**). The spiked concentration levels of carbofuran were 0.40, 0.80, 1.00 and 20 mg kg^{-1}, respectively. The first recovery experiment was conducted in seven replicates at 0.40 mg kg^{-1} spiking concentration level. Similarly, the second recovery experiment was conducted in seven replicates at 0.80 mg kg^{-1} spiking concentration level. The third recovery experiment was conducted by spiking 1.00 mg kg^{-1} of carbofuran standard solution and the forth recovery experiment was conducted in seven replicates at 20 mg kg^{-1} spiking concentration level. The standard deviation (SD) was calculated by considering the results of seven replicate analysis and the relative standard deviation (RSD) was calculated from standard deviation and average value of the replicate studies. The % recovery was calculated by considering the spiked and recovered concentrations of carbofuran (**Table 10**). The results of recovery experiments with seven (n=7) replications at four different spiking concentrations (0.4, 0.8, 1.0 and 20 mg kg^{-1}) were ranged from 92.52 ± 0.01 to 103.14 ± 2.41 % (RSD ≤ 12%).

Carbofuran residues in the sample extracts were identified by comparing the retention times (**Figure 23**) with those of the pure standards (**Figure 22**) and quantified by extrapolation of corresponding sample peak areas with those from standard calibration curves prepared using pure carbofuran. The carbofuran peak in turmeric sample was confirmed by co-injection. The

area of target peak after co-injection was increased. Among 46 samples, carbofuran residue was found in 37. The amount of carbofuran residues (**Table 11**) were found to be in the range of 2.5 ± 0.07 to 23.1 ± 0.30 mg kg^{-1} in the packet samples and 2.06 ± 0.14 to 7.8 ± 0.32 mg kg^{-1} in loose samples. In addition to LC-PDA analysis, two of the extracted samples were analyzed by LC-MS/MS (ionization technique was EI and mode was positive) for reconfirmation (**Figure 24**). The carbofuran peak was found to be at 4.19 min. After heating in a water bath for 30 min, no residue was found.

Table 11: Carbofuran Residue (Mean ± SD, mg kg^{-1}) in Commercial Packet and Loose Turmeric Powder Samples

Packet sample			Loose sample	
Sample Code	**Company Name**	**Mean ±SD (mg kg^{-1} ; n=3)**	**Sample Code**	**Mean ± SD (mg kg^{-1} ; n=3)**
P1	Pran	11.4 ± 0.58	U 1	BDL*
P 2	,,	4.4 ± 0.22	U 2	BDL
P 3	,,	6.9 ± 0.77	U 3	6.1 ± 0.76
P 4	,,	5.1 ± 0.10	U 4	6.4± 0.25
P 5	,,	BDL	U 5	4.9± 0.13
P 6	,,	2.6± 0.30	U 6	BDL
P 7	,,	13.0± 0.17	U 7	2.06 ± 0.14
P 8	,,	12.3± 0.24	U 8	BDL
P 9	,,	16.5± 1.11	U 9	7.8± 0.32
P 10	,,	23.1± 0.30		
P 11	,,	20.0± 0.13		
P 12	,,	BDL		
P13	,,	BDL		
P 14	,,	20.2± 0.27		
P 15	,,	19.8± 0.39		
P 16	,,	4.8 ± 0.68		
P 17	,,	2.5 ± 0.07		
P 18	Radhuni	13.0 ± 0.57		
P 19	,,	8.8 ± 0.35		
P 20	,,	9.6 ± 0.11		
P 21	,,	7.4 ± 0.09		
P 22	,,	11.7 ± 0.62		
P 23	,,	5.4 ± 0.25		
P 24	,,	2.5 ± 0.15		
P 25	Rani	6.3 ± 0.68		
P 26	,,	BDL		
P 27	,,	8.1 ± 0.06		
P28	Rupchanda	8.1 ± 1.53		
P29	,,	8.4 ± 1.24		
P 30	,,	5.3 ± 0.19		
P31	,,	6.0 ± 0.52		
P32	Arku	6.5 ± 0.09		
P33	,,	BDL		
P34	,,	9.9 ± 0.54		
P35	BD	7.8± 0.94		
P36	Fresh	8.2 ± 0.08		
P37	Pure	21.0 ± 0.35		

BDL* = Below Detection Limit

3.2.1.2 Discussion

Carbofuran is being used in Bangladesh in the storage of spices in order to protect them from pest attack. The presence of residual amounts of carbofuran over the MRL value would be an important health concern considering its toxicity. Hence, a total of 46 turmeric powder samples purchased from local markets were analyzed to see the residual amount of carbofuran (**Figure 25**) for the purpose of food safety. The extraction was carried out by QuEChERS method (Anastassiades *et al.*; 2003) and was analyzed by LC-PDA.

Turmeric rhizome is very susceptible to attack by nematodes (Meloidogyne species, Radopholus similis and Pratylenchus coffeae). So, there is a probability of losing the bright golden yellow colour of the rhizome which can affect market value. Nematode-free rhizome is also essential for the purpose of fresh planting. Carbofuran is allowed to use in soil for cultivation of turmeric rhizome (Ravichandra N.G., 2014). But it is not recommended to apply carbofuran on the dried/polished turmeric to prevent storage pests. From the earlier published data, the presence of organochlorine pesticide residue were found in spices (turmeric, chili, coriander, and black pepper) that were analyzed by gas liquid chromatography. Residues of hexachlorocyclohexane (HCH) and 2, 2-bis (p-chlorophenyl)-1, 1, 1-trichloroethane (DDT) were detected in chili, turmeric, corlander and black pepper **(**Kaphalia et al. 1990).

The pesticide residue levels in spices were studied in various levels (George *et al.;* 2013; Johnson *et al*.; 2014). Presence of pesticides that are banned was also found in a study conducted by Kerala Agricultural University (KAU), Vellayani. The majority of the MRL exceedance was found in cardamom (spice) samples. The pesticide residue which was most frequently found above MRL in cardamom was quinalphos **(**Naseema *et al.*, 2014**)**.

3.2.1.2.1 Method Performance for Turmeric Powder Samples

Method performance was assessed by evaluating quality parameters, such as selectivity, limits of detection (LOD) and quantification (LOQ), linearity, repeatability and recovery .The selectivity of the method was evaluated by injecting extracted blank samples. The absence of

signals above a signal-to-noise ratio of at the retention times of the target compounds proved that the method is free of interferences. The different concentrations (5.0, 4.0, 2.0, 1.0, 0.5, 0.2, 0.1, 0.05, 0.04, 0.02 and 0.01 mg L^{-1}) of carbofuran were injected separately to the LC-PDA. The results showed good linearity with correlation coefficients (r^2) of > 0.99 (**Figure 22a, 22b and 22c**). The limit of detection (LOD) was 0.01 mg kg^{-1}at a signal-to-noise ratio of 3 and the limit of quantification (LOQ) was 0.03 mg kg^{-1}at a signal to noise ratio of 10 (**Table 10)**. The typical LC-PDA chromatograms are shown in **Figure 21 & 23**. The standard deviation in all the analysis was in the range of 0.06 to 1.53 % (< 4.00 %) which were proved the excellent repeatability of the study. The efficiency of the method has been evaluated separately by spiking blank turmeric samples with known concentrations of carbofuran working solution (0.4, 0.8, 1.0 and 20 mg kg^{-1}). The recoveries for spiked turmeric samples ranged from 92.52 ± 0.01 to 103.14 ± 2.41 % and the relative standard deviations ranged from 3.33 to 11.70 %. All values indicated good accuracy and repeatability. The recovery and relative standard deviations are shown in **Table 10**.

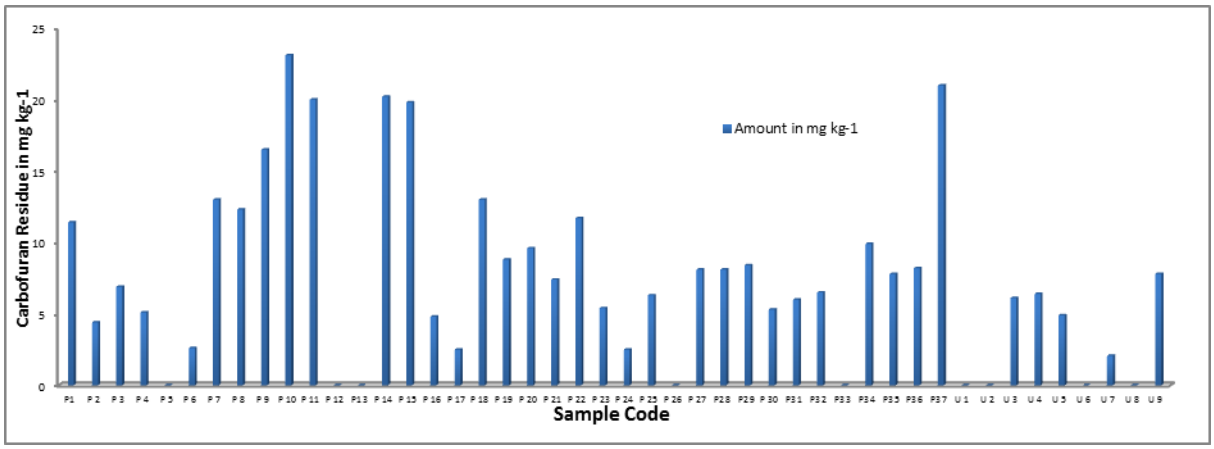


Figure 25: Amount of carbofuran (mg kg^{-1}) in turmeric samples

Carbofuran residues in the sample extracts were identified by comparing the retention times with those of the pure standards and quantified by extrapolation of corresponding sample peak areas with those from standard calibration curves prepared using pure carbofuran. The peak of carbofuran was confirmed initially by co-injection. The co-injection was done by adding 2 mg L^{-1} standard carbofuran (0.50 mL) solution with the sample (0.50 mL) which gave peak for the target. The peak area was increased and the target was confirmed. Among 46 samples, carbofuran residue was found in 37. The amount of carbofuran residues (**Table 11**) were found to be in the range of 2.5 ± 0.07 to 23.1 ± 0.30 mg kg^{-1} in the packet samples

and 2.06 ± 0.14 to 7.8 ± 0.32 mg kg^{-1} in loose samples. For reconfirmation, two of the extracted samples were analyzed using liquid chromatography (LC-MS/MS) with Turbo Ion Spray tandem mass spectrometry in positive ionization mode (**Figure 24**). The m/z ratio for carbofuran was at 223 → 166, and the major fragment ions observed in product spectrum were at m/z 207 and 166 and 123.8, respectively (**Figure 24b**). The CXL (Codex maximum residue limit) of carbofuran in spices (roots or rhizome) is 0.1 mg kg^{-1} (European Food Safety Authority, 2014).The residue of carbofuran was present in most of the turmeric powder samples (37 out of 46) in both packet and open form. The high residue in packet samples than loose sample may be due to more oxidation in open form than closed packed form. There was no residue after heating in a water bath for 30 min. The major degradants of carbofuran are the 3-hydroxy carbofuran and 7-phenol products resulting from hydrolysis and oxidation (United States Environmental Protection Agency, 2008) which were not analyzed in the present study. In our traditional cooking system, the temperature is raised up to about few hundred degrees, so the cooked food where the turmeric powder is used is safe but still there is risk for the person who are handling.

PART-C

Studies of Dissipation Patterns of Diazinon and Carbosulfan in Four Different Vegetable Samples

2.4 Experimental

2.4.1 Preparation of Standard Solution of Diazinon

Preparation of primary standard solution

The primary standard solution (100 mg L^{-1}) of diazinon was made by dissolving 10 mg of certified standard diazinon in 100 mL n-hexane.

The prepared solution (in 100 mL amber bottle) was labelled indicating name of the standard, concentration and the date of preparation. The meniscuses of the solution was marked with permanent black ink and stored in the freezer (-20 °C) away from the sample storing area until further use.

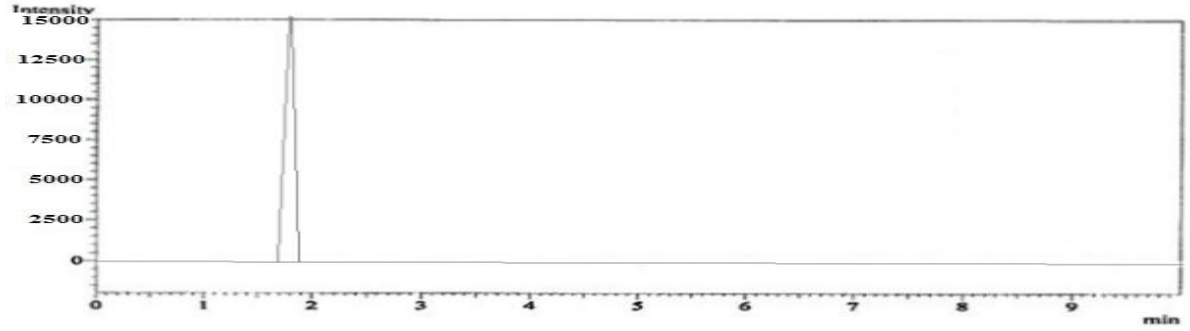
Preparation of middle and working standard solutions

The primary standard solution of diazinon was diluted with n-hexane to the middle standard solution (20 mg L^{-1}). The middle standard (20 mg L^{-1}) was diluted with n-hexane to get 0.5, 0.2, 0.1, 0.05, 0.02, 0.01, 0.005, 0.002 and 0.001 mg L^{-1} working standard solutions to make calibration curves.

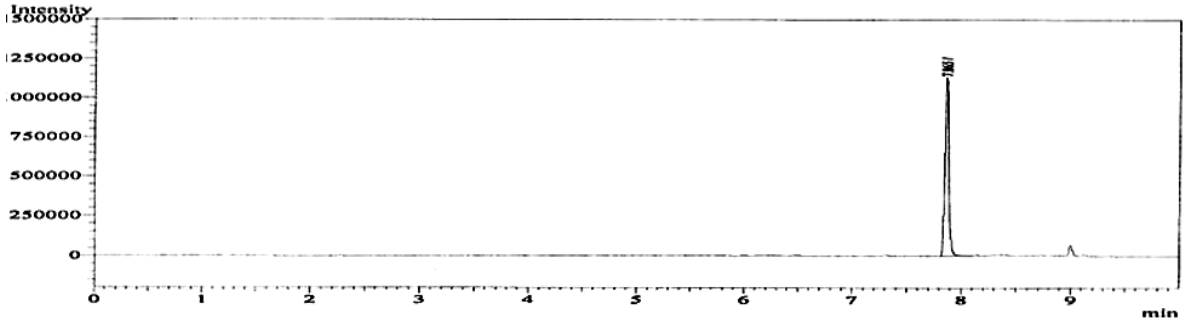
2.4.2 Preparation of Calibration Curves

The GC system was conditioned at its maximum operating temperature until a smooth base line was obtained. n-Hexane was injected as solvent blank and the retention time of the solvent blank was at 1.8 min (**Figure 26a**). The working standard solution of 0.5, 0.2, 0.1, 0.05, 0.02, 0.01, 0.005, 0.002 and 0.001 mg L^{-1} of diazinon were injected gradually into GC-ECD from the lowest to the highest concentration level. The peak of diazinon was obtained at 7.8 min (**Figure 26b**). From the ten chromatograms (one for solvent bank and nine concentration levels of diazinon); limit of detection (LOD) and limit of quantification (LOQ)

were found out to be 0.001 and 0.003 mg L^{-1}, respectively (**Table 12**). The calibration curves at different concentration levels were made using MS Excel software by plotting area of the eluted standard vs concentration. Four calibration curves are presented in **Figure 27**. Linear range of elution and correlation coefficient are given in **Table 12**.



(a)



(b)

Figure 26: GC-ECD chromatograms of blank solvent (a) and certified standard Diazinon (b)

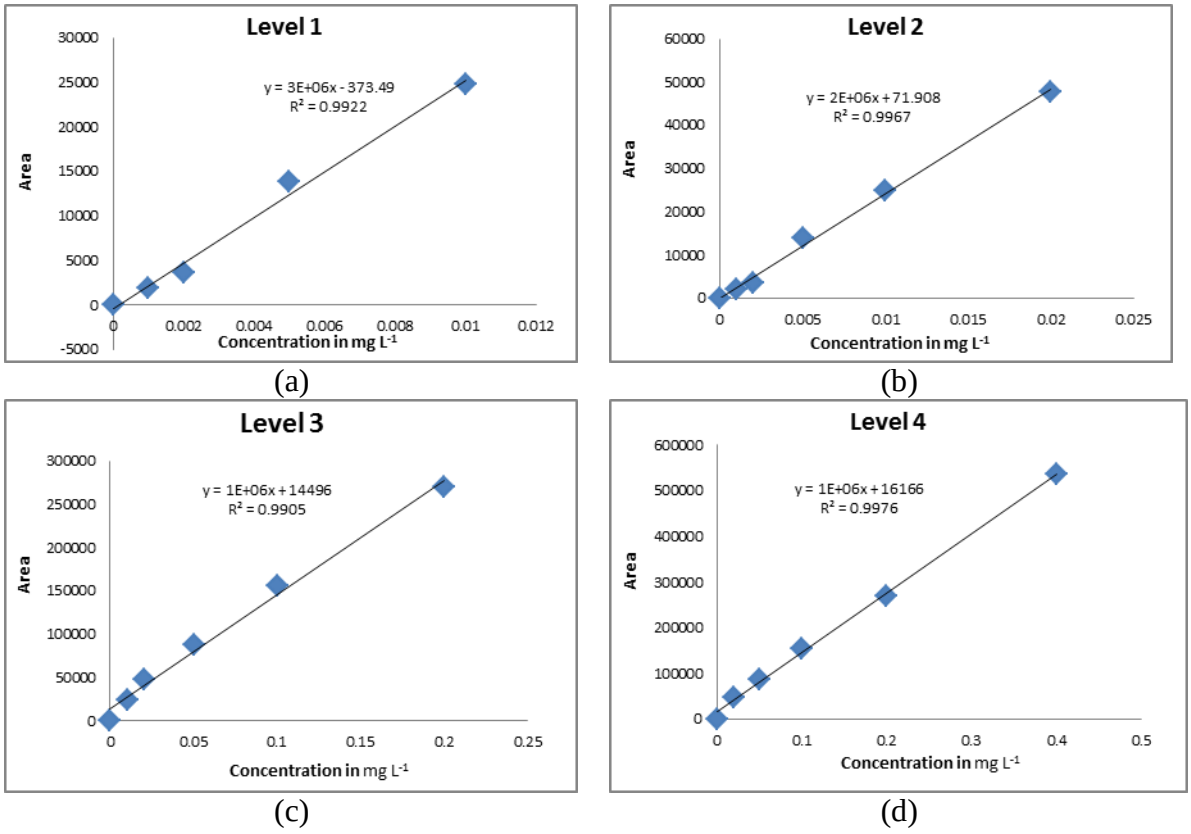


Figure 27: Calibration curves of diazinon in four levels (a, b, c and d)

2.4.3 Application of Diazinon in Tomato

The tomato samples were grown in four different experimental fields. Three experimental fields were for application of diazinon and one experimental field was for control. The commercial diazinon (Shabion 60 EC) of 2 mL was diluted with 1L of water and was sprayed by a hand sprayer in the three matured tomato fields at BARI (**Figure 28**).

(a)

(b)

(c)

(d)

(e)

(f)

Figure 28: Control field of cauliflower (a), Application of pesticides at BARI (b) and Collection of vegetable samples from experimental field (c, d, e & f)

2.4.3.1 Harvesting of Tomato

The tomato samples were randomly harvested from the diazinon treated three plots and were kept in plastic polyethylene jip locked bags. The samples were harvested at 0 (2 hours), 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 days after spray and the harvested samples (~ 1 kg) were kept in chill box and immediately brought to the Laboratory of Chemistry, Dhaka University. The samples were coded as TDN 0 - TDN 15, respectively. Control sample was harvested from the untreated experimental field in the same way as done for treated sample.

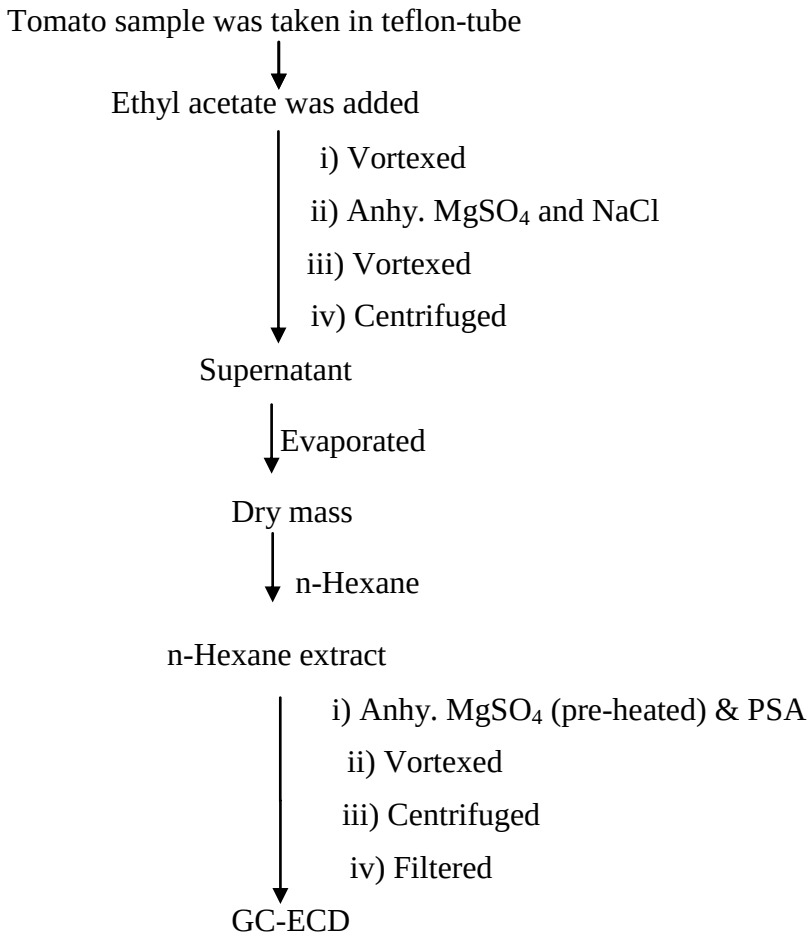
2.4.3.2 Homogenization and Storage of Tomato Sample

The tomato samples were chopped into small pieces and homogenized by a kitchen blender. The homogenized tomato samples (10 g) were kept in a 3x16 screw-cap teflon tubes (50 mL) and stored in a freezer at -20^0 C until analysis.

2.4.3.3 Extraction and Clean-up of Tomato Samples

The homogenized frozen tomato samples (10 g) were taken out from the freezer and thawed at room temperature, 20 mL of ethyl acetate was added to it and vortexed for 2 min. The pre-heated anhydrous magnesium sulphate (6.0 g) and sodium chloride (1.5 g) were added to the tube and vortexed for 1 min and was centrifuged for 5 min at 5000 rpm. From the supernatant 10 mL was transferred into a round bottom flask (100 mL) and evaporated to dryness. The dried material was reconstituted with n-hexane (5 mL) (**Scheme-5**).

The n-hexane extract (2 mL) was transferred into a screw-cap test tube, 750 mg anhydrous preheated $MgSO_4$ and 150 mg PSA were added to it. The test tube was vortexed for 30 s and centrifuged for 5 min at 5000 rpm. The supernatant was taken and filtered through LC sample filter (0.25 µm) and transferred into vial to analyse by GC-ECD having an auto-injector (**Scheme-5**). The results are given in **Table 13**.



Scheme 5: Extraction and clean-up procedure for tomato samples

2.4.3.4 Analysis of Cleaned Extract of Tomato by GC-ECD

Quantification of residue of diazinon was done on a gas chromatograph (GC-2010 Shimadzu) equipped with ^{63}Ni Electron Capture detector (ECD) and an auto injector was attached with it. Nitrogen was used as carrier and make up gas. Separations were performed on Non-polar (HP-5 MS) capillary column of 30 m long x 250 µm i.d. x 0.25 µm film thicknesses from Agilent, USA. Temperature was programed at 120ºC; hold for 1 min, rise at 20 °C min^{-1} to 260 °C; post run for 2 min. The injector and detector temperatures were set at 220 and 280 °C, respectively. All injections were made in splitless-split mode with 1 µL injection volume. The chromatograms of standard diazinon and cleaned extract of tomato are given in **Figure 29**.

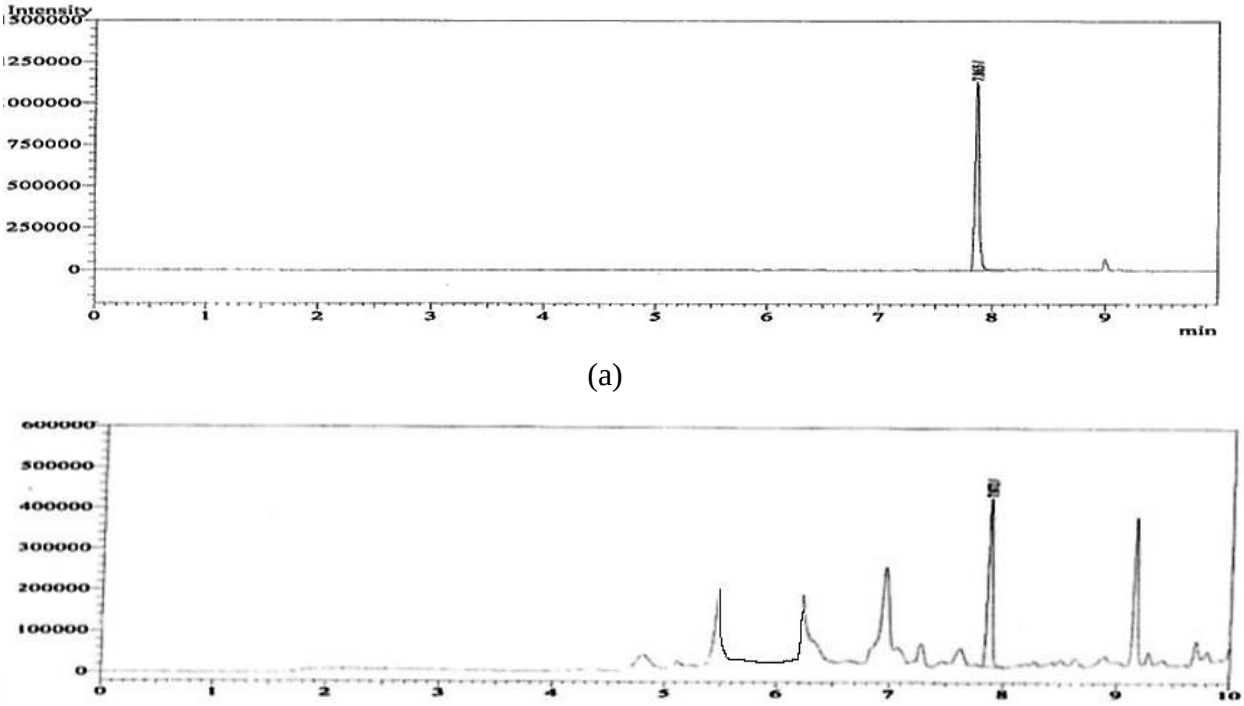


Figure 29: GC-ECD chromatograms of standard diazinon (a) and tomato extract (b)

2.4.3.5 Control Sample of Tomato

The control tomato samples were chopped and homogenized as the treated samples and kept in freezer at -20^0 C.

2.4.3.6 Recovery Experiment of Diazinon in Tomato

The homogenized control tomato sample (10 g) was taken in a screw cap Teflon tube (50 mL), spiked with 1 mL of certified standard diazinon of 15, 5 and 0.2 mg L^{-1} concentrations. The spiked samples were vortexed for 0.5 min and were allowed to stand for 30 min at room temperature. The recovery experiments were conducted in 4 replicates at three different spiking concentrations of 0.02, 0.50 and 1.50 mg kg^{-1} (**Table 17**). Then the spiked control samples (4 x 3) were extracted and cleaned up by the same procedure as was done for experimental tomato sample (**Scheme 5**). The cleaned extract was analyzed by GC-ECD. The results are given in **Table 17**. Standard deviations and relative standard deviations were

calculated. Similarly four recovery experiments were done at three spiking concentration levels and analysis was done under the same condition. Percent recovery was calculated and the results are given in **Table 17**.

2.4.4 Application of Diazinon in Cauliflower

The cauliflower samples were grown in the four different experimental fields. Three experimental fields were for application of diazinon and one experimental field was for control. The commercial diazinon (Shabion 60 EC) of 2 mL was diluted with 1L of water and was sprayed by a hand sprayer in three matured cauliflower fields at BARI (**Figure 28**). No pesticide was sprayed in the control plot.

2.4.4.1 Harvesting of Cauliflower

The cauliflower samples were randomly harvested from the diazinon treated three plots and were kept in plastic polyethylene jip locked bags. The samples were harvested at 0 (2 hours), 1, 4, 5, 6, 9, 10 and 11 days after spray and the harvested samples (~ 1 kg) were kept in chill box and immediately brought to the Laboratory of Chemistry, Dhaka University. The samples were coded as CDN 0 to CDN 11, respectively. Control sample was harvested from the untreated experimental field same way as done for the treated sample.

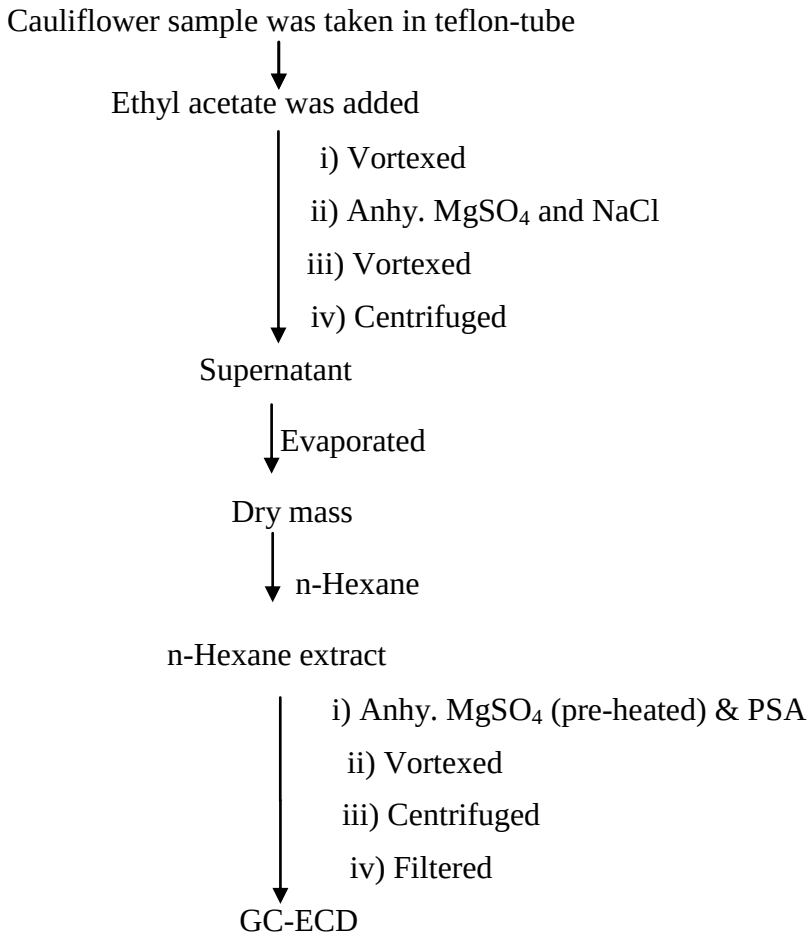
2.4.4.2 Homogenization and Storage of Cauliflower Sample

The fruit parts of cauliflower samples were chopped into small pieces and homogenized by a kitchen blender. The homogenized cauliflower samples (10 g) were kept in a 3x8 screw-cap teflon tubes (50 mL) and stored in a freezer at -20^0 C until analysis.

2.4.4.3 Extraction and Clean-up of Cauliflower Samples

The homogenized frozen cauliflower samples (10 g) were taken out from the freezer and thawed at room temperature, 20 mL of ethyl acetate was added to it and vortexed for 2 min. The pre-heated anhydrous magnesium sulphate (6.0 g) and sodium chloride (1.5 g) were added to the tube and vortexed for 1 min and was centrifuged for 5 min at 5000 rpm. From the supernatant 10 mL was transferred into a round bottom flask (100 mL) and evaporated to dryness. The dried material was reconstituted with n-hexane (5 mL) (**Scheme-6**).

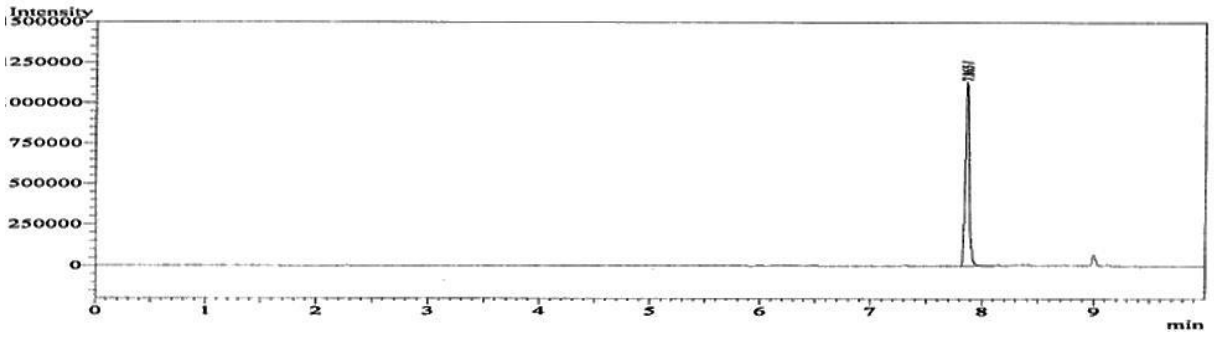
The n-hexane extract (2 mL) was transferred into a screw-cap test tube, 750 mg anhydrous preheated $MgSO_4$ and 150 mg PSA were added to it. The test tube was vortexed for 30 s and centrifuged for 5 min at 5000 rpm. The supernatant was taken and filtered through LC sample filter (0.25 µm) and transferred into vial to analyse by GC-ECD having an auto-injector (**Scheme-6**). The results are given in **Table 14**.



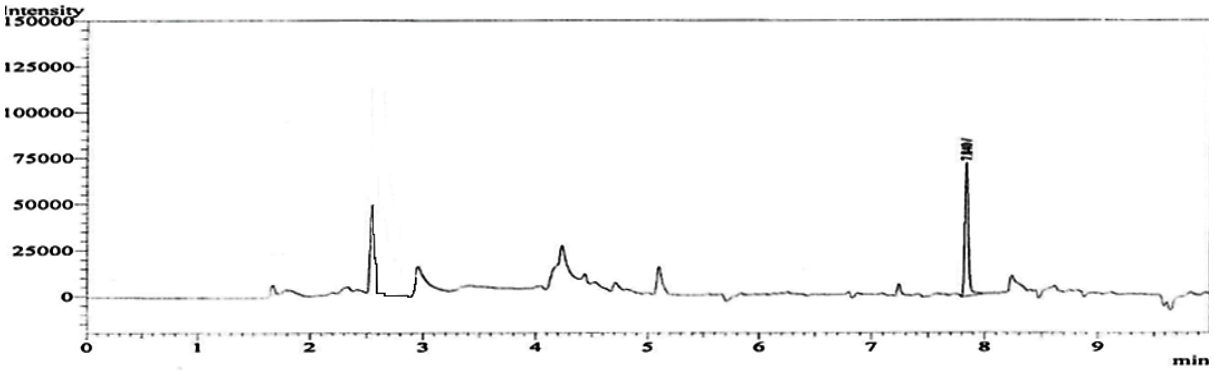
Scheme 6: Extraction and clean-up procedure for cauliflower samples

2.4.4.4 Analysis of Cleaned Extract of Cauliflower by GC-ECD

The residue of diazinon was quantified by a gas chromatograph (GC-2010 Shimadzu) equipped with ^{63}Ni Electron Capture detector (ECD). Nitrogen was used as carrier and make up gas. Separations were performed on Non-polar (HP-5 MS) capillary column of 30 m long x 250 µm i.d. x 0.25 µm film thicknesses from Agilent, USA. Auto injector was used for the determination of the pesticide residue in the samples. Temperature was programed at 120ºC; hold for 1 min, rise at 20 °C min^{-1} to 260 °C; post run for 2 min. The injector and detector temperatures were set at 220 and 280 °C, respectively. The injections were made in splitless-split mode with 1 µL injection volume. The chromatograms of standard diazinon and cauliflower extract are given in **Figure 30**.



(a)



(b)

Figure 30: GC-ECD chromatograms of standard diazinon (a) and cleaned cauliflower extract (b)

2.4.4.5 Control Sample of Cauliflower

The control samples of cauliflower were collected from the respective untreated control field. The samples were chopped and homogenized as the treated samples and stored separately from treated samples in another freezer at -20^0 C.

2.4.4.6 Recovery Experiment of Diazinon in Cauliflower

The homogenized control cauliflower sample (10 g) was taken in a screw cap teflon tube (50 mL), spiked with 1 mL of certified standard diazinon of 40, 20 and 0.5 mg L^{-1} concentrations. The spiked samples were vortexed for 0.5 min and were allowed to stand for 30 min at room temperature. The recovery experiments were conducted in 4 replicates at three different spiking concentrations of 0.05, 2.00 and 4.00 mg kg^{-1} (**Table 17**). Then the spiked control samples (4x3) were extracted and cleaned up by the same procedure as was done for experimental cauliflower sample (**Scheme 6**). The cleaned extract was analyzed by GC-ECD. The results are given in **Table 17**. Standard deviations and relative standard deviations were calculated. Similarly, four recovery experiments were done at three spiking concentration levels and analysis was done under same condition. Percent recovery was calculated and the results are given in **Table 17**.

2.4.5 Application of Diazinon in Eggplant

The eggplant samples were grown in the four different experimental fields. Three experimental fields were for application of diazinon and one experimental field was for control. The commercial diazinon (Shabion 60 EC) of 2 mL was diluted with 1L of water and was sprayed by a hand sprayer in the three matured eggplant fields at BARI (**Figure 28**).

2.4.5.1 Harvesting of Eggplant Sample

The eggplant samples were randomly harvested from the diazinon treated three plots and were kept in plastic polyethylene jip locked bags. The samples were harvested at 0 days, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 days days and the harvested samples (~ 1 kg) were

kept in chill box and immediately brought to the Laboratory of Chemistry, Dhaka University. The samples were coded as EPDN 0 to EPDN 15, respectively. Control sample was harvested from the untreated experimental field same way as done for treated sample.

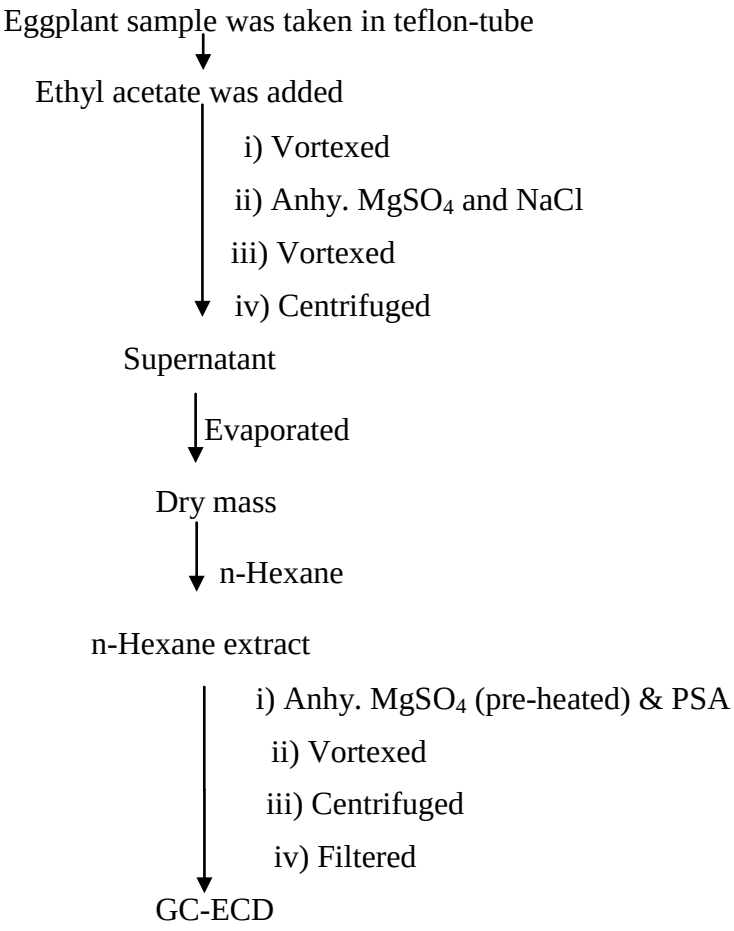
2.4.5.2 Homogenization and Storage of Eggplant Sample

The eggplant samples were chopped into small pieces and homogenized by a kitchen blender. The homogenized eggplant samples (10 g) were kept in a 3x16 screw-cap teflon tubes (50 mL) and stored in a freezer at -20^0 C until analysis.

2.4.5.3 Extraction and Clean-up of Eggplant Samples

The homogenized frozen eggplant samples (10 g) were taken out from the freezer and thawed at room temperature, 20 mL of ethyl acetate was added to it and vortexed for 2 min. The pre-heated anhydrous magnesium sulphate (6.0 g) and sodium chloride (1.5 g) were added to the tube and vortexed for 1 min and was centrifuged for 5 min at 5000 rpm. From the supernatant 10 mL was transferred into a 100 mL of round bottom flask and evaporated to dryness. The dried material was reconstituted with n-hexane (5 mL) (**Scheme-7**).

The n-hexane extract (2 mL) was transferred into a screw-cap test tube, 750 mg anhydrous preheated $MgSO_4$ and 150 mg PSA were added to it. The test tube was vortexed for 30 s and centrifuged for 5 min at 5000 rpm. The supernatant was taken and filtered through LC sample filter (0.25 µm) and transferred into vial to analyse by GC-ECD having an auto-injector (**Scheme-7**). The results are given in **Table 15**.



Scheme 7: Extraction and clean-up procedure for eggplant samples

2.4.5.4 Analysis of Cleaned Extract of Eggplant by GC-ECD

The quantitative analysis of diazinon residue in eggplant samples were conducted by gas chromatograph (GC-2010 Shimadzu) equipped with ^{63}Ni Electron Capture, (EC) detector. A non-polar (HP-5 MS) capillary column of 30 m long x 250 µm i.d. x 0.25 µm film thicknesses from Agilent, USA was used to carry out the separation. Nitrogen was used as both carrier and make up gas. The injector and detector temperatures were 220 °C and 280 °C, respectively. All injections were made in splitless-split mode and injection volume was 1µL. The oven temperature was programmed as: initial temperature of 120 °C hold for 1 minute; increased at 20 °C min^{-1} to 260 °C; hold for 2 min. Identification of residues was achieved by running samples and external reference standards in GC and then comparing the corresponding retention times. The chromatograms of standard diazinon and cleaned eggplant extract are given in **Figure 31**.

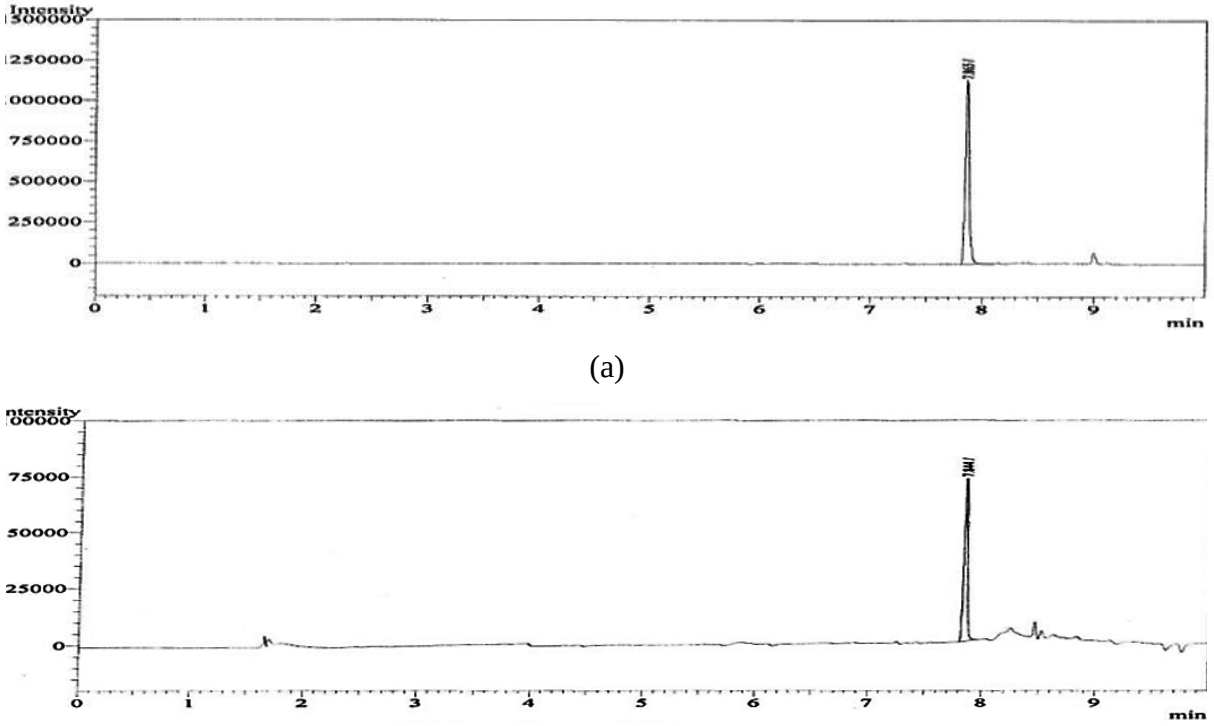


Figure 31: GC-ECD chromatograms of standard diazinon (a) and cleaned eggplant extract (b)

2.4.5.5 Control Sample of Eggplant

The control samples of eggplant were collected from the respective untreated control field. The samples were chopped and homogenized as the treated samples and stored in freezer at -20^0 C away from the Diazinon treated sample storing area until further use.

2.4.5.6 Recovery Experiment of Diazinon in Eggplant

The homogenized control eggplant sample (10 g) was taken in a screw cap teflon tube (50 mL), spiked with 1 mL of certified standard diazinon of 10, 5 and 0.2 mg L^{-1}. The spiked samples were vortexed for 0.5 min and were allowed to stand for 30 min at room temperature. The recovery experiments were conducted in 4 replicates at three different spiking concentrations of 0.02, 0.50 and 1.00 mg kg^{-1} (**Table 17**). Then the spiked control

sample was extracted and cleaned up by the same procedure as was done for experimental eggplant sample (**Scheme 7**). The cleaned extract was analyzed by GC-ECD. The results are given in **Table 17**. Standard deviations and relative standard deviations were calculated. Similarly, four recovery experiments were done at three spiking concentration levels and analysis was done under same condition. Percent recovery was calculated and the results are given in **Table 17**.

2.4.6 Application of Diazinon in Bean

The bean samples were grown in the four different experimental fields. Three experimental fields were for application of diazinon and one experimental field was for control. The commercial diazinon (Shabion 60 EC) of 2 mL was diluted with 1L of water and was sprayed by a hand sprayer in the three matured bean fields at BARI.

2.4.6.1 Harvesting of Bean

The bean samples were randomly harvested from the diazinon treated three plots and were kept in plastic polyethylene jip locked bags. The samples were harvested at 0 days, 1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14 and 15 days after spray and the harvested samples (~ 1 kg) were kept in chill box and immediately brought to the Laboratory of Chemistry, Dhaka University. The samples were coded as BDN 0 to BDN 15, respectively. Control sample was harvested from the untreated experimental field same way as done for treated sample.

2.4.6.2 Homogenization and Storage of Bean

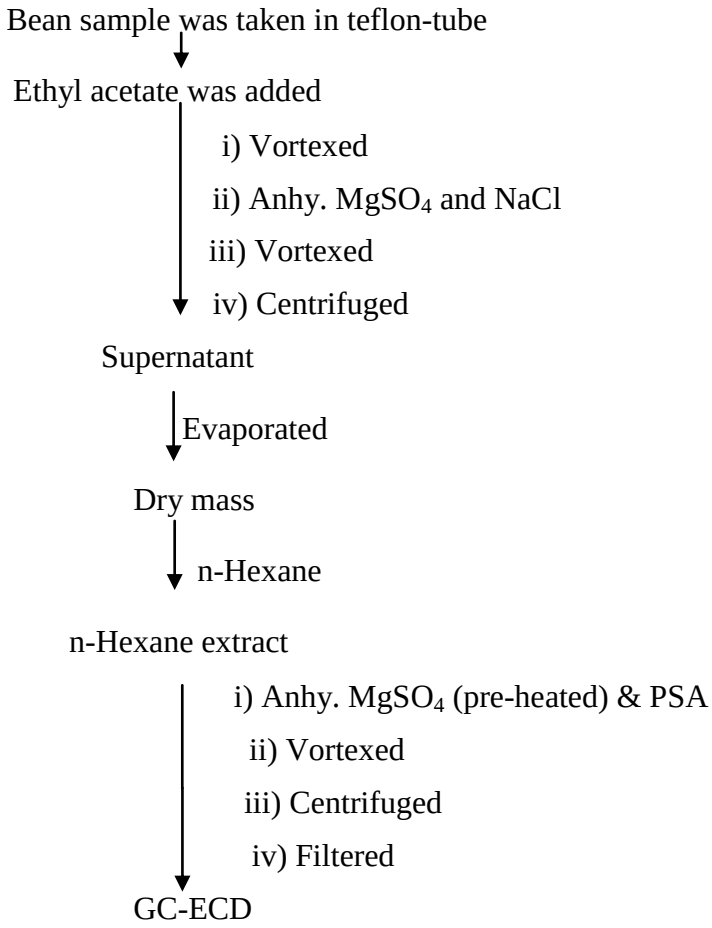
The bean samples were chopped into small pieces and homogenized by a kitchen blender. The homogenized bean samples (10 g) were kept in a 3x15 screw-cap teflon tubes (50 mL) and stored in a freezer at -20^0 C until analysis.

2.4.6.3 Extraction and Clean-up of Bean Samples

The homogenized frozen bean samples (10 g) were taken out from the freezer and thawed at room temperature, 20 mL of ethyl acetate was added to it and vortexed for 2 min. The pre-

heated anhydrous magnesium sulphate (6.0 g) and sodium chloride (1.5 g) were added to the tube and vortexed for 1 min and was centrifuged for 5 min at 5000 rpm. From the supernatant 10 mL was transferred into a 100 mL of round bottom flask and evaporated to dryness. The dried material was reconstituted with n-hexane (5 mL) (**Scheme-8**).

The n-hexane extract (2 mL) was transferred into a screw-cap test tube, 750 mg anhydrous preheated $MgSO_4$ and 150 mg PSA were added to it. The test tube was vortexed for 30 s and centrifuged for 5 min at 5000 rpm. The supernatant was taken and filtered through LC sample filter (0.25 µm) and transferred into vial to analyse by GC-ECD having an auto-injector (**Scheme-8**). The results are given in **Table 16**.



Scheme 8: Extraction and clean-up procedure for bean samples

2.4.6.4 Analysis of Cleaned Extract of Bean by GC-ECD

The residue of diazinon was quantified by a gas chromatograph (GC-2010 Shimadzu) equipped with ^{63}Ni Electron Capture detector (ECD). Nitrogen was used as carrier and make up gas. Separations were performed on Non-polar (HP-5 MS) capillary column of 30 m long x 250 µm i.d. x 0.25 µm film thicknesses from Agilent, USA. Auto injector was used. Temperature was programed at 120ºC; hold for 1 min, rise at 20 °C min^{-1} to 260 °C; post run for 2 min. The injector and detector temperatures were set at 220 and 280 °C, respectively. All injections were made in splitless-split mode with 1 µL injection volume. The chromatograms of standard diazinon and cleaned extract of bean are given in **Figure 32**.

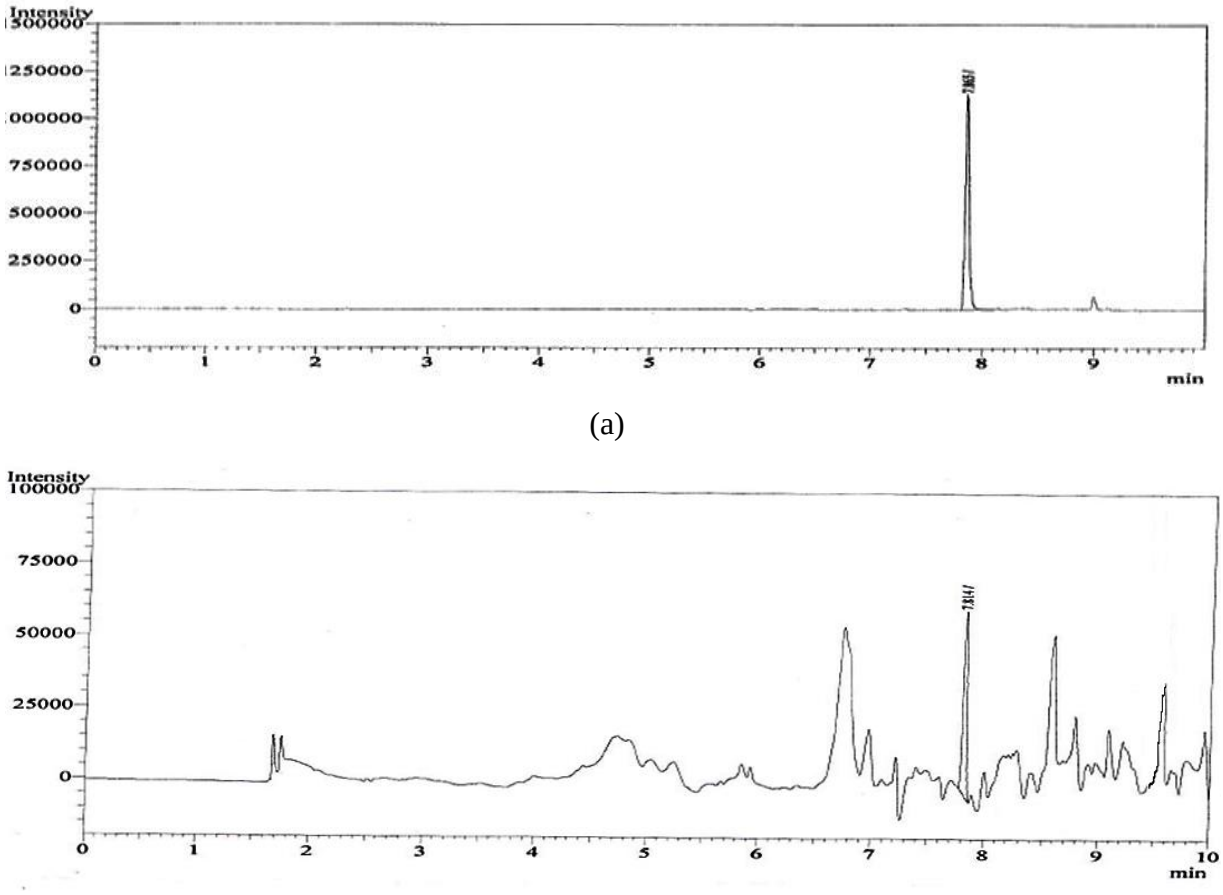


Figure 32: GC-ECD chromatograms of standard diazinon (a) and cleaned extract of bean (b)

2.4.6.5 Control Sample of Bean

The control samples of bean were collected from the respective untreated control field. The samples were chopped and homogenized as the treated samples and stored separately from treated samples in another freezer at -20^0 C.

2.4.6.6 Recovery Experiment of Diazinon in Bean

The homogenized control bean sample (10 g) was taken in a screw cap teflon tube (50 mL), spiked with 1 mL of certified standard diazinon of 1.5, 0.1 and 0.05 mg L^{-1}concentrations. The spiked samples were vortexed for 0.5 min and were allowed to stand for 30 min at room temperature. The recovery experiments were conducted in 4 replicates at three different spiking concentrations of 0.005, 0.01 and 0.15 mg kg^{-1} (**Table 17**). Then the spiked control samples (4x3) were extracted and cleaned up by the same procedure as was done for experimental bean sample (**Scheme 8**). The cleaned extract was analyzed by GC-ECD. Standard deviations and relative standard deviations were calculated. Similarly four recovery experiments were done at three spiking concentration levels and analysis was done under same condition. Percent recovery was calculated and the results are given in **Table 17**.

2.5.0 Application of Carbosulfan in Tomato

The tomato samples were grown in the four different experimental fields. Three experimental fields were for application of carbosulfan and one experimental field was for control. The commercial carbosulfan (Marshal 20 EC) of 1.5 mL was diluted with 1L of water and was sprayed by a hand sprayer in the three matured tomato fields at BARI (**Figure 28**).

2.5.1 Preparation of Standard Solution

Preparation of Primary Standard Solution

The primary standard solution (100 mg L^{-1}) of carbosulfan was prepared by dissolving 10 mg of certified standard carbosulfan sample in 100 mL of n-hexane.

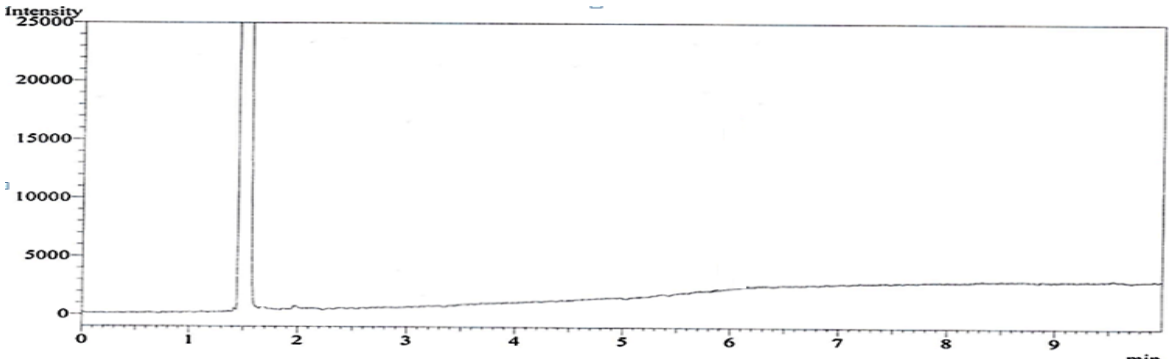
The prepared solution in 100 mL amber bottle was labelled indicating name of the standard, concentration and the date of preparation. The meniscuses of the solution were marked with permanent black ink and stored in the freezer (-20 °C) away from the sample storing area until further use.

Preparation of Middle and Working Standard Solutions

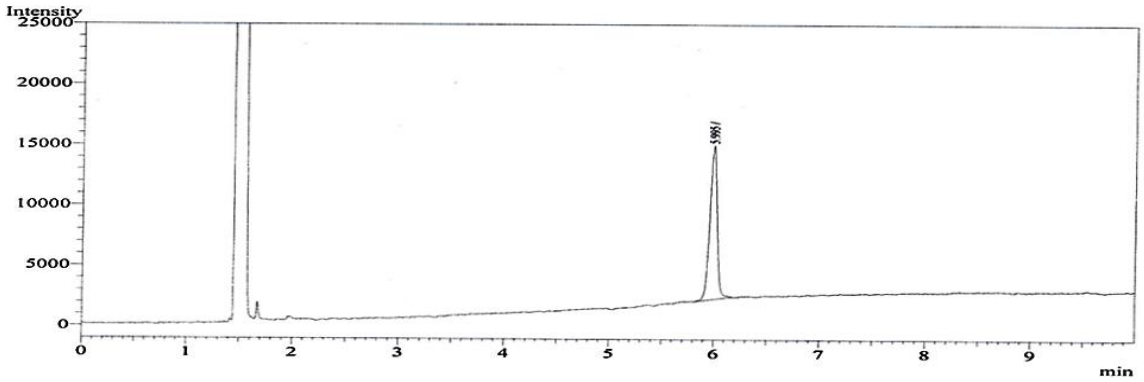
The middle (20 mg L^{-1}) and working standard solution of carbosulfan (5.0, 2.5, 1.25, 0.625, 0.312, 0.156 and 0.10 mg L^{-1}) were made by dilution with n hexane.

2.5.2 Preparation of Calibration Curves of Carbosulfan

The GC system was conditioned at its maximum operating temperature until a smooth base line was obtained. n-Hexane was injected as solvent blank and the retention time of the solvent blank was at 1.5 min (**Figure 33a**). The working standard solutions of 5.0, 2.5, 1.25, 0.625, 0.312, 0.156 and 0.10 mg L^{-1} of carbosulfan were injected gradually into GC-FID from the lowest to the highest concentration level. The retention time of carbosulfan was found to be at 5.9 min (**Figure 33b**). The limit of detection (LOD) and quantification (LOQ) were found out to be at 0.1 and 0.3 mg L^{-1}, respectively for carbosulfan (**Table 18**). From the eight chromatograms of carbosulfan (one for solvent bank and seven concentration levels of carbosulfan) calibration curves were made using MS Excel software by plotting area of the eluted standard vs concentration. Two calibration curves are presented in (**Figure 34**). Linear range of elution and correlation coefficient are given in **Table 18**.

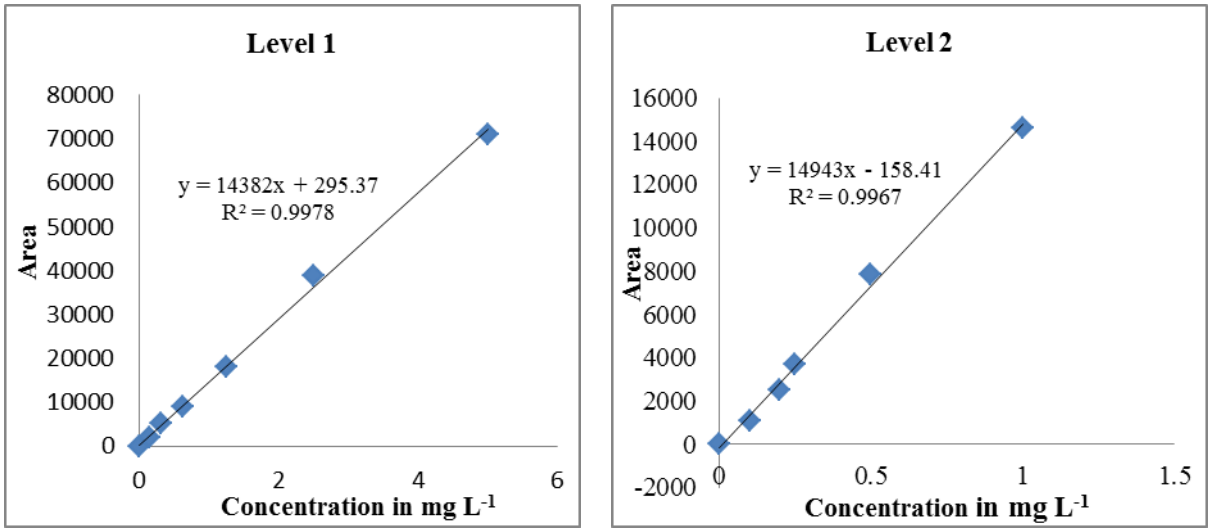


(a)



(b)

Figure 33: GC-FID chromatograms of blank solvent (a) and certified standard carbosulfan (b)



(a) (b)

Figure 34: Calibration curves of carbosulfan in two levels (a and b)

2.5.2.1 Harvesting of Tomato

The tomato samples were randomly harvested from the carbosulfan treated three plots and were kept in plastic polyethylene jip locked bags. The samples were harvested at 0 (2 hours), 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 and 15 days after spray and the harvested samples (~ 1 kg) were kept in chill box and immediately brought to the Laboratory of Chemistry, Dhaka University. The samples were coded as TCS 0 to TCS 15, respectively. Control sample was harvested from the untreated experimental field same way as done for treated sample.

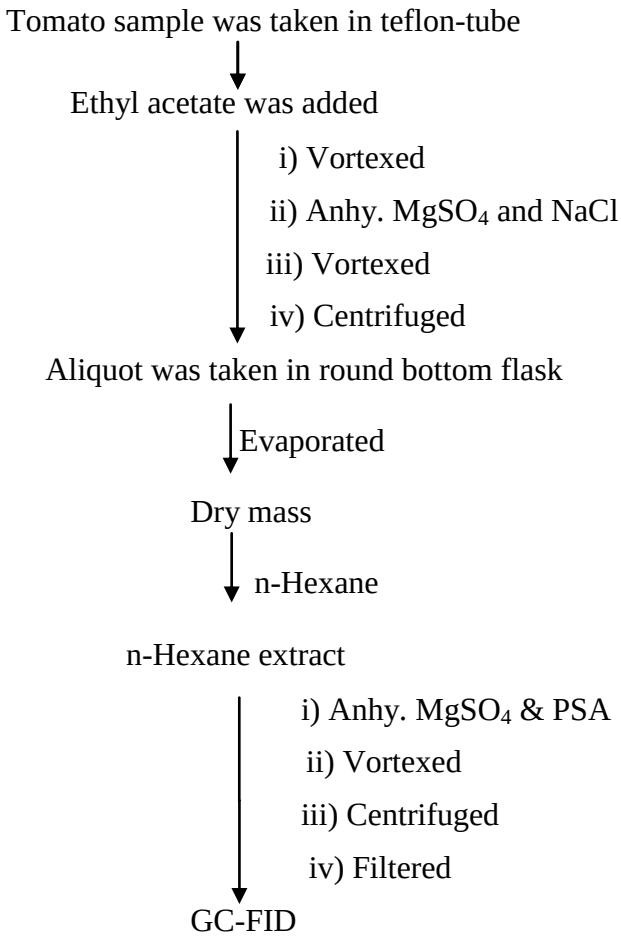
2.5.2.2 Homogenization and Storage of Tomato Sample

The tomato samples were chopped into small pieces and homogenized by a kitchen blender. The homogenized tomato samples (10 g) were kept in a 3x16 screw-cap teflon tubes (50 mL) and stored in a freezer at -20^0 C until analysis.

2.5.2.3 Extraction and Clean-up of Tomato Samples

The homogenized frozen tomato samples (10 g) were taken out from the freezer and thawed at room temperature, 20 mL of ethyl acetate was added to it and vortexed for 2 min. The pre-heated anhydrous magnesium sulphate (6.0 g) and sodium chloride (1.5 g) were added to the tube and vortexed for 1 min and was centrifuged for 5 min at 5000 rpm. From the supernatant 10 mL was transferred into a round bottom flask (100 mL) and evaporated to dryness. The dried material was reconstituted with n-hexane (5 mL) (**Scheme-9**).

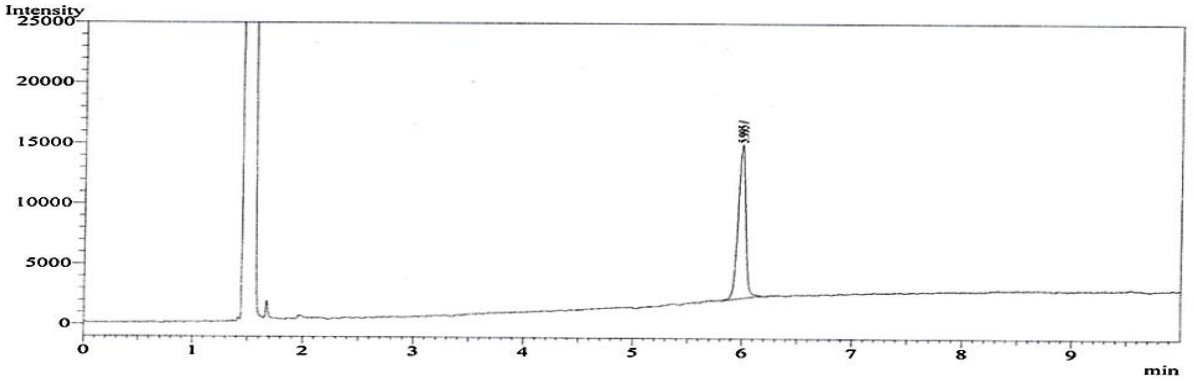
The n-hexane extract (2 mL) was transferred into a screw-cap test tube, 750 mg anhydrous preheated $MgSO_4$ and 150 mg PSA were added to it. The test tube was vortexed for 30 s and centrifuged for 5 min at 5000 rpm. The supernatant was taken and filtered through LC sample filter (0.25 µm) and transferred into vial to analyse by GC-FID having an auto-injector (**Scheme-9**). The results are given in **Table 19**.



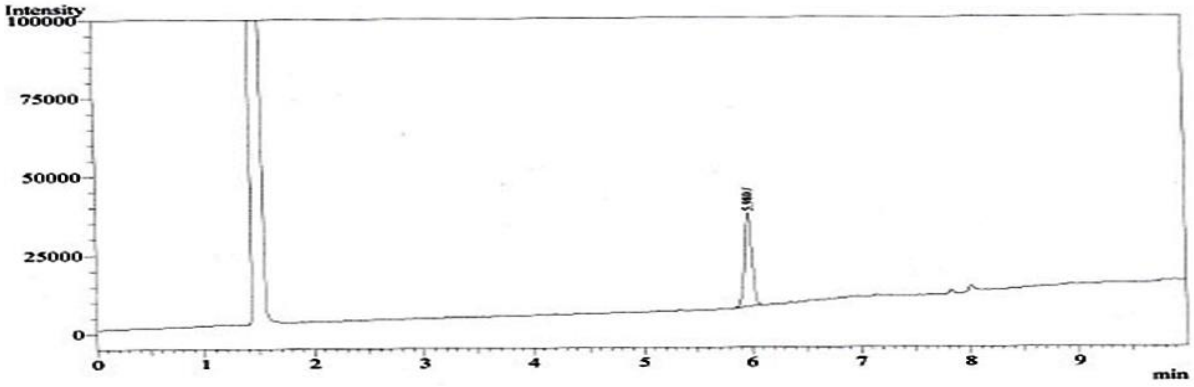
Scheme 9: Extraction and clean-up procedure for tomato samples

2.5.2.4 Analysis of Cleaned Extract of Tomato by GC-FID

The analysis of residue of carbofuran in tomato was done by using gas chromatography (GC) equipped with a flame ionization detector (FID). Nitrogen was used as carrier and makeup gas. Hydrogen and air were used for flame. Separations were performed on HP-5 (30 m long, 0.25 inner diameter & film thickness 0.25 µm) capillary WCOT quartz column. Temperature program was employed as follows: initial temperature of 120 °C; hold for 1 min, increased at 25 °C min^{-1} to 270 °C; post run for 3 min. The detector and injector temperatures were 290 °C and 250 °C, respectively. The split ratio was 70:30 and auto injector was used with injection volume of 1 µL. The injection mode was splitless-split. The chromatograms of standard carbosulfan and tomato extract are given in **Figure 35**.



(a)



(b)

Figure 35: GC-FID chromatograms of standard carbosulfan (a) and tomato extract (b)

2.5.2.5 Control Sample

The control sample of tomato was collected from control field. Homogenization, extraction and clean-up of the control sample were done as **Section 2.5.2.2** and **Scheme 9**. The control sample was stored separately from carbosulfan treated samples in freezer at -20^0 C.

2.5.2.6 Recovery Experiment

The control tomato sample (10 g) was taken into a 50 mL teflon tube and 1 mL of 25 mg L^{-1} solution of carbosulfan was added. The spiked tomato sample was vortexed for 0.5 min and allowed to stay for 30 min at room temperature. Then the samples (5x2) were extracted and

cleaned up by the same procedure as was done for experimental tomato sample (**Scheme 9**). The cleaned extract was diluted 10 times and passed through GC-FID. Five recovery experiments were done at two different spiking concentration levels (2.50 and 4.00 mg kg^{-1}). The results are given in **Table 20**. Standard deviation and relative standard deviation were calculated. Similarly another five recovery experiments were done at 4.00 mg kg^{-1} spiking concentration level by spiking 1 mL of 40 mg L^{-1} of standard carbosulfan, extracted and cleaned following exactly same procedure and analysis were done under same condition. Percent recovery was calculated and the results are given in **Table 20**.

3.3 Results and Discussion

Extensively use of pesticides in modern agriculture to combat plant pests has begun to receive much attention because pesticide residues in food commodities may be hazardous to human health. It is well known that the intervals between application of pesticides and harvest for human consumption are critical period. The residues of such pesticides on and in crop should be estimated to give recommendation about the safety consumable time to avoid such hazards. Among the vegetables grown in Bangladesh, tomato, cauliflower, eggplant and bean are high valued and popular. It has been reported that these vegetable are severely attacked by insect pests and farmers sprayed insecticides quite frequently even every day (Anon., 2000). Therefore, the deposition and residue levels and rates of dissipation of diazinon and carbosulfan insecticides on tomato, cauliflower, eggplant and bean under field conditions were studied. Also the time intervals between application and harvest (waiting time) for human consumption were estimated.

3.3.1 Site Selection

Collaboration was made with Entomology Division, Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur, Dhaka for experimental field **(Figure 36)**. Total twenty plots were selected in BARI for the present studies **(Figure 37)**. Sixteen plots were for the study of dissipation pattern of diazinon in tomato, cauliflower, eggplant and bean and four plots were for the study of dissipation pattern of carbosulfan in tomato. Four plots were for each vegetable (1 was control field and 3 were selected for diazinon/carbosulfan application).

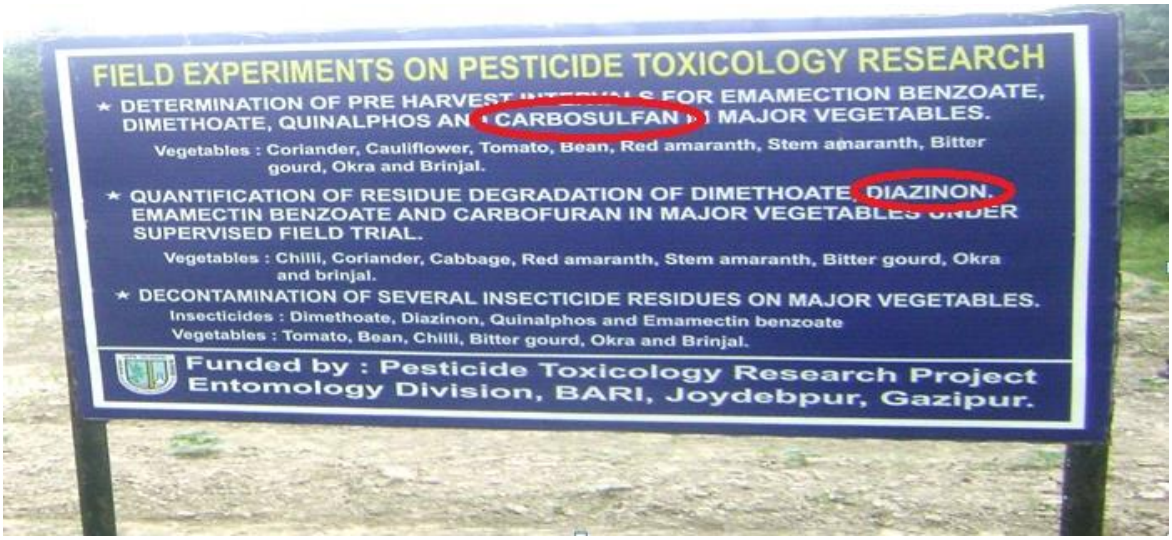


Figure 36: Experimental vegetable field in Bangladesh Agricultural Research Institute

3.3.2 Plantation

The studies on the dissipation of synthetic insecticides namely, diazinon in tomato, cauliflower, eggplant and bean and carbosulfan in tomato were carried out through field experiments during winter season in 2014. Diazinon (Shabion 60 EC) and carbosulfan (Marshal 20 EC) pesticides were considered for application. Tomato, cauliflower, eggplant and bean seed-lings were transplanted on raised beds at the experimental fields of BARI. Irrigation and all cultural practices of selected 20 plots were carried out by technical persons of BARI. Tomato, cauliflower, eggplant and bean were grown in the experimental fields of Agricultural Research Institute (BARI), Joydebpur, Gazipur, Dhaka.

Figure 37: Site selection for **e**xperimental vegetable plot in Bangladesh Agricultural Research Institute

3.3.3 Result of Dissipation Patterns of Diazinon

In this study, samples were extracted by using QuEChERS method (Anastassiades *et al*., 2003) after some modifications as described in the experimental **Section 2.4.3.3** and analysed by GC-ECD. Samples were extracted in three replicates and standard deviations were calculated. The study revealed that under open field conditions, the initial deposits (2 hours after spraying) of diazinon (2 mL /L) were 4.02 ± 0.37, 1.66 ± 0.24, 1.29 ± 0.09 and 0.152 ± 0.007 mg kg^{-1} for cauliflower, tomato, eggplant and bean, respectively. The residues of diazinon in tomato, bean, cauliflower and eggplant were found in the range of 0.02 ± 0.01 to 1.66 ± 0.24, 0.005 ± 0.001 to 0.152 ± 0.007, 0.03 ± 0.01 to 4.02 ± 0.37, and 0.02 ± 0.01 to 1.29 ± 0.09 mg kg^{-1}, respectively.

Table 12: Linear Range, Correlation Coefficient (r^2), LOD and LOQ of Diazinon

Linear range ($mg\ L^{-1}$)	Diazinon		
	r^2	Limit of detection (LOD)	Limit of quantification (LOQ)
		($mg\ L^{-1}$)	
0.001-0.01	0.9922	0.001	0.003
0.001-0.02	0.9967		
0.01-0.20	0.9905		
0.02-0.40	0.9976		

3.3.4 Method Validation

3.3.4.1. Specificity

Diazinon was detected at 7.86 min (retention time) (**Figure 25b**) through the procedure of **Section 2.4.2**. Specificity was confirmed by injecting control vegetable extract and no matrix peaks was found to interfere with the retention time of diazinon.

3.3.4.2. Linearity

Linearity test was made by plotting calibration curves with different standard concentrations (0.5, 0.2, 0.1, 0.05, 0.02, 0.01, 0.005, 0.002 and 0.001 µg mL^{-1}) versus the respective area of the peaks obtained. The linear regression equations were obtained with r^2 0.922, 0.9967, 0.9905 and 0.9976.

Table 13: Residues of Diazinon in Tomato Matrix at Various Time Intervals

Sample code	Time interval / Day	Average amount ± SD (mg kg^{-1}; n = 3)	Dissipation (%)
TDN 0	0	1.66 ± 0.24	0
TDN 1	1	0.84 ± 0.06	49
TDN 2	2	0.66 ± 0.02	60
TDN 3	3	0.46 ± 0.07	72
TDN 4	4	0.42 ± 0.01	75
TDN 5	5	0.35 ± 0.02	79
TDN 6	6	0.29 ± 0.02	82
TDN 7	7	0.17 ± 0.01	89
TDN 8	8	0.12 ± 0.01	92
TDN 9	9	0.09 ± 0.01	94
TDN 10	10	0.08 ± 0.01	95
TDN 11	11	0.05 ± 0.01	97
TDN 12	12	0.04 ± 0.01	98
TDN 13	13	0.03 ± 0.01	98
TDN 14	14	0.02 ± 0.01	99
TDN 15	15	BDL*	

*BDL – Below Detection Limit

3. 3.4.3. Detection and Quantification Limit

The limit of detection (LOD) and limit of quantification (LOQ**)** were determined by dilution of working standard solution of diazinon with n-hexane and injected up to the concentration where the peak heights of the standards were same to the noise level. The limit of detection (LOD) was determined using a signal-to-noise ratio of 3 with reference to the background noise obtained for the blank sample and the limits of quantification (LOQ) were determined with a signal-to-noise ratio of 10. The limit of detection and the limit of quantification were found to be 0.001 and 0.003 mg L^{-1}, respectively (**Table 12**).

3.3.4.4. Precision, Repeatability, and Recovery Percentage

The mean recovery percentage was found to be 99 to 105 % for tomato at 0.02, 0.50 and 1.50 mg kg^{-1} spiking concentration levels. For cauliflower the mean recovery at 0.05, 2.00 and 4.00 mg kg^{-1} spiking levels was 97 to 104%. The recoveries at 0.005, 0.01 and 0.15 mg kg^{-1} spiking levels were 89 to 108 % for bean. For eggplant, the mean recovery was 93 to

104 % at 0.02, 0.50 and 1.00 mg kg^{-1} spiking levels. The standard deviations of recovery percentages for four vegetable were in the range of 0.01 to 0.17 % and thus the repeatability of the method was excellent.

Table 14: Residues of Diazinon in Cauliflower Matrix at Various Time Intervals

Sample code	Time interval / Day	Average amount ± SD (mg kg^{-1}; n = 3)	Dissipation (%)
CDN 0	0	4.02 ± 0.37	0
CDN 1	1	3.76 ± 0.63	6
CDN 4	4	3.05 ± 0.42	24
CDN 5	5	2.14 ± 0.09	46
CDN 6	6	1.87 ± 0.18	53
CDN 9	9	0.57 ± 0.07	71
CDN 10	10	0.48 ± 0.03	88
CDN 11	11	0.30 ± 0.01	92
CDN 12	12	0.28 ± 0.01	93
CDN 13	13	0.11 ± 0.01	97
CDN 14	14	0.08 ± 0.01	98
CDN 15	15	0.03 ± 0.01	99

3.3.4.5 Residue of Diazinon in Tomato, Cauliflower, Eggplant and Bean and Waiting Period and Half-life

The maximum residue limit (MRL) of diazinon on cauliflower, tomato, eggplant and bean has been fixed by CODEX is 0.5 mg kg^{-1}. The diazinon residues declined to a level below the maximum residue limits within 3, 3 and 10 days for eggplant, tomato and cauliflower, respectively. The residue of diazinon was below the maximum residue limit even at 0 day (two hours after spraying) for bean. The waiting periods for safe consumption of cauliflower, tomato, bean and eggplant are 10, 3, 0 and 3 days, respectively.

Table 15: Residues of Diazinon in Eggplant Matrix at Various Time Intervals

Sample code	Time interval / Day	Average amount ± SD (mg kg^{-1}; n = 3)	Dissipation (%)
EPDN 0	0	1.29 ± 0.09	0
EPDN 1	1	0.83 ± 0.05	35
EPDN 2	2	0.52 ± 0.01	60
EPDN 3	3	0.14 ± 0.02	89
EPDN 4	4	0.09 ± 0.01	93
EPDN 5	5	0.02 ± 0.01	99
EPDN 6	6	BDL*	
EPDN 7	7	BDL	
EPDN 8	8	BDL	
EPDN 9	9	BDL	
EPDN 10	10	BDL	
EPDN 11	11	BDL	

*BDL – Below Detection Limit

3.3.4.5.1 Half-life of Diazinon on Tomato

The degradation kinetics of diazinon in tomato was determined by plotting residue concentration against time (**Figure 38**). The dissipation of diazinon residues on tomato was followed first-order kinetics. Confirmation of the first-order kinetics was made graphically from the linearity of the plots of residue against time. The persistence of this pesticide is expressed in terms of half-life $t_{1/2}$, i.e., time for disappearance of pesticide to 50 % of its initial concentration. Half-life ($t_{1/2}$) of the total diazinon residues was observed to be 2.23 days.

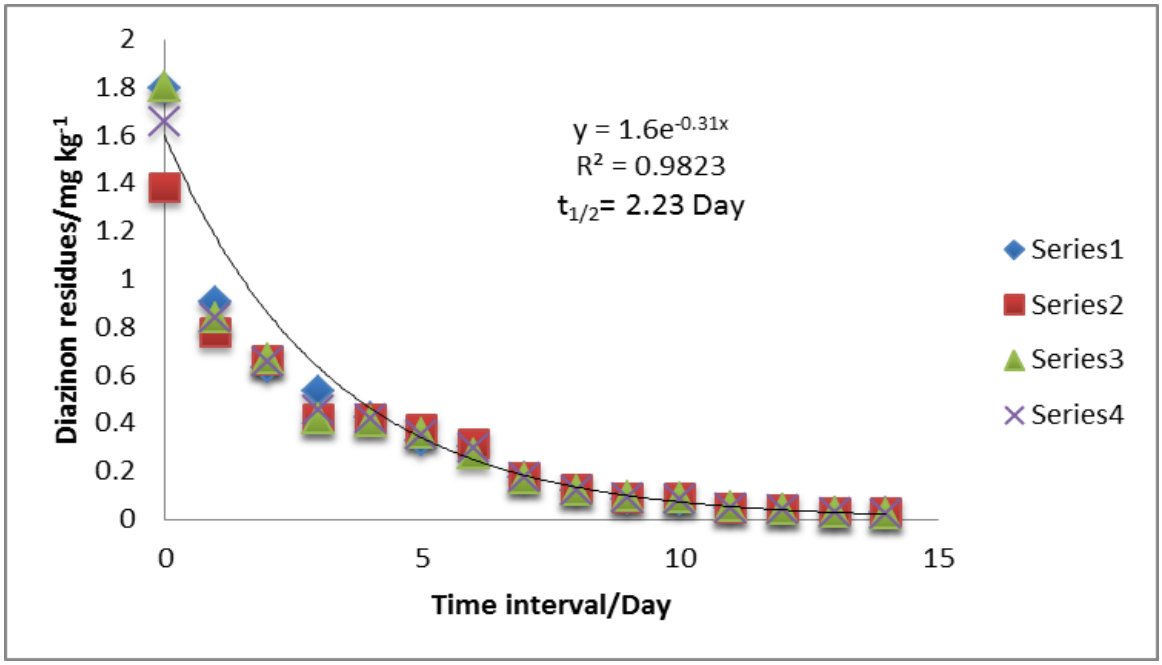


Figure 38: Dissipation curve of diazinon in tomato

3.3.4.5.2 Half-life of Diazinon on Cauliflower

The degradation kinetics of diazinon in cauliflower was determined by plotting residue concentration against time (**Figure 39**). The dissipation of diazinon residues on cauliflower was followed first-order kinetics. Confirmation of the first-order kinetics was made graphically from the linearity of the plots of residue against time. The persistence of this pesticide is expressed in terms of half-life $t_{1/2}$, i.e., time for disappearance of diazinon to 50 % of its initial concentration. Half-life ($t_{1/2}$) of the total diazinon residues was observed to be 2.63 days.

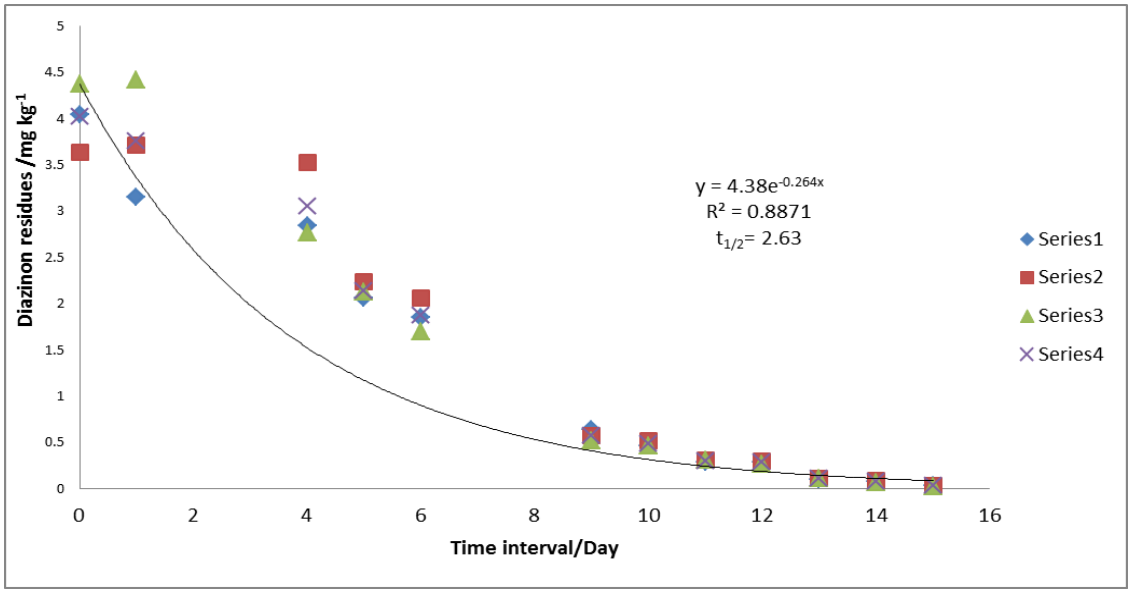


Figure 39: Dissipation curve of diazinon in cauliflower

Table 16: Residues of Diazinon in Bean Matrix at Various Time Intervals

Sample code	Time interval / Day	Average amount ± SD (mg kg^{-1}; n = 3)	Dissipation (%)
BDN 0	0	0.152 ± 0.007	0
BDN 1	1	0.052 ± 0.007	66
BDN 2	2	0.046 ± 0.002	70
BDN 3	3	0.036 ± 0.002	76
BDN 4	4	0.032 ± 0.004	79
BDN 5	5	0.022 ± 0.003	85
BDN 6	6	0.015 ± 0.001	90
BDN 7	7	0.012 ± 0.002	92
BDN 9	9	0.009 ± 0.001	94
BDN 10	10	0.007 ± 0.001	95
BDN 11	11	0.006 ± 0.001	96
BDN 12	12	0.005 ± 0.001	97
BDN 13	13	BDL*	
BDN 14	14	BDL	
BDN 15	15	BDL	

*BDL – Below Detection Limit

3.3.4.5.3 Half-life of Diazinon on Eggplant

The degradation kinetics of diazinon in eggplant was determined by plotting residue concentration against time (**Figure 40**). The dissipation of diazinon residues on eggplant was followed first-order kinetics. Confirmation of the first-order kinetics was made graphically from the linearity of the plots of residue against time. The persistence of this pesticide is expressed in terms of half-life $t_{1/2}$, i.e., time for disappearance of diazinon to 50 % of its initial concentration. Half-life ($t_{1/2}$) of the total diazinon residues was observed to be 0.90 days.

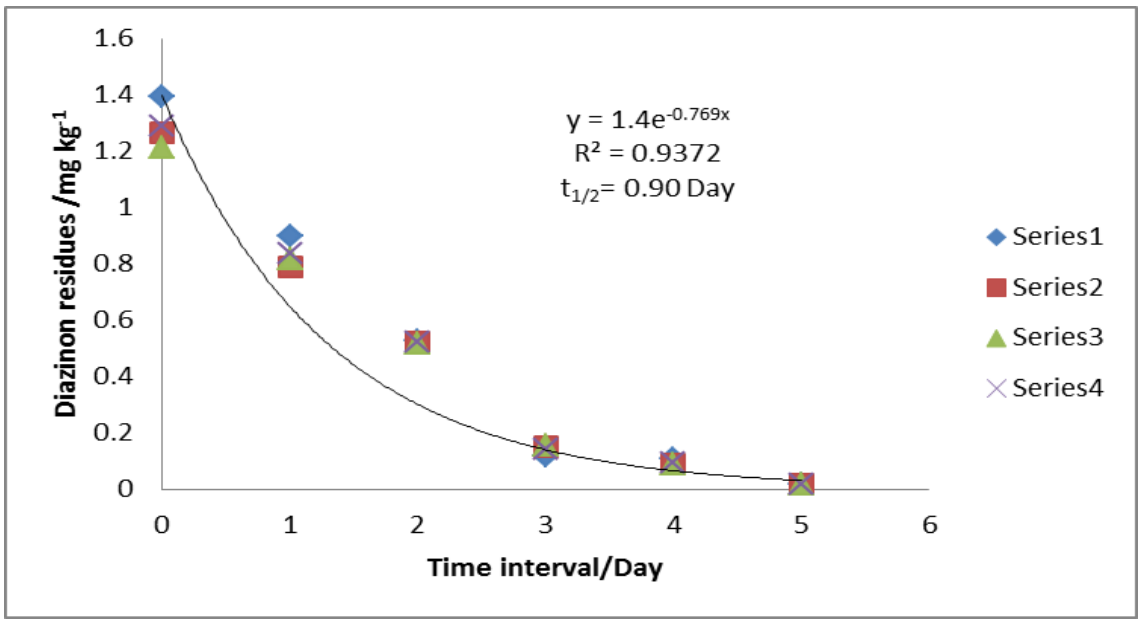


Figure 40: Dissipation curve of diazinon in eggplant

3.3.4.5.4 Half-life of Diazinon on Bean

The degradation kinetics of diazinon in bean was determined by plotting residue concentration against time (**Figure 41**). The dissipation of diazinon residues on bean was followed first-order kinetics. Confirmation of the first-order kinetics was made graphically from the linearity of the plots of residue against time. The persistence of this pesticide is expressed in terms of half-life $t_{1/2}$, i.e., time for disappearance of diazinon to 50 % of its initial concentration. Half-life ($t_{1/2}$) of the total diazinon residues was observed to be 1.12 days.

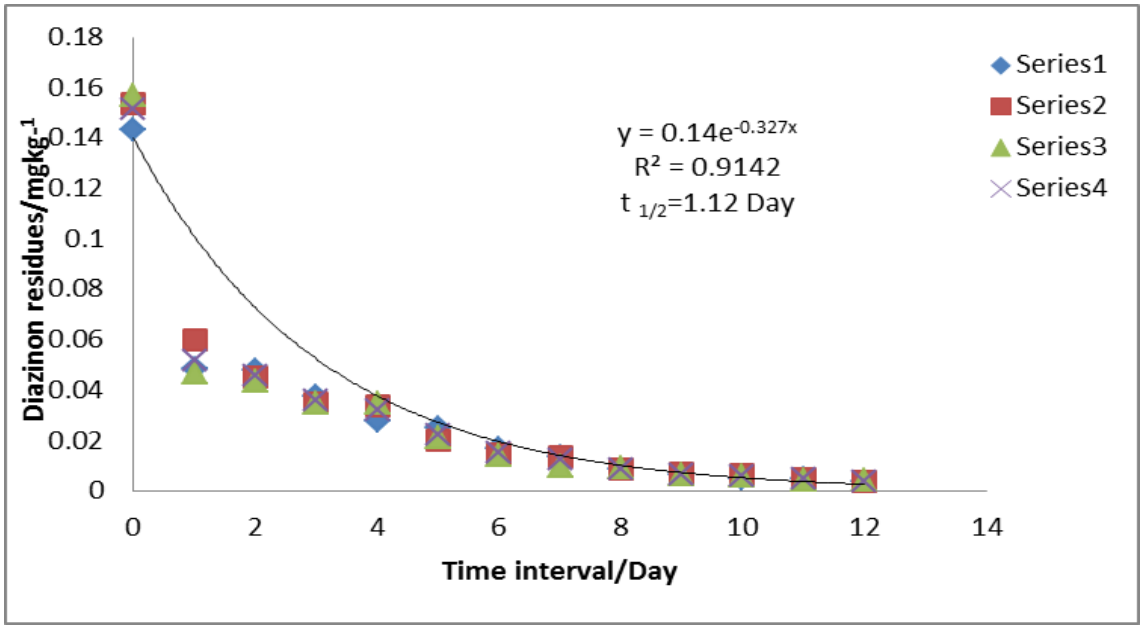


Figure 41: Dissipation curve of diazinon in bean

Table 17: Recovery Results of Diazinon Residues on Tomato, Cauliflower, Bean and Egg plant

Pesticide/ Vegetable	Spiking level (mg kg^{-1})	Recovery (%)	Average accuracy ± SD* (%)	Precision (RSD** %)
Diazinon in tomato (n = 4)	0.02	96.96, 95.05, 103.24, 100.92	99.04 ± 0.01	3.75
	0.50	105.62, 109.16, 100.48, 105.83	105.27 ± 0.02	3.40
	1.50	107.76, 111.89, 101.95, 98.50	105.03 ± 0.09	5.67
Diazinon in Cauliflower (n = 4)	0.05	108.93, 100.23, 99.14, 108.87	104.29 ± 0.01	5.12
	2.00	102.63, 99.03, 96.72, 98.56	99.24 ± 0.05	2.49
	4.00	104.02, 96.24, 95.37, 94.93	97.64 ± 0.17	4.39
Diazinon in Bean (n = 4)	0.005	91.64, 97.46, 75.93, 94.24	89.82 ± 0.01	10.64
	0.01	110.23, 117.16, 102.35, 100.89	107.66 ± 0.01	7.01
	0.15	114.58, 94.98, 110.95, 111.49	108.00 ± 0.01	8.17
Diazinon in Eggplant (n = 4)	0.02	100.18, 107.33, 106.45, 105.78	104.94 ± 0.01	3.08
	0.50	103.16, 102.67, 100.92, 99.45	101.55 ± 0.01	1.68
	1.00	93.02, 95.24, 93.39, 104.46	93.88 ± 0.05	5.57

*SD = Standard deviation and **RSD = Relative standard deviation

3.3.4.6 Dissipation Studies

The modified QuEChERS method gave an excellent recovery within the limit of 99 to 105 % for tomato, 97 to 104 % for cauliflower, 89 to 108 % for bean and 93 to 104 % for eggplant with relative standard deviations in the range of 1.68 to10.64 %. The method was evaluated by doing recovery experiments with four replicates in three concentration levels. The dissipation of pesticide residues in/on crops depends on the climatic conditions, type of application, plant species, dosage, the interval between application and harvest. The present study was done in winter vegetable of Bangladesh. Diazinon residues in tomato, cauliflower, eggplant and bean after each treatment are shown in **Table 13, 14, 15 and 16**. The dissipation of diazinon was 100% at 15, 14, 12 and 5 days for cauliflower, tomato, bean and eggplant samples, respectively.

The results can be compared with the published result of dissipation of diazinon in eggplant (Khabir *et al*, 2008). The initial residue of diazinon in eggplant was 1.29 ± 0.09 mg kg^{-1} and was above MRL value up to 2 days and half-life was 0.90 days. In Khabir *et al.* studies the initial residue of diazinon in eggplant was 2.23 mg kg^{-1} and was above MRL value up to 3 days. Another study of dissipation of diazinon in chinese cabbage was done under green house (Sathya *et al.*, 2006) but the present study was done in open field and dissipation patterns of pesticide residues in the crop under greenhouse conditions are quite different from those in the open-air. In another report (Adnan *et al*., 1987) it was found that diazinon residue was above MRL upto 8 days in sweet pepper grown in green house. Residue of diazinon in plants, in animal tissues, or even in the soil is not highly persistent (FAO-WHO, 1975). From this study, it was revealed that residue of diazinon in eggplant, bean, tomato and cauliflower could be detected up to 5, 12, 14 and 15 days, respectively. These results were more or less agreed with the observation reported by Geigy (Geigy, 1956-67). He reported that the diazinon residue level after spraying the field dose, were < 0.1 mg kg^{-1} in cabbage at 7 days, 0.4 mg kg^{-1} in cauliflower at 5 days and < 0.1 mg kg^{-1} in cucumber at 7 days.

The residue of diazinon at 0 day (**Figure 42)** was found to be followed the following order:

Cauliflower (4.02 ± 0.37 mg kg^{-1}) > Tomato (1.66 ± 0.24 mg kg^{-1}) > Eggplant(1.29 ± 0.09 mg kg^{-1}) > Bean (0.152 ± 0.007mg kg^{-1})

In cauliflower, the pesticide was reached the target point properly. Cauliflower is growing in upper direction and its surface is large and not smooth. So, the pesticide can accommodate easily. But the skin of both eggplant and tomato is smooth compare to cauliflower. Eggplant and tomato are growing in down direction. The skin of eggplant is more thick and hard and its leaves are also large compare to tomato. Thus the residue was higher in tomato than eggplant. In bean, the pesticide was reached the target point less properly compare to other three vegetables.

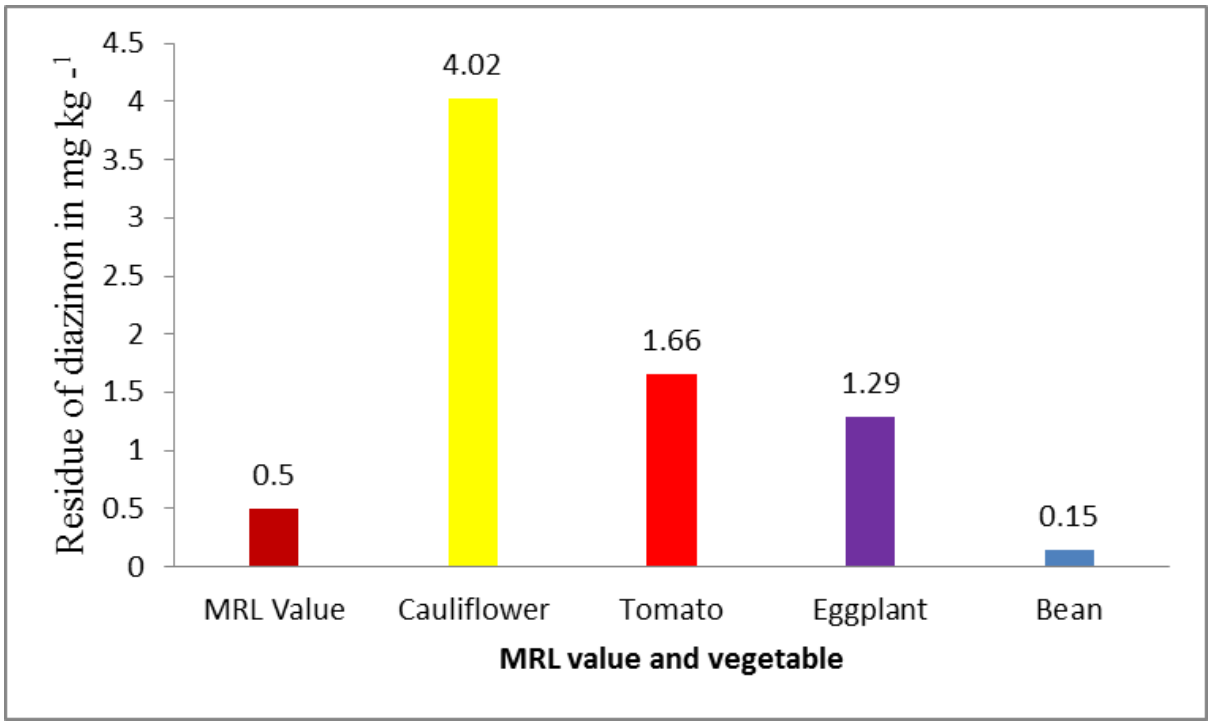


Figure 42: The MRL value and the residue of diazinon in cauliflower, tomato, eggplant and bean samples at 0 day (2 hours after spraying**)**

Pesticide residues in food pose a significant health effect. To provide adequate food for growing population, the usage of pesticide is necessary but dissemination of information regarding food safety, pesticide handling and good agricultural practices (GAP) among farmers is also a dire need. Moreover, good agricultural practice is the important and effective tools in minimizing pesticide residues in food commodities. In Bangladesh Ministry of Agriculture, it was found that the referred dose of diazinon for eggplant is 1mL L^{-1} but no dose was fixed for tomato, bean and cauliflower. Therefore, this study will provide adequate

information for safe harvesting period and required dose of diazinon for tomato, eggplant, bean and cauliflower growing under open field conditions.

3.3.5.1 Result of Dissipation Pattern of Carbosulfan in Tomato Samples

The study of dissipation of carbosulfan residues on tomato was carried out in open field condition. The samples were prepared as **Section 2.5.2.3** and was analysed as **Section 2.5.2.4**. The initial deposits of carbosulfan was found to be 9.43 ± 0.16 mg kg^{-1} at 2 hours after spraying, which degraded to BDL by 15th day (**Table 19**). The initial deposits dissipated to 6.17 ± 0.07, 5.75 ± 0.37, 4.90 ± 0.29, 4.45 ± 0.02, 4.33 ± 0.41, 4.05 ± 0.20, 3.86 ± 0.24, 2.96 ± 0.47, 2.25 ± 0.29, 2.04 ± 0.24, 1.95 ± 0.26, 1.68 ± 0.25 and 1.19 ± 0.06 mg kg^{-1}, at 1, 2, 3 ,4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14 days after spraying, respectively. The dissipation pattern showed constant decrease of residues from first day to 14th day (**Figure 43**).

3.3.5.2 Limit of Detection (LOD) and Limit of Quantification (LOQ) of Carbosulfan

The limit of detection (LOD) was determined using a signal-to-noise ratio of 3 with reference to the background noise obtained for the blank sample and the limits of quantification (LOQ) were determined with a signal-to-noise ratio of 10. The limit of detection and the limit of quantification were found to be 0.10 and 0.30 mg L^{-1}, respectively (**Table 18**).

Table 18: Linear Range, Correlation Coefficient (r^2), LOD and LOQ of Carbosulfan

Linear range (mg L^{-1})	Carbosulfan		
	r^2	Limit of Detection (LOD)	Limit of Quantification (LOQ)
		(mg L^{-1})	
0.10-1.25	0.9967	0.10	0.30
0.10-5.00	0.9978		

The LOD and LOQ were 0.10 and 0.30 mg kg^{-1}, respectively (**Table 18**). Calibration curves were linear over the calibration ranges with coefficient of determinants 0.9978 and 0.9967 for carbosulfan (**Figure 34**). According to Europion Union, the MRL value of carbosulfan in tomato is 0.05 mg kg-^{1}. The residue of carbosulfan in tomato was found to be above the MRL value (0.05 mg kg^{-1}) up to 14 days (9.43 ± 0.16 to 1.19 ± 0.06 mg kg^{-1}).

Table 19: Residues of Carbosulfan in Tomato Matrix at Various Time Intervals

Sample code	Time interval / Day	Average amount ± SD (mg kg^{-1}; n = 3)	Dissipation (%)
TCS 0	0	9.43 ± 0.16	0
TCS 1	1	6.72 ± 0.45	29
TCS 2	2	6.17 ± 0.07	35
TCS 3	3	5.75 ± 0.37	39
TCS 4	4	4.90 ± 0.29	48
TCS 5	5	4.45 ± 0.02	53
TCS 6	6	4.33 ± 0.41	54
TCS 7	7	4.05 ± 0.20	57
TCS 8	8	3.86 ± 0.24	59
TCS 9	9	2.96 ± 0.47	69
TCS 10	10	2.25 ± 0.29	76
TCS 11	11	2.04 ± 0.24	78
TCS 12	12	1.95 ± 0.26	79
TCS 13	13	1.68 ± 0.25	82
TCS 14	14	1.19 ± 0.06	87
TCS 15	15	BDL*	100

*BDL – Below Detection Limit

3.3.5.3 Repeatability and Recovery Percentage

For the study of repeatability and extraction efficiency, control tomato samples (10 g) were spiked with 1 mL of certified standard carbosulfan of 25 and 40 mg L^{-1} concentration levels with five replications for each. The mean recovery of carbosulfan in tomato with five replications was 98 to 102 % at 2.50 and 4.00 mg kg^{-1} spiking levels. The standard deviations of recovery percentages were 0.25 to 0.35 %. The relative standard deviation was 10.15 and 8.64 % (**Table 20**).

Table 20: Recovery Result of Carbosulfan Residues on Tomato

Pesticide/ Vegetable	Spiking level (mg kg^{-1})	Average accuracy ± SD (%)	Precision (RSD %)
Carbosulfan in tomato (n = 5)	2.50	98.52 ± 0.25	10.15
	4.00	102.03 ± 0.35	8.64

*SD = Standard deviation and **RSD = Relative standard deviation

3.3.5.4 Half-life of Carbosulfan on Tomato

The degradation kinetics of carbosulfan in tomato was determined by plotting residue concentration against time (**Figure 43**). The dissipation of carbosulfan residues on tomato was followed first-order kinetics. Confirmation of the first-order kinetics was made graphically from the linearity of the plots of residue against time. The persistence of this pesticide is expressed in terms of half-life $t_{1/2}$, i.e., time for disappearance of pesticide to 50 % of its initial concentration. Half-life ($t_{1/2}$) of the total carbosulfan residues was observed to be 5.25 days.

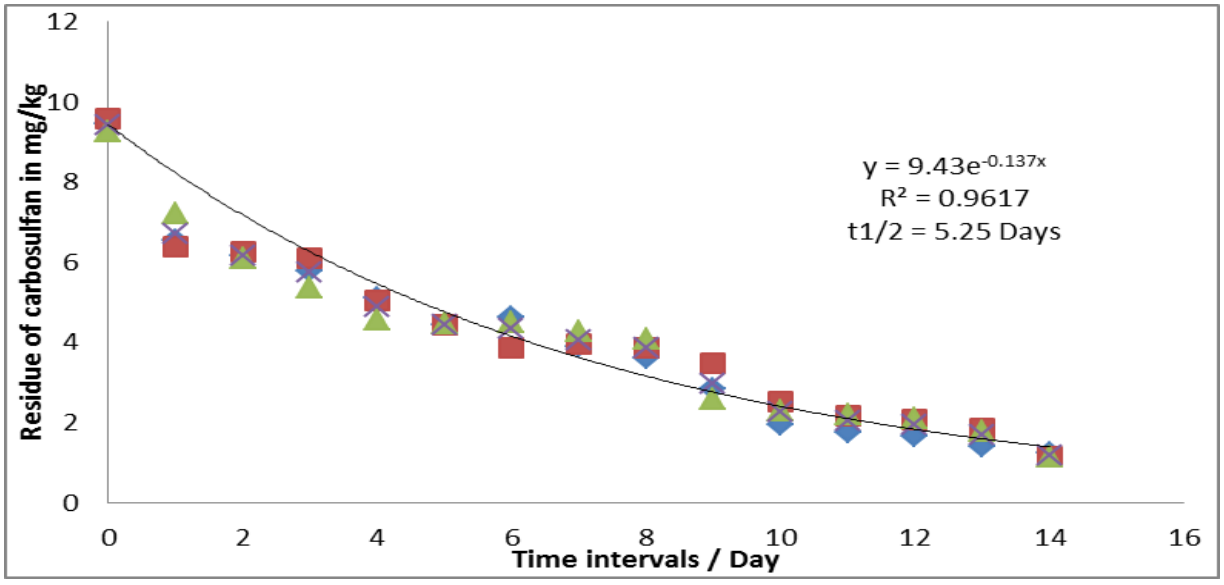


Figure 43: Dissipation curve of carbosulfan in tomato

3.3.5.5 Discussion

The initial deposit of carbosulfan in tomato as determined two hours after treatment was 9.43 ± 0.16 mg kg^{-1}. This amount was dropped to 6.72 ± 0.45 mg kg^{-1} indicating 29 % loss after one day from application. Then the residues of carbosulfan were decreased gradually and at 14 days after application of carbosulfan, the residue was found to be 1.19 ± 0.06 mg kg^{-1} indicating 87 % loss (**Table 19**).

The extraction efficiency was evaluated by recovery experiment. The mean recovery of carbosulfan in tomato was 98 to 102 % at 2.50 and 4.00 mg kg^{-1} spiking levels with five replications (**Table 20**). The standard deviations of recovery percentages were 0.25 to 0.35 %; thus the repeatability of the method was excellent. The relative standard deviation was 10.15 and 8.64 % mean excellent efficiency of the method.

According to Bates (1979) data on residues of pesticides in treated crops are required for the premarket registration of pesticides and for setting maximum residues limits (toxicologically acceptable level) to protect the consumer against the possible health hazards of exposure to pesticides. The maximum residue limits (MRLs) for carbosulfan in tomato is 0.05 mg kg^{-1} (Codex Alimentarius Commission 2004). According to the maximum residue limits (MRLs), and the determined residues of these insecticides in tomato, the approximate waiting time value (preharvest interval) for carbosulfan residues in tomato could not be determined because the insecticide residues through the sampling period were exceeded the Codex MRL value up to 14 days .

It is clear from the present study that the initial deposits of carbosulfan on tomato was high and the calculated half-life value ($t_{1/2}$) for carbosulfan in tomato was 5.25 days (**Figure 43**). The ratio of the leaf surface to its weight is high enough to result in receiving higher initial deposits of the applied pesticide (Ahmed *et al*. 1991 and Dogheim 1966). The influence of plant varieties on the deposition and dissipation of pesticides was discussed by Lee and Cheng (1983).

El-Sayed *et al*. (1976) stated that the amounts of deposits depended on the rate of application, the nature of the treated surface and the relation between the treated surface and its weight.

The degradation and disappearance of carbosulfan may be due to many factors such as weathering, metabolic conversions or other degradation processes. There were many factors, including plants, pesticides, and environments, which affected the dissipation of pesticides on crops. Christensen (2004) reported that the decline of pesticides may be due to biological, chemical or physical processes, or if still in the field, due to dilution by growth of the crop. Besides, plant growth is also responsible to certain extent for decreasing the pesticide residue concentrations due to growth dilution effect (Walgenbah *et al*., 1991). In addition, the rapid dissipation of originally applied pesticide is dependent on a variety of environmental factors such as sunlight and temperature (Lichtenstein, 1972). However, high temperature is reported to the major factor in reducing the pesticides from plant surfaces (Awad *et al*., 1967). Light plays an important role in the behaviour of pesticide in the environment (Zepp and Cline, 1977). The fate of carbosulfan has been investigated in plants and reported by FAO/WHO (1984). In general carbosulfan, carbofuran, 3- hydroxycarbofuran and 3-ketocarbofuran are the principle carbamate residues in plants with relative amounts varying from crop to crop and with time. The persistence of carbosulfan and its cholinesterase inhibiting metabolites on apple leaves after three foliar sprays each of 1.1 kg a.i./ha was investigated by Leppert (1982). Residues of 3-hydroxycarbofuran were approximately one-hundredth and one-tenth of the carbosulfan and carbofuran residues, respectively, after 1 day from application and were at about the same level (0.03 mg / cm^2) on day 21, residues of 3-ketocarbofuran were ≤ 0.01 mg/kg throughout the 21day study. In plants carbosulfan is typically the same parent compound at or near the last day of application, while thereafter carbofuran and/or 3-hydroxy-carbofuran tend to be the predominant carbamate residues in many commodities, the latter primarily as a conjugate, conjugation increases with time (FAO/WHO, 1984).

Residues of carbosulfan detected in tomato at 0 to 14 days, when these pesticides were applied on the vegetative parts of tomato plants are evidence that penetrate movement and translocated carbosulfan, through plant tissues. Several investigators had studied the absorption and translocation of carbosulfan through plant tissues after application. Umetsu *et al*., (1979) found that carbonyl-14C-labelled carbosulfan residues remained at point of application (up to 9 days) when the carbosulfan was applied near the tips of corn and cotton leaves, but were translocated to the whole leaf within 24 hour when it was applied to the base of a cotton leaf. In whole-plant autoradiography studies (Capps, 1980) of carbofuran showed the same movement with residues concentrating in leaf tips and roots after soil treatment with 14C-ring-labelled compounds.

From literature survey, it was found that the dissipation of carbosulfan in eggplant was studied by Kabir *et al* (2008). Carbosulfan (Sunsalfan 20 EC) @1.5 mL L^{-1} of water was used in open field condition. Carbosulfan residue in the eggplant was 0.88 mg kg^{-1}at 0 day. The dissipation was studied up to 7 days and the quantities were over MRL up to 3 days. Trevisan *et al.* (2004) reported that the residue level of carbosulfan in citrus decreased rapidly and being not found in samples after 7 days. In another study, Varca *et al.* (1998) reported that in rice leaves, carbosulfan residue lasted till 7 days.

The results of the study indicated that carbosulfan applied to tomato under open field condition required longer pre-harvest interval to allow its residues to dissipate to the safe level. To avoid the longer interval the dose 1.5 mL L^{-1} used in the experimental field should be reduced.

PART-D

Analysis of Pesticide Residues in Four Different Vegetable Samples from Market

2.5 Experimental

2.5.1 Collection of Vegetable Samples

Four different vegetable namely bean, eggplant, cauliflower and tomato samples (n = 45) were purchased from the different markets of Dhaka City and Noakhali and Kurigram districts of Bangladesh during 7 to 26 April 2016. All the samples were kept in zip locked plastic bags; labelled then carried to the laboratory in chill box (**Figure 44**). The samples were chopped into small pieces and homogenized by a kitchen blender. The homogenized samples (10 g) were kept in a 3x45 screw-cap teflon tubes (50 mL) and stored in a freezer at -20^0 C until analysis. Name of the vegetable samples with Code No., place and date of collections are presented in **Table 21, 22, 23 & 24** for tomato, cauliflower, eggplant and bean samples, respectively.

Figure 44: Labeling of market vegetable samples

2.5.2 Preparation of Standard Solutions (Chlorpyrifos, Cypermethrin, Diazinon, Fenvalerate and Quinalphos)

The primary standard solutions (100 µg mL^{-1}) of chlorpyrifos, cypermethrin, diazinon, fenvalerate and quinalphos were separately prepared by dissolving standard reference sample in n-hexane. All the prepared solutions in 100 mL amber bottles were labelled indicating name of each of the standard, concentration and the date of preparation. The meniscuses of the solutions were marked with permanent black ink and stored in the freezer (-20 °C) away from the sample storing area until further use.

Mixture of chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos

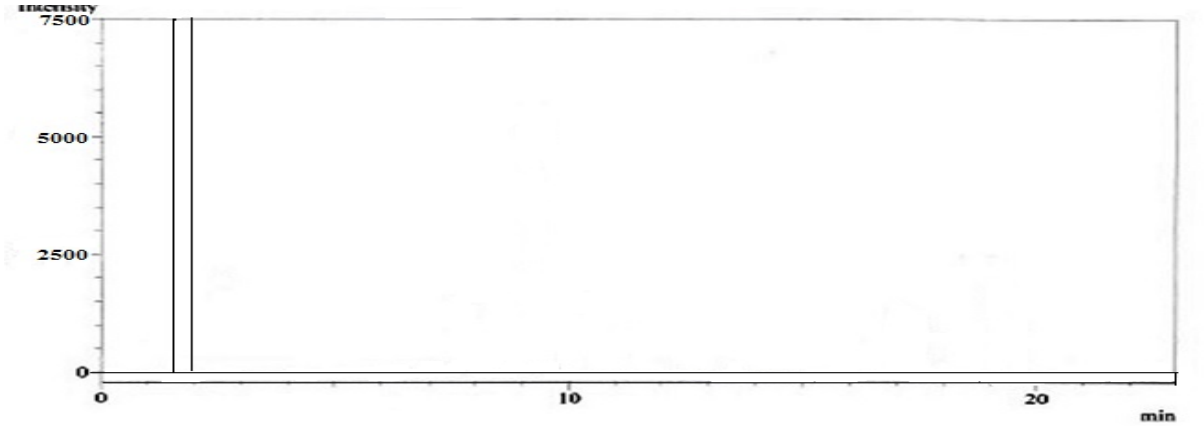
The middle (20 µg mL^{-1}) standard solution mixture of chlorpyrifos, cypermethrin, diazinon , fenvalerate, and quinalphos were made by taking 20 mL of primary standard solution (100 µg mL^{-1}) from each five and then mixture of working standard solutions (0.25, 0.10, 0.025, 0.010, 0.005, 0.0025 and 0.0010 µg mL^{-1}) were made with n-hexane. All solutions were labelled with permanent ink indicating name of the standard, concentration and date of preparation and stored in a freezer (-20 °C) away from the pesticide residue laboratory.

2.5.3 Analytical Conditions of Gas Chromatograph-Electron Capture Detector (GC-ECD)

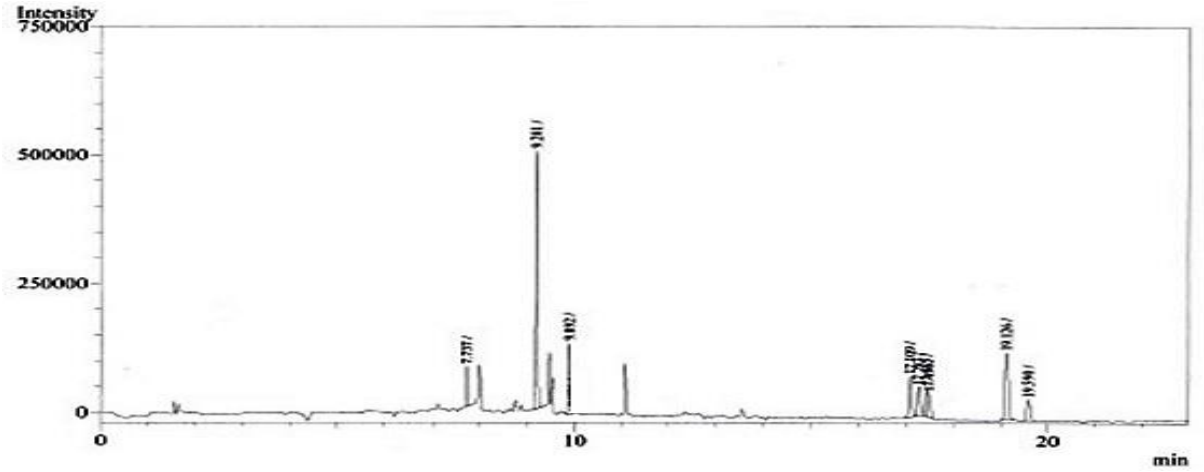
Quantification of residue of chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos were done on a gas chromatograph (GC-2010 Shimadzu) equipped with ^{63}Ni Electron Capture detector (ECD). Nitrogen was used as carrier and make up gas. Separations were performed on Non-polar (HP-5 MS) capillary column of 30 m long x 250 µm i.d. x 0.25 µm film thicknesses from Agilent, USA. Auto injector was used for the determination of the pesticides residue in the vegetable samples. Temperature was programed at 120ºC; hold for 1 min, rise at 20 °C min^{-1} to 220 °C (0 min); rise at 10 °C min^{-1} to 270 °C. The injector and detector temperatures were set at 220 and 290 °C, respectively. The column flow rate was set as 1mL min^{-1}. All injections were made in splitless-split mode with 1 µL injection volume.

2.5.4 Preparation of Calibration Curves

Carrier gas was passed until smooth base line was obtained. n Hexane was injected as solvent blank and the retention time of the solvent blank was at 1.8 min (**Figure 45a**). The working standard solutions of 0.25, 0.10, 0.025, 0.010, 0.005, 0.0025 and 0.0010 mg L^{-1} of mixture of chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos were injected gradually into GC-ECD from the lowest to the highest concentration level.



(a)



(b)

Figure 45: Chromatograms of solvent blank (a) and standard mixture of chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos (b)

The peaks of diazinon, chlorpyrifos, quinalphos, cypermethrin and fenvalerate were obtained at 7.7, 9.2, 9.9, 17.5(four peaks) and 19.5(two peaks) min, respectively (**Figure 45b**). From the eight chromatograms of mixture of chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos (one for solvent bank and seven concentration levels of mixture of chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos).

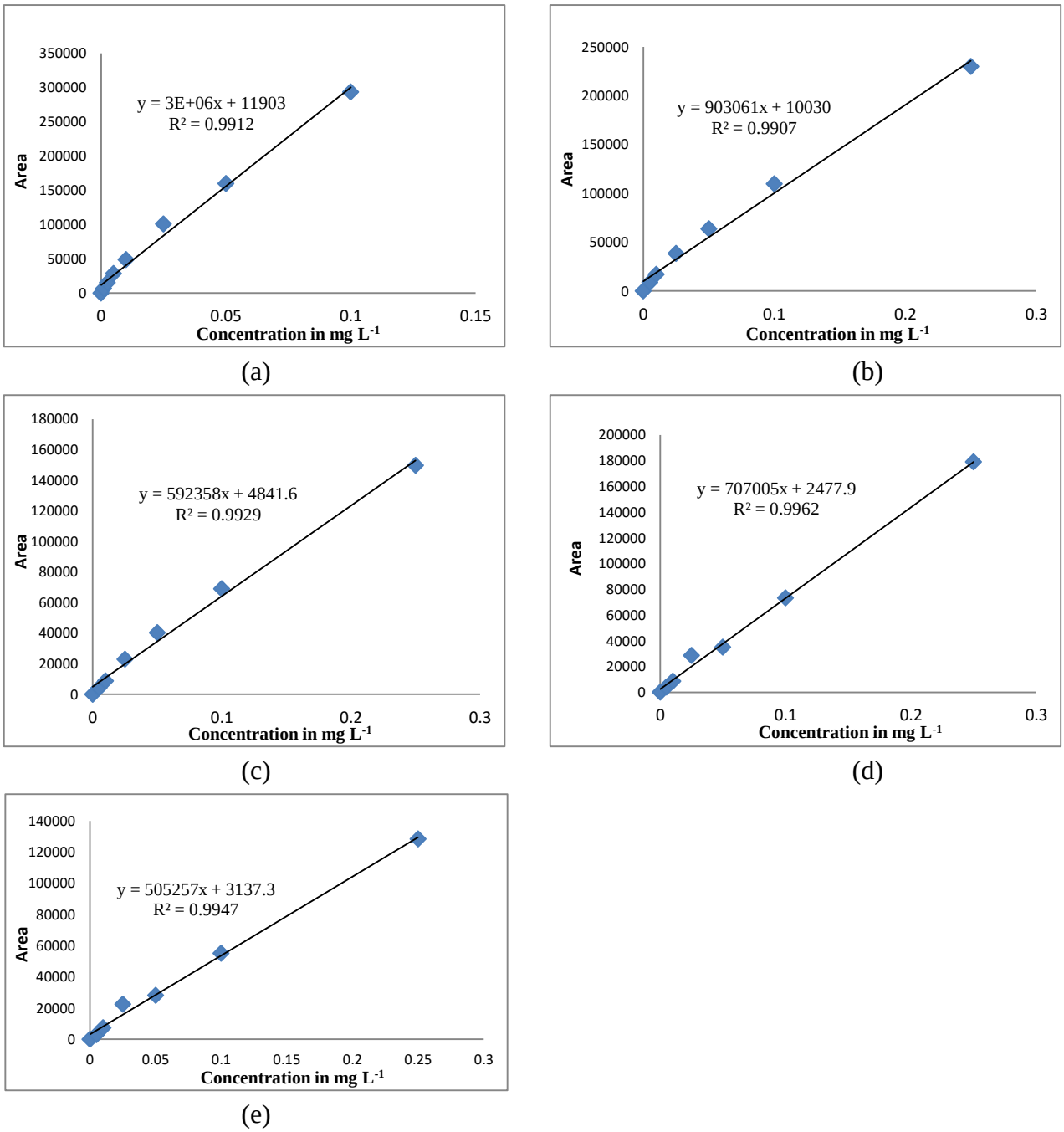


Figure 46: Calibration curves of chlorpyrifos (a), quinalfos (b), diazinon (c), cypermethrin (d) and fenvalerate (e)

The limit of detection (LOD) and quantification (LOQ) were found to be 0.50 & 1.65, 5.0 & 16.5, 2.50 & 8.25, 5.0 & 16.5 and 2.50 & 8.25 μg L^{-1} for chlorpyrifos, cypermethrin, diazinon, fenvalerate and quinalphos, respectively (**Table 25**). The calibration curves at different concentration levels were made using MS Excel software by plotting area of the eluted standards vs concentration. The calibration curves are presented in (**Figure 46**). Linear range of elution and correlation coefficient are given in **Table 25**.

2.5.5 Collection of Tomato Samples

Twelve tomato samples were purchased from different markets of Dhaka city, Kurigram and Noakhali districts and coded as STM 1 to STM 12 (**Table 21**).

Table 21: Sampling Date and Code No. of Tomato Samples from Different Markets of Dhaka, Kurigram and Noakhali Districts

Name of vegetable	Collection place	Date of sampling	Sample code
Tomato	Kaptan Bazar	10/04//16	STM 1
	Kawran Bazar	11/04/16	STM 2
	Hatirpool Bazar	12/04/16	STM 3
	Middle Badda Bazar	07/04/16	STM 4
	New Market	18/04/16	STM 5
	Mirpur-1	22/04/16	STM 6
	Kachukhet Cantonment	22/04/16	STM 7
	Polashi	18/04/16	STM 8
	Anando Bazar	18/04/16	STM 9
	Chowmani Bazar, Noakhali	20/04/16	STM 10
	Savar	24/04/16	STM 11
	Kurigram	26/04/16	STM 12

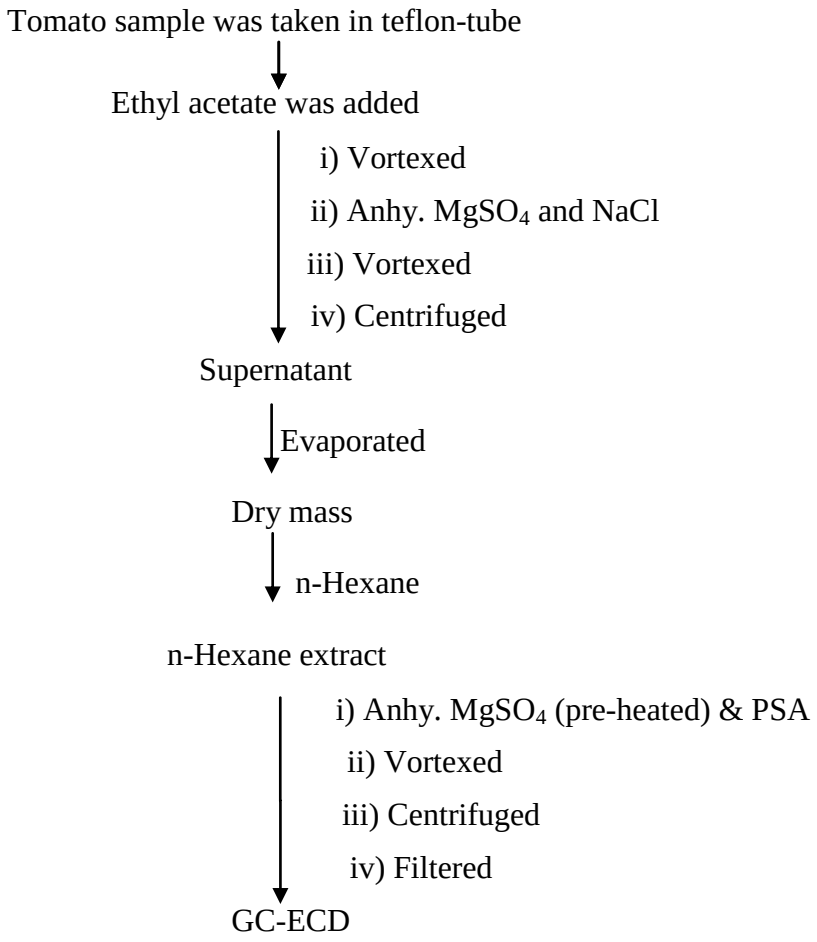
2.5.6 Homogenization and Storage of Tomato Sample

The tomato samples were chopped into small pieces and homogenized by a kitchen blender. The homogenized tomato samples (10 g) were kept in a 3x12 screw-cap teflon tubes (50 mL) and stored in a freezer at -20^0 C until analysis.

2.5.7 Extraction and Clean-up of Tomato Samples

The homogenized frozen tomato samples (10 g) were taken out from the freezer and thawed at room temperature, 20 mL of ethyl acetate was added to it and vortexed for 2 min. The pre-heated anhydrous magnesium sulphate (6.0 g) and sodium chloride (1.5 g) were added to the tube and vortexed for 1 min and was centrifuged for 5 min at 5000 rpm. From the supernatant 10 mL was transferred into a 100 mL of round bottom flask and evaporated to dryness. The dried material was reconstituted with n hexane (5 mL) (**Scheme-10**).

The n-hexane extract (2 mL) was transferred into a screw-cap test tube, 750 mg anhydrous preheated $MgSO_4$ and 150 mg PSA were added to it. The test tube was vortexed for 30 s and centrifuged for 5 min at 5000 rpm. The supernatant was taken and filtered through LC sample filter (0.25 µm) and transferred into vial to analysed by GC-ECD having auto-injector (**Scheme-10**). The results are given in **Table 26**.



Scheme 10: Extraction and clean-up procedure for tomato samples

2.5.8 Control Sample of Tomato

The control tomato samples from BARI experimental control field were chopped and homogenized as the treated samples and kept in freezer at -20^0 C.

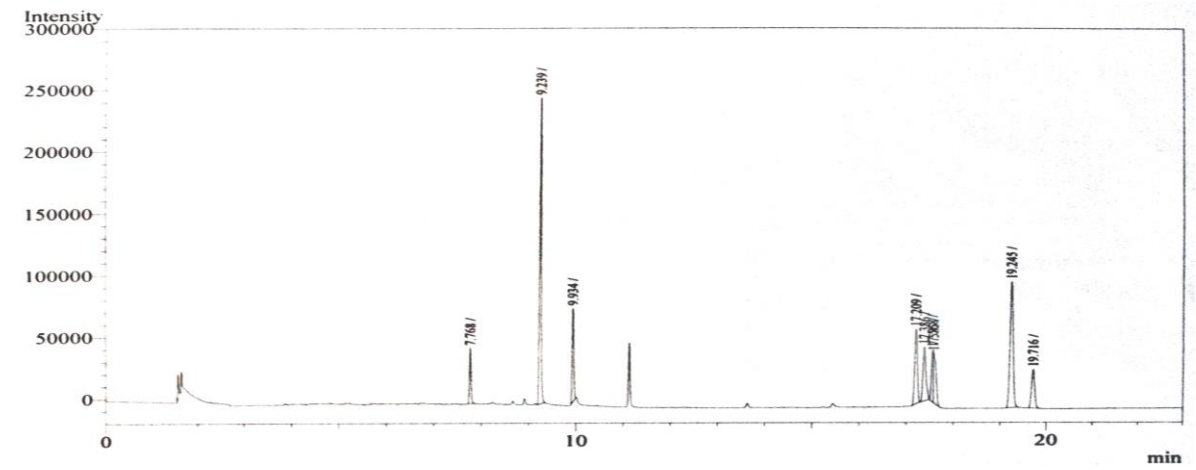
2.5.9 Recovery Experiment of Chlorpyrifos, Cypermethrin, Diazinon, Fenvalerate and Quinalphos in Tomato

The homogenized control tomato sample (10 g) was taken in a screw cap Teflon tube (50 mL), spiked with certified standard solution of mixture of chlorpyrifos, cypermethrin, diazinon, fenvalerate and quinalphos. The spiked samples were vortexed for 0.5 min and were allowed to stand for 30 min at room temperature. The recovery experiments were conducted in 5 replicates at spiking concentrations of 0.05 mg kg^{-1} for chlorpyrifos and 0.10 mg kg^{-1} for cypermethrin, diazinon, fenvalerate and quinalphos, respectively (**Table 30**).

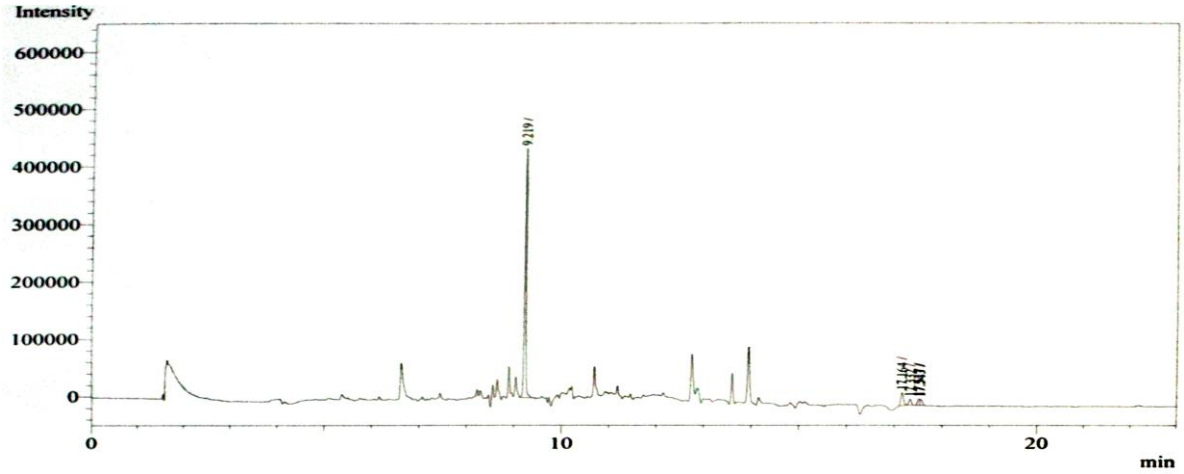
Then the spiked control sample was extracted and cleaned up by the same procedure as was done for experimental tomato sample (**Scheme 10**). The cleaned extract was analyzed by GC-ECD. The results are given in **Table 30**. Standard deviation and relative standard deviation were calculated. Percent recovery was calculated and the results are given in **Table 30**.

2.6.0 Analysis of Cleaned Extract of Tomato by GC-ECD

Quantification of residue of chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos was done on a gas chromatograph (GC-2010 Shimadzu) equipped with ^{63}Ni Electron Capture detector (ECD). Nitrogen was used as carrier and make up gas. Separations were performed on Non-polar (HP-5 MS) capillary column of 30 m long x 250 µm i.d. x 0.25 µm film thicknesses from Agilent, USA. Auto injector was used for the determination of the pesticide residue in the tomato samples. Temperature was programmed as 120 °C (1 min hold), increased at 20 °C min^{-1} to 220 °C (0 min hold), increased at 10 °C min^{-1} to 270 °C. The injector and detector temperatures were 220 °C and 290 °C, respectively. The injector and detector temperatures were set at 220 and 290 °C, respectively. All injections were made in splitless-split mode with 1 µL injection volume. The GC-ECD chromatograms of standard mixture of chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos and cleaned tomato extract are given in **Figure 47**.



(a)



(b)

Figure 47: GC-ECD chromatograms of standard mixture of chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos (a) and tomato extract (b)

2.6.1 Collection of Cauliflower Samples

Eleven cauliflower samples were purchased from different markets of Dhaka city and Kurigram district and coded as SCF 1 to SCF 11 (**Table 22**).

Table 22: Sampling Date and Code No. of Cauliflower Samples from Different Markets of Dhaka City and Kurigram District

Name of vegetable	Collection place	Date of sampling	Sample code
Cauliflower	Kaptan Bazar	10/04//16	SCF 1
	Kawran Bazar	11/04/16	SCF 2
	Hatirpool Bazar	12/04/16	SCF 3
	Middle Badda Bazar	07/04/16	SCF 4
	New Market	18/04/16	SCF 5
	Mirpur-1	22/04/16	SCF 6
	Kachukhet Cantonment	22/04/16	SCF 7
	Polashi	18/04/16	SCF 8
	Anando Bazar	18/04/16	SCF 9
	Savar	24/04/16	SCF 10
	Kurigram	26/04/16	SCF 11

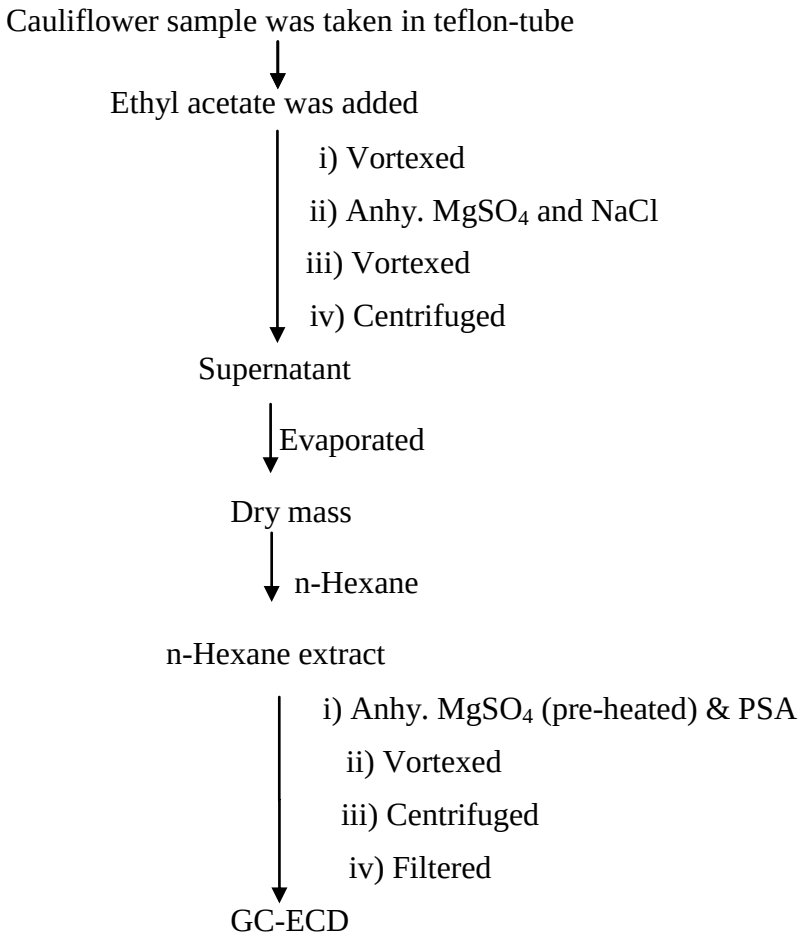
2.6.2 Homogenization and Storage of Cauliflower Sample

The cauliflower samples were chopped into small pieces and homogenized by a kitchen blender. The homogenized cauliflower samples (10 g) were kept in a 3x11 screw-cap teflon tubes (50 mL) and stored in a freezer at -20^0 C until analysis.

2.6.3 Extraction and Clean-up of Cauliflower Samples

The homogenized frozen cauliflower samples (10 g) were taken out from the freezer and thawed at room temperature, 20 mL of ethyl acetate was added to it and vortexed for 2 min. The pre-heated anhydrous magnesium sulphate (6.0 g) and sodium chloride (1.5 g) were added to the tube and vortexed for 1 min and was centrifuged for 5 min at 5000 rpm. From the supernatant 10 mL was transferred into a 100 mL of round bottom flask and evaporated to dryness. The dried material was reconstituted with n hexane (5 mL) (**Scheme-11**).

The n-hexane extract (2 mL) was transferred into a screw-cap test tube, 750 mg anhydrous preheated $MgSO_4$ and 150 mg PSA were added to it. The test tube was vortexed for 30 s and centrifuged for 5 min at 5000 rpm. The supernatant was taken and filtered through LC sample filter (0.25 µm) and transferred into vial to analysed by GC-ECD having auto-injector (**Scheme-11**). The results are given in **Table 27**.

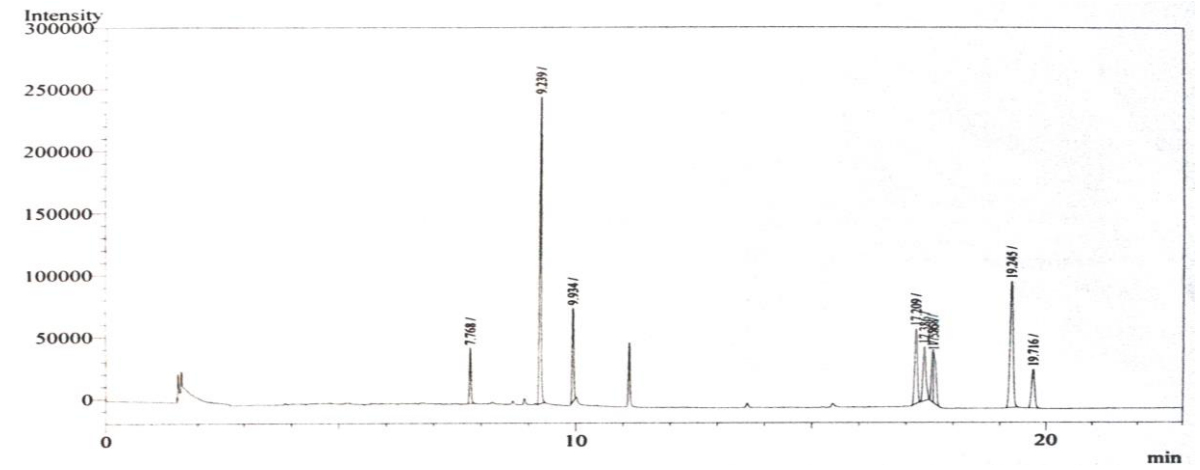


Scheme 11: Extraction and clean-up procedure for cauliflower sample

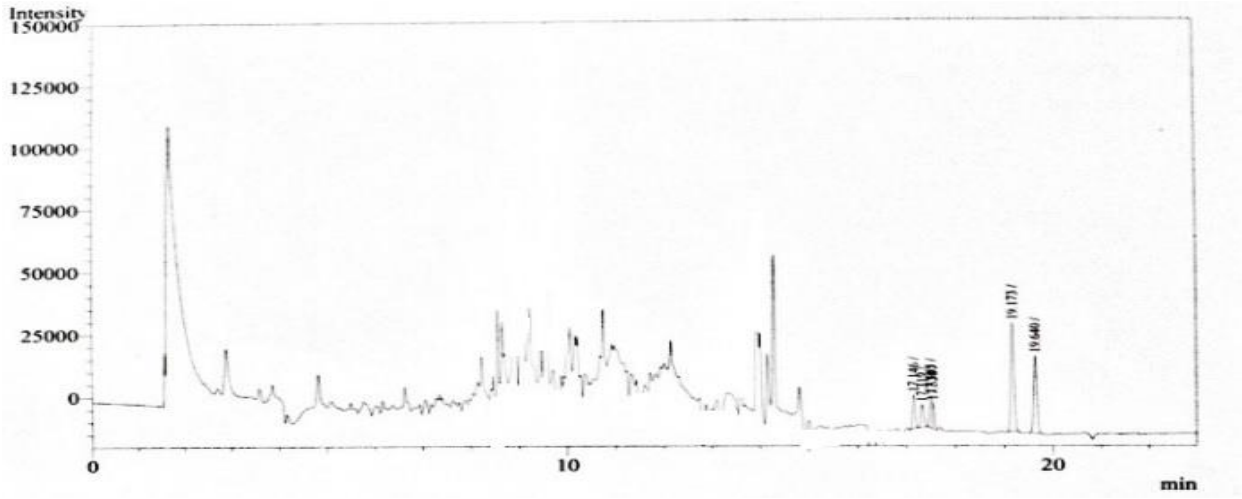
2.6.4 Analysis of Cleaned Extract of Cauliflower by GC-ECD

Quantification of residue of chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos was done on a gas chromatograph (GC-2010 Shimadzu) equipped with ^{63}Ni Electron Capture detector (ECD). Nitrogen was used as carrier and make up gas. Separations were performed on Non-polar (HP-5 MS) capillary column of 30 m long x 250 µm i.d. x 0.25 µm film

thicknesses from Agilent, USA. Auto injector was used for the determination of the pesticide residue in the cauliflower samples. Temperature was programmed as 120 °C (1 min hold), increased at 20 °C min^{-1} to 220 °C (0 min hold), increased at 10 °C min^{-1} to 270 °C. The injector and detector temperatures were 220 °C and 290 °C, respectively. The injector and detector temperatures were set at 220 and 290 °C, respectively. All injections were made in splitless-split mode with 1 µL injection volume. The GC-ECD chromatograms of standard mixture of chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos and cauliflower extract are given in **Figure 48**.



(a)



(b)

Figure 48: GC-ECD chromatograms of standard mixture of chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos (a) and cauliflower extract (b)

2.6.5 Collection of Eggplant Samples

Twelve cauliflower samples were purchased from different markets of Dhaka city and Kurigram and Noakhali districts and coded as SEP 1 to SEP12 (**Table 23**).

Table 23: Sampling Date and Code No. of Eggplant Samples from Different Markets of Dhaka, Kurigram and Noakhali Districts

Name of vegetable	Collection place	Date of sampling	Sample code
Eggplant	Kaptan Bazar	10/04//16	SEP 1
	Kawran Bazar	11/04/16	SEP 2
	Hatirpool Bazar	12/04/16	SEP 3
	Middle Badda Bazar	07/04/16	SEP 4
	New Market	18/04/16	SEP 5
	Mirpur-1	22/04/16	SEP 6
	Kachukhet Cantonment	22/04/16	SEP 7
	Polashi	18/04/16	SEP 8
	Anando Bazar	18/04/16	SEP 9
	Chowmani Bazar, Noakhali	20/04/16	SEP 10
	Savar	24/04/16	SEP 11
	Kurigram	26/04/16	SEP 12

2.6.6 Homogenization and Storage of Eggplant Sample

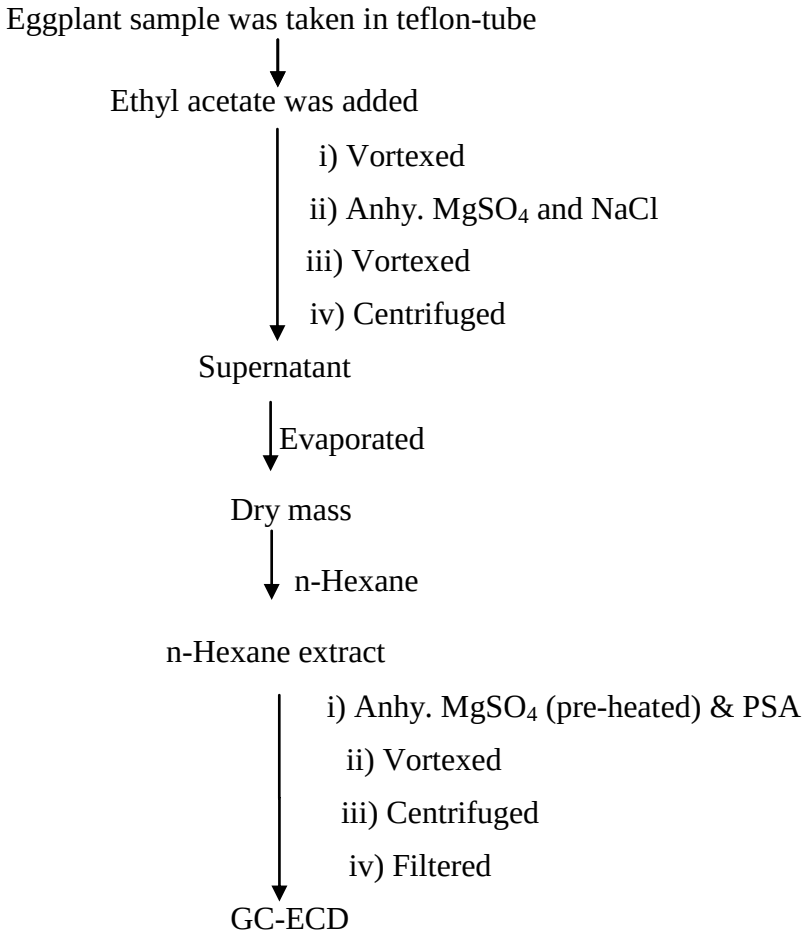
The eggplant samples were chopped into small pieces and homogenized by a kitchen blender. The homogenized eggplant samples (10 g) were kept in a 3x12 screw-cap teflon tubes (50 mL) and stored in a freezer at -20^0 C until analysis.

2.6.7 Extraction and Clean-up of Eggplant Sample

The homogenized frozen eggplant samples (10 g) were taken out from the freezer and thawed at room temperature, 20 mL of ethyl acetate was added to it and vortexed for 2 min. The pre-

heated anhydrous magnesium sulphate (6.0 g) and sodium chloride (1.5 g) were added to the tube and vortexed for 1 min and was centrifuged for 5 min at 5000 rpm. From the supernatant 10 mL was transferred into a 100 mL of round bottom flask and evaporated to dryness. The dried material was reconstituted with n hexane (5 mL) (**Scheme-12**).

The n-hexane extract (2 mL) was transferred into a screw-cap test tube, 750 mg anhydrous preheated $MgSO_4$ and 150 mg PSA were added to it. The test tube was vortexed for 30 s and centrifuged for 5 min at 5000 rpm. The supernatant was taken and filtered through LC sample filter (0.25 µm) and transferred into vial to analysed by GC-ECD having auto-injector (**Scheme-12**). The results are given in **Table 28**.



Scheme 12: Extraction and clean-up procedure for eggplant samples

2.6.8 Control Sample of Eggplant

The control eggplant samples from BARI experimental control field were chopped and homogenized as the treated samples and kept in freezer at -20^0 C.

2.6.9 Recovery Experiment of Chlorpyrifos, Cypermethrin, Diazinon, Fenvalerate and Quinalphos in Eggplant

The homogenized control eggplant sample (10 g) was taken in a screw cap Teflon tube (50 mL), spiked with certified standard solution of mixture of chlorpyrifos, cypermethrin, diazinon, fenvalerate and quinalphos. The spiked samples were vortexed for 0.5 min and were allowed to stand for 30 min at room temperature. The recovery experiments were conducted in 6 replicates at spiking concentrations of 0.05 mg kg^{-1} for chlorpyrifos and 0.10 mg kg^{-1} for cypermethrin, diazinon and fenvalerate and 0.15 mg kg^{-1} for quinalphos, respectively (**Table 30**). Then the spiked control sample was extracted and cleaned up by the same procedure as was done for experimental eggplant sample (**Scheme 12**). The cleaned extract was analyzed by GC-ECD. The results are given in **Table 30**. Standard deviation and relative standard deviation were calculated. Percent recovery was calculated and the results are given in **Table 30**.

2.7.0 Analysis of Cleaned Extract of Eggplant by GC-ECD

Quantification of residue of chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos was done on a gas chromatograph (GC-2010 Shimadzu) equipped with ^{63}Ni Electron Capture detector (ECD). Nitrogen was used as carrier and make up gas. Separations were performed on Non-polar (HP-5 MS) capillary column of 30 m long x 250 µm i.d. x 0.25 µm film thicknesses from Agilent, USA. Auto injector was used for the determination of the pesticide residue in the eggplant samples. Temperature was programmed as 120 °C (1 min hold), increased at 20 °C min^{-1} to 220 °C (0 min hold), increased at 10 °C min^{-1} to 270 °C. The injector and detector temperatures were 220 °C and 290 °C, respectively. The injector and detector temperatures were set at 220 and 290 °C, respectively. All injections were made in splitless-split mode with 1 µL injection volume. The chromatograms of standard mixture of

chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos and eggplant extract are given in **Figure 49**.

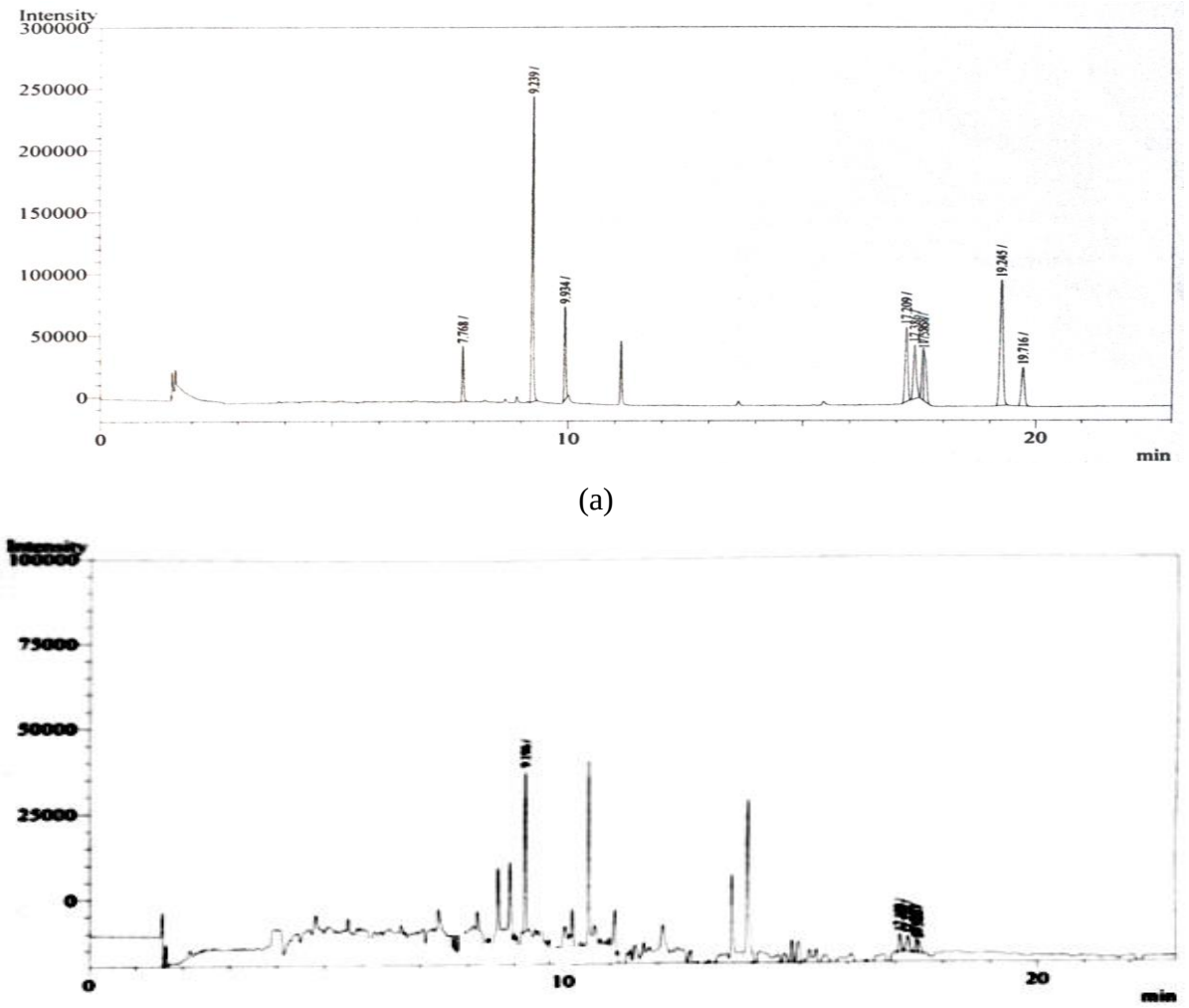


Figure 49: GC-ECD chromatograms of standard mixture of chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos (a) and eggplant extract (b)

2.7.1 Collection of Bean Samples

Ten bean samples were purchased from different markets of Dhaka city and Noakhali district and coded as SB 1 to SB10 (**Table 24**).

Table 24: Sampling Date and Code No. of Bean Samples from Different Markets of Dhaka City and Noakhali District

Name of vegetable	Collection place	Date of sampling	Sample code
Bean	Kaptan Bazar	10/04//16	SB 1
	Kawran Bazar	11/04/16	SB 2
	Hatirpool Bazar	12/04/16	SB 3
	Middle Badda Bazar	07/04/16	SB 4
	New Market	18/04/16	SB 5
	Mirpur-1	22/04/16	SB 6
	Kachukhet Cantonment	22/04/16	SB 7
	Polashi	18/04/16	SB 8
	Anandobazar	18/04/16	SB 9
	Chowmani Bazar, Noakhali	20/04/16	SB 10

2.7.2 Homogenization and Storage of Bean Sample

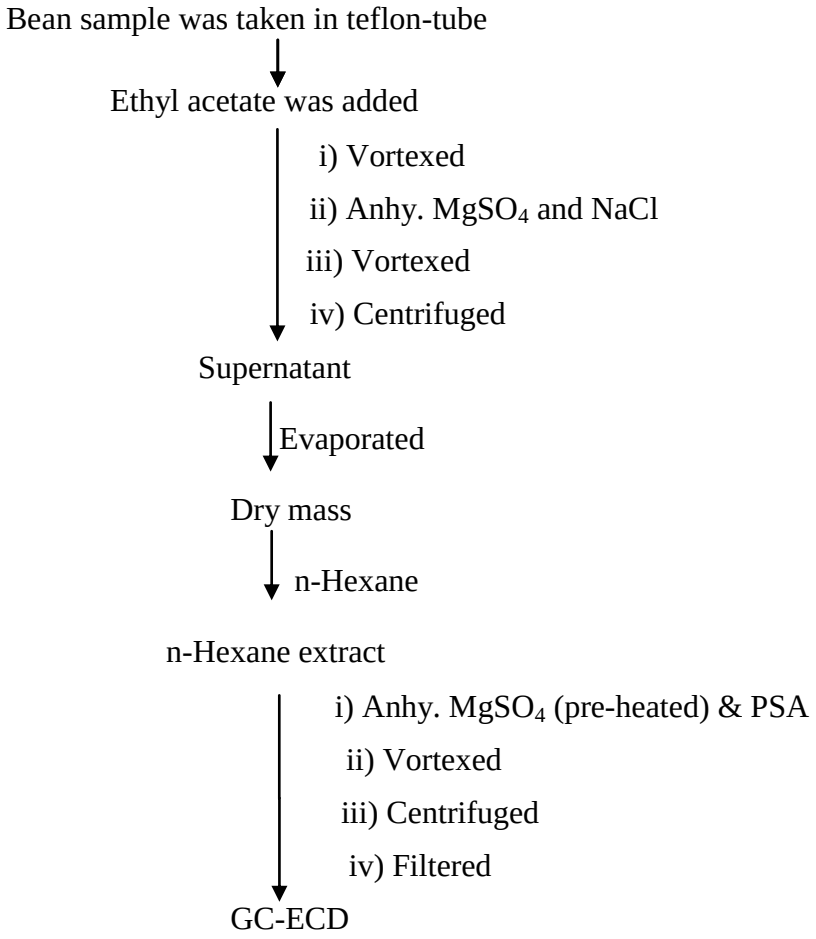
The bean samples were chopped into small pieces and homogenized by a kitchen blender. The homogenized bean samples (10 g) were kept in a 3x10 screw-cap teflon tubes (50 mL) and stored in a freezer at -20^0 C until analysis.

2.7.3 Extraction and Clean-up of Bean Samples

The homogenized frozen bean samples (10 g) were taken out from the freezer and thawed at room temperature, 20 mL of ethyl acetate was added to it and vortexed for 2 min. The pre-heated anhydrous magnesium sulphate (6.0 g) and sodium chloride (1.5 g) were added to the tube and vortexed for 1 min and was centrifuged for 5 min at 5000 rpm. From the supernatant 10 mL was transferred into a 100 mL of round bottom flask and evaporated to dryness. The dried material was reconstituted with n hexane (5 mL) (**Scheme-13**).

The n-hexane extract (2 mL) was transferred into a screw-cap test tube, 750 mg anhydrous preheated $MgSO_4$ and 150 mg PSA were added to it. The test tube was vortexed for 30 s and

centrifuged for 5 min at 5000 rpm. The supernatant was taken and filtered through LC sample filter (0.25 µm) and transferred into vial to analysed by GC-ECD having auto-injector (**Scheme-13**). The results are given in **Table 29**.



Scheme 13: Extraction and clean-up procedure for bean samples

2.7.4 Control Sample of Bean

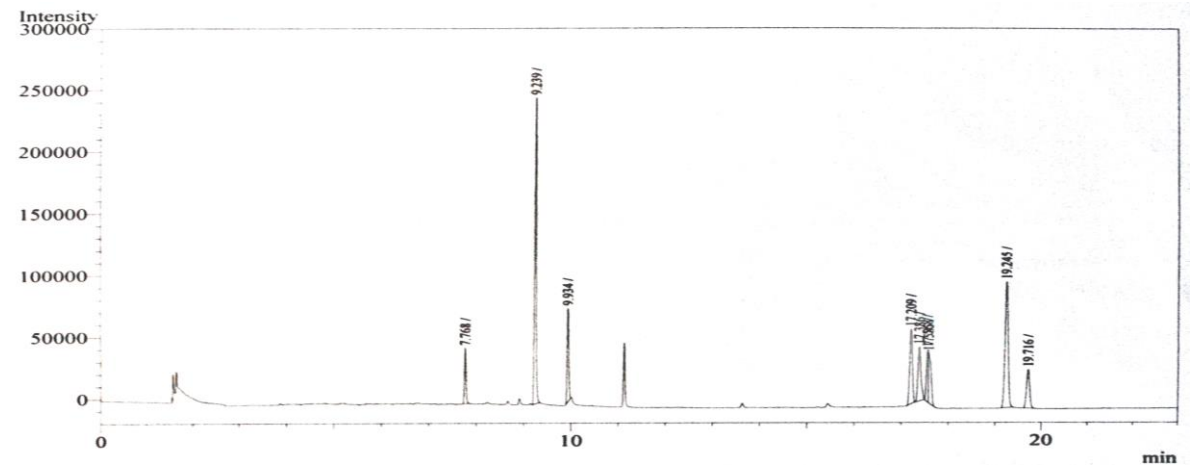
The control bean samples from BARI experimental control field were chopped and homogenized as the treated samples and kept in freezer at -20^0 C.

2.7.5 Recovery Experiment of Chlorpyrifos, Cypermethrin, Diazinon, Fenvalerate and Quinalphos in Bean

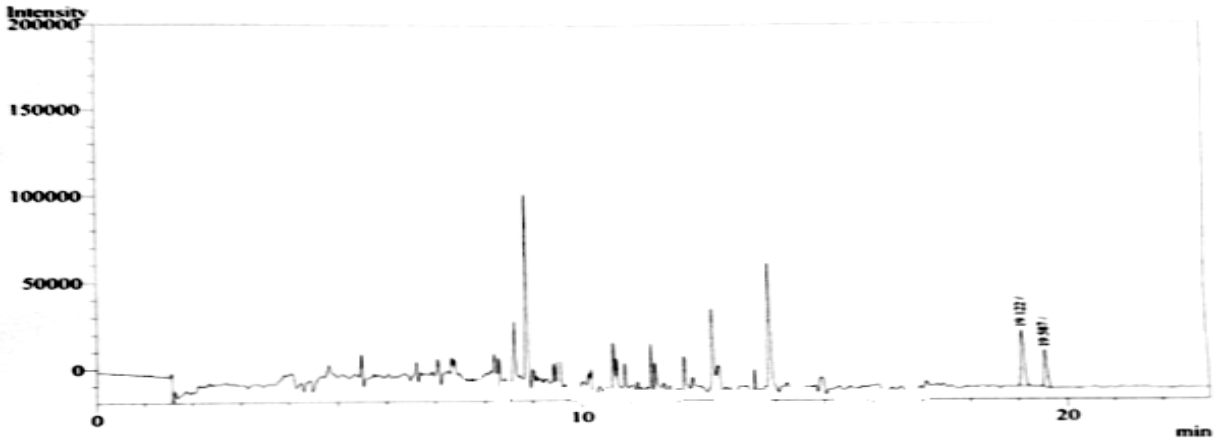
The homogenized control bean sample (10 g) was taken in a screw cap Teflon tube (50 mL), spiked with certified standard solution of mixture of chlorpyrifos, cypermethrin, diazinon, fenvalerate and quinalphos. The spiked samples were vortexed for 0.5 min and were allowed to stand for 30 min at room temperature. The recovery experiments were conducted in three replicates at spiking concentrations of 0.15 mg kg^{-1} for chlorpyrifos, diazinon and fenvalerate and 0.10 mg kg^{-1} for cypermethrin and 0.20 mg kg^{-1} for quinalphos, respectively (**Table 30**). Then the spiked control sample was extracted and cleaned up by the same procedure as was done for experimental bean sample (**Scheme 12**). The cleaned extract was analyzed by GC-ECD. The results are given in **Table 30**. Standard deviation and relative standard deviation were calculated. Percent recovery was calculated and the results are given in **Table 30**.

2.7.6 Analysis of Cleaned Extract of Bean by GC-ECD

Quantification of residue of chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos was done on a gas chromatograph (GC-2010 Shimadzu) equipped with ^{63}Ni Electron Capture detector (ECD). Nitrogen was used as carrier and make up gas. Separations were performed on Non-polar (HP-5 MS) capillary column of 30 m long x 250 µm i.d. x 0.25 µm film thicknesses from Agilent, USA. Auto injector was used for the determination of the pesticide residue in the bean samples. Temperature was programmed as 120 °C (1 min hold), increased at 20 °C min^{-1} to 220 °C (0 min hold), increased at 10 °C min^{-1} to 270 °C. The injector and detector temperatures were 220 °C and 290 °C, respectively. The injector and detector temperatures were set at 220 and 290 °C, respectively. All injections were made in splitless-split mode with 1 µL injection volume. The GC-ECD chromatograms of standard mixture of chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos and bean extract are given in **Figure 50**.



(a)



(b)

Figure 50: GC-ECD chromatograms of standard mixture of chlorpyrifos, cypermethrin, diazinon, fenvalerate, and quinalphos (a) and bean extract (b)

3.4 Results and Discussion

3.4.1 Results of the Analysis of Market Vegetable Samples

Four different vegetable (bean, eggplant, cauliflower and tomato) samples (45) were purchased from the markets of Dhaka, Noakhali and Kurigram districts. Cypermethrin, chlorpyrifos, diazinon, fenvalerate and quinalphos were detected in some of them. Quantification of residues was done on a gas chromatograph (GC) with an electron capture detector (ECD). Calibration curves were linear with coefficient of determinant $r^2 \geq 0.9912$, 09962, 0.9929, 0.9947 and 0.9907 for chlorpyrifos, cypermethrin, diazinon, fenvalerate and quinalphos, respectively (**Figure 46**).

Table 25: Linear Ranges, Correlation Coefficients (r^2), Limit of Detection (LOD) and Limit of Quantification (LOQ) of Chlorpyrifos, Cypermethrin, Diazinon, Fenvalerate and Quinalphos

Pesticides	Linear range (mg L^{-1})	Linearity (r^2)	LOD (µg L^{-1})	LOQ (µg L^{-1})
Chlorpyrifos	0.001-0.25	0.9912	0.50	1.65
Cypermethrin	0.005 – 0.25	0.9962	5.00	16.5
Diazinon	0.005 -0.25	0.9929	2.50	8.25
Fenvalerate	0.005 -0.25	0.9947	5.00	16.5
Quinalphos	0.005-0.25	0.9907	2.50	8.25

The limit of detection (LOD) and quantification (LOQ) were 0.50 and 1.65 µg L^{-1} for chlorpyrifos, 5.0 and 16.5 µg L^{-1} for cypermethrin, 2.50 and 8.25 µg L^{-1} for diazinon, 5.0 and 16.5 µg L^{-1} for fenvalerate and 2.50 and 8.25 µg L^{-1} for quinalphos, respectively (**Table 25**).

In tomato samples (**Table 26**), the residue of chlorpyriphos was detected in 9 out of 12 samples in the range of 0.01 ± 0.01 to 0.33 ± 0.02 mg kg^{-1} (MRL 0.2 mg kg^{-1}) and cypermethrin was detected in 3 samples in the range of 0.05 ± 0.01 to 0.32 ± 0.04 mg kg^{-1} (MRL 0.5 mg kg^{-1}).

Table 26: Pesticide Residues (Average amount ± SD) in Different Tomato Samples Analyzed by GC-ECD

Sample code	Chlorpyrifos (mg kg^{-1}; n = 3)	Cypermethrin (mg kg^{-1}; n = 3)	Diazinon (mg kg^{-1}; n = 3)	Fenvalerate (mg kg^{-1}; n = 3)	Quinalphos (mg kg^{-1}; n = 3)
STM 1	0.02 ± 0.01	ND*	ND	ND	ND
STM 2	0.33 ± 0.02	0.32 ± 0.04	ND	ND	ND
STM 3	0.01 ± 0.01	ND	ND	ND	ND
STM 4	0.01 ± 0.01	ND	ND	ND	ND
STM 5	0.01 ± 0.01	ND	ND	ND	ND
STM 6	ND	ND	ND	ND	ND
STM 7	0.01 ± 0.01	ND	ND	ND	ND
STM 8	0.02 ± 0.01	ND	ND	ND	ND
STM 9	0.03 ± 0.01	ND	ND	ND	ND
STM 10	0.01 ± 0.01	ND	ND	ND	ND
STM 11	ND	0.06 ± 0.01	ND	ND	ND
STM 12	ND	0.05 ± 0.01	ND	ND	ND

*ND – Not Detected

In cauliflower samples (**Table 27**), the residue of chlorpyriphos was detected in 10 out of 11samples in the range of 0.01 ± 0.01 to 0.79 ± 0.02 mg kg^{-1} (MRL 0.05 mg kg^{-1}), cypermethrin was detected in 3 samples in the range of 0.09 ± 0.01 to 0.74 ± 0.16 mg kg^{-1} (MRL 1.0 mg kg^{-1}) and quinalphos was detected in 4 samples in the range of 0.07 ± 0.01 to 0.49 ± 0.08 mg kg^{-1} (MRL 0.2 mg kg^{-1}).

Table 27: Pesticide Residues (Average amount ± SD) in Different Cauliflower Samples Analyzed by GC-ECD

Sample code	Chlorpyrifos (mg kg^{-1}; n = 3)	Cypermethrin (mg kg^{-1}; n = 3)	Diazinon (mg kg^{-1}; n = 3)	Fenvalerate (mg kg^{-1}; n = 3)	Quinalphos (mg kg^{-1}; n = 3)
SCF 1	0.22 ± 0.02	0.09 ± 0.01	ND*	ND	ND
SCF 2	0.60 ± 0.05	0.74 ± 0.16	ND	ND	ND
SCF 3	0.79 ± 0.02	0.34 ± 0.06	ND	ND	ND
SCF 4	0.75 ± 0.04	ND	ND	ND	0.27 ± 0.05
SCF 5	0.01 ± 0.01	ND	ND	ND	ND
SCF 6	0.03 ± 0.01	ND	ND	ND	ND
SCF 7	ND	ND	ND	ND	ND
SCF 8	0.01 ± 0.01	ND	ND	ND	0.07 ± 0.01
SCF 9	0.02 ± 0.01	ND	ND	ND	ND
SCF 10	0.15 ± 0.01	ND	ND	ND	0.43 ± 0.08
SCF 11	0.12 ± 0.02	ND	ND	ND	0.49 ± 0.08

*ND – Not Detected

Among the 12 eggplant samples (**Table 28**), the residue of chlorpyriphos was detected in 4 samples 0.02 ± 0.01 and 0.05 ± 0.01 mg kg^{-1} (MRL 0.5 mg kg^{-1}) and cypermethrin was detected in 2 samples in the range of 0.04 ± 0.01 to 0.13 ± 0.01 mg kg^{-1} (MRL 0.2 mg kg^{-1}).

Table 28: Pesticide Residues (Average amount ± SD) in Different Eggplant Samples Analyzed by GC-ECD

Sample code	Chlorpyrifos (mg kg^{-1}; n = 3)	Cypermethrin (mg kg^{-1}; n = 3)	Diazinon (mg kg^{-1}; n = 3)	Fenvalerate (mg kg^{-1}; n = 3)	Quinalphos (mg kg^{-1}; n = 3)
EP 1	ND*	ND	ND	ND	ND
EP 2	ND	0.04 ± 0.01	ND	ND	ND
EP 3	ND	ND	ND	ND	ND
EP 4	ND	ND	ND	ND	ND
EP 5	ND	ND	ND	ND	ND
EP 6	0.02 ± 0.01	0.13 ± 0.01	ND	ND	ND
EP 7	ND	ND	ND	ND	ND
EP 8	ND	ND	ND	ND	ND
EP 9	ND	ND	ND	ND	ND
EP 10	0.05 ± 0.01	ND	ND	ND	ND
EP 11	0.02 0.01	ND	ND	ND	ND
EP 12	0.04 ± 0.01	ND	ND	ND	ND

*ND – Not Detected

Out of 10 bean samples (**Table 29**), the residue of chlorpyriphos was detected in 3 samples (0.01 ± 0.01 mg kg^{-1}; MRL 0.01 mg kg^{-1}), cypermethrin was detected in 5 samples in the range of 0.05 ± 0.01 to 0.74 ± 0.09 mg kg^{-1} (MRL 0.05 mg kg^{-1}) and fenvalerate was detected in 3 samples in the range of 0.39 ± 0.05 to 0.55 ± 0.04 mg kg^{-1} (MRL 1.0 mg kg^{-1}).

Table 29: Pesticide Residues (Average amount ± SD) in Different Bean Samples Analyzed by GC-ECD

Sample code	Chlorpyrifos (mg kg^{-1}; n = 3)	Cypermethrin (mg kg^{-1}; n = 3)	Diazinon (mg kg^{-1}; n = 3)	Fenvalerate (mg kg^{-1}; n = 3)	Quinalphos (mg kg^{-1}; n = 3)
SB 1	ND*	ND	ND	ND	ND
SB 2	ND	ND	ND	ND	ND
SB 3	ND	0.19 ± 0.01	ND	0.55 ± 0.04	ND
SB 4	ND	ND	ND	ND	ND
SB 5	ND	0.74 ± 0.09	ND	ND	ND
SB 6	ND	0.05 ± 0.01	ND	ND	ND
SB 7	0.01 ± 0.01	0.14 ± 0.01	ND	ND	ND
SB 8	0.01 ± 0.01	0.58 ± 0.07	ND	0.39 ± 0.05	ND
SB 9	0.01 ± 0.01	ND	ND	ND	ND
SB 10	ND	ND	ND	0.49 ± 0.08	ND

*ND – Not Detected

The recoveries for chlorpyrifos were 98.48 ± 2.73, 99.57 ± 6.98 and 88.51 ± 2.64 % , for cypermethrin were 79.65 ± 5.56 , 86.29 ± 7.33 and 97.43 ± 8.52 %, for diazinon were 109.92 ± 2.33 , 101.41 ± 4.72 and 106.78 ± 3.55 %, for fenvalerate were 90.88 ± 2.15 , 84.10 ± 8.91 and 90.04 ± 9.29% and for quinalphos were 78.28 ± 4.85 , 85.15 ± 7.72 and 85.28 ± 2.32 % in tomato, eggplant and bean, respectively (**Table 30**).

Table 30: Recovery Results of Chlorpyrifos, Cypermethrin, Diazinon, Fenvalerate and Quinalphos Residues in Tomato, Bean and Egg plant

Vegetable	Pesticides	Spiking level (mg kg^{-1})	Average accuracy ± SD* (%)	Precision (RSD** %)
Tomato (n = 5)	Chlorpyrifos	0.05	98.48 ± 2.73	2.77
	Cypermethrin	0.10	79.65 ± 5.56	6.98
	Diazinon	0.10	109.92 ± 2.33	2.12
	Fenvalerate	0.10	90.88 ± 2.15	2.36
	Quinalphos	0.10	78.28 ± 4.85	6.20
Eggplant (n = 6)	Chlorpyrifos	0.05	99.57 ± 6.98	7.01
	Cypermethrin	0.10	86.29 ± 7.33	8.49
	Diazinon	0.10	101.41 ± 4.72	4.65
	Fenvalerate	0.10	84.10 ± 8.91	10.60
	Quinalphos	0.15	85.15 ± 7.72	9.07
Bean (n = 3)	Chlorpyrifos	0.15	88.51 ± 2.64	2.98
	Cypermethrin	0.1	97.43 ± 8.52	8.74
	Diazinon	0.15	106.78 ± 3.55	3.33
	Fenvalerate	0.15	90.04 ± 9.29	10.32
	Quinalphos	0.20	85.28 ± 2.32	2.72

*SD = Standard deviation and **RSD = Relative standard deviation

3.4.2 Discussion

3.4.2.1 Method Validation for Market Vegetable Samples

The QuEChERS method (Anastassiades *et al*., 2003) with some modifications was used for analysis of pesticide residue in market vegetable samples. The analytical method was validated in terms of selectivity, linearity, sensitivity and recovery.

Selectivity was assessed by injecting standard mixture of pesticides, blank matrices and blank matrices spiked with the mixture of pesticides simultaneously and by checking their retention times. The chromatograms of blank samples were checked and no interference peak was observed at the retention time of chlorpyrifos, cypermethrin, diazinon, fenvalerate and

quinalphos. The target peaks were obtained at 7.7, 9.2, 9.9, 17.5 and 19.5 min for diazinon, chlorpyrifos, quinalphos, cypermethrin and fenvalerate, respectively. But four peaks were obtained for cypermethrin and two peaks were obtained for fenvalerate. The four peaks for cypermethrin due to its four cis isomers which are more biologically active than trans. Similarly, the two peaks for two isomers of fenvalerate.

Linearity of the quantifications was evaluated by constructing calibration curves. The amount of chlorpyrifos, cypermethrin, diazinon, fenvalerate and quinalphos in the spiked and market vegetable samples were determined via calibration curve. Calibration curve at different ranges were made for each five pesticide in order to quantify them in the spiked and market vegetable samples in the proper range. The linearity was excellent for each pesticide with coefficient of determinant $r^2 \geq 0.9912$, 09962, 0.9929, 0.9947 and 0.9907 for chlorpyrifos, cypermethrin, diazinon, fenvalerate and quinalphos, respectively (**Figure 46**).

The limit of detection (LOD) was determined using a signal-to-noise ratio of 3 with reference to the background noise obtained for the blank sample, whereas the limit of quantification (LOQ) was determined with a signal-to-noise ratio of 10. The LOD was found to be 0.50 µg L^{-1} for chlorpyrifos, 2.50 µg L^{-1} for diazinon and quinalphos and 5.0 µg L^{-1} for cypermethrin and fenvalerate, respectively. LOQ was determined 1.65 µg L^{-1} for chlorpyrifos, 8.25 µg L^{-1} for diazinon and quinalphos and 16.5 µg L^{-1} for cypermethrin and fenvalerate, respectively (**Table 25**). The different values of LOD and LOQ (**Figure 51**) for five different pesticides were due to their different sensitivities in GC–ECD. Among five pesticides the sensitivity was the highest for chlorpyrifos then for diazinon and quinalphos. The sensitivity was lowest for cypermethrin and fenvalerate.

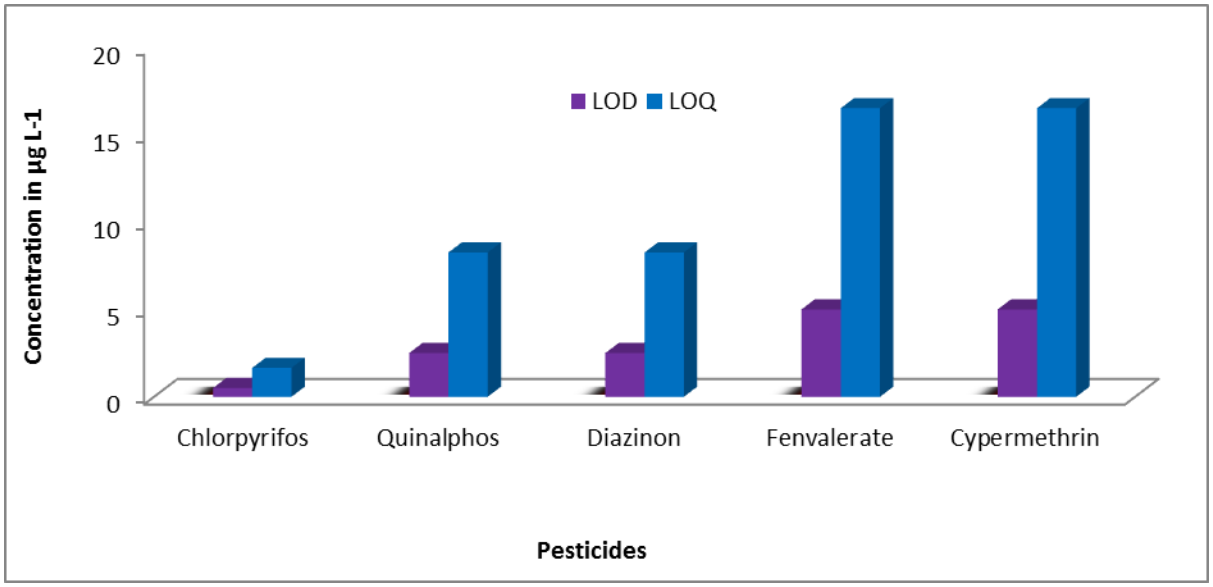


Figure 51: LOD and LOQ of Chlorpyrifos, Quinalphos, Diazinon, Fenvalerate and Cypermethrin

The **extraction efficiency** of the analytical procedure was evaluated via recovery experiments (**Table 30**). The average recovery of chlorpyrifos in tomato (n=5) was 98.48 ± 2.73% and in eggplant (n=6) was 99.57 ± 6.98 % at spiking level of 0.05 mg kg^{-1}. In bean (n=3), the average recovery of chlorpyrifos was 88.51 ± 2.64 % at spiking level of 0.15 mg kg^{-1}. The average recovery of cypermethrin in tomato (n=5) was 79.65 ± 5.56 %, in eggplant (n=6) was 86.29 ± 7.33 % and in bean (n=3) was 97.43 ± 8.52 % at spiking level of 0.10 mg kg^{-1}. The average recovery of diazinon in tomato (n=5) was 109.92 ± 2.33 % and in eggplant (n=6) was 101.41 ± 4.72 % at spiking level of 0.10 mg kg^{-1}. In bean (n=3), the average recovery of chlorpyrifos was 106.78 ± 3.55 % at spiking level of 0.15 mg kg^{-1}. For fenvelarate, the average recovery in tomato (n=5) was 90.88 ± 2.15 % and in eggplant (n=6) was 84.10 ± 8.91 % at spiking level of 0.10 mg kg^{-1}. In bean (n=3), the average recovery of fenvelarate was 90.04 ± 9.29 % at spiking level of 0.15 mg kg^{-1}. For quinalphos the average recovery in tomato (n=5), eggplant (n=6) and bean (n=3) were 78.28 ± 4.85 , 85.15 ± 7.72 and 85.28 ± 2.32 % at the spiking level of 0.10,0.15 and 0.20 mg kg^{-1}, respectively.

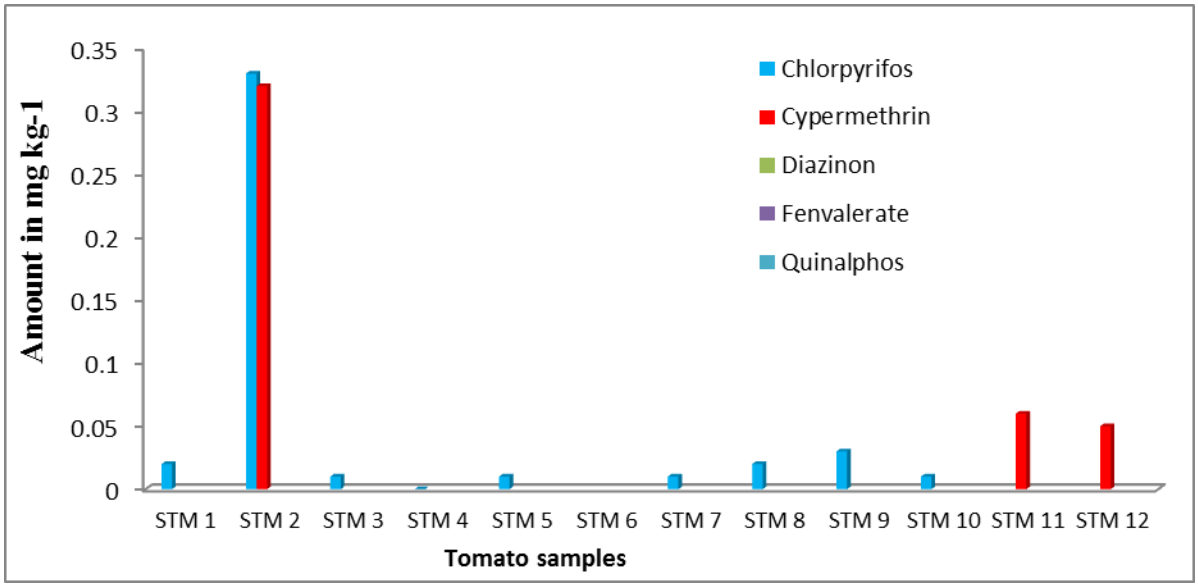


Figure 52: Pesticide Residues in Different Tomato Samples

The four vegetable samples cauliflower, eggplant, tomato and bean are very popular winter vegetables in Bangladesh. Pesticide residue in food has become a consumer's safety issue and the consumers have the right to know how much pesticide get incorporated in the food they eat. The detection, identification and quantification of pesticide in the food are becoming the public interest. However, very little references are available on the presence of pesticides in vegetables in Bangladesh (J. A. Khatoon *et al*, 2004).Therefore, this study was undertaken to assess the quantity of residue of five frequently used pesticides in cauliflower, eggplant, tomato and bean.

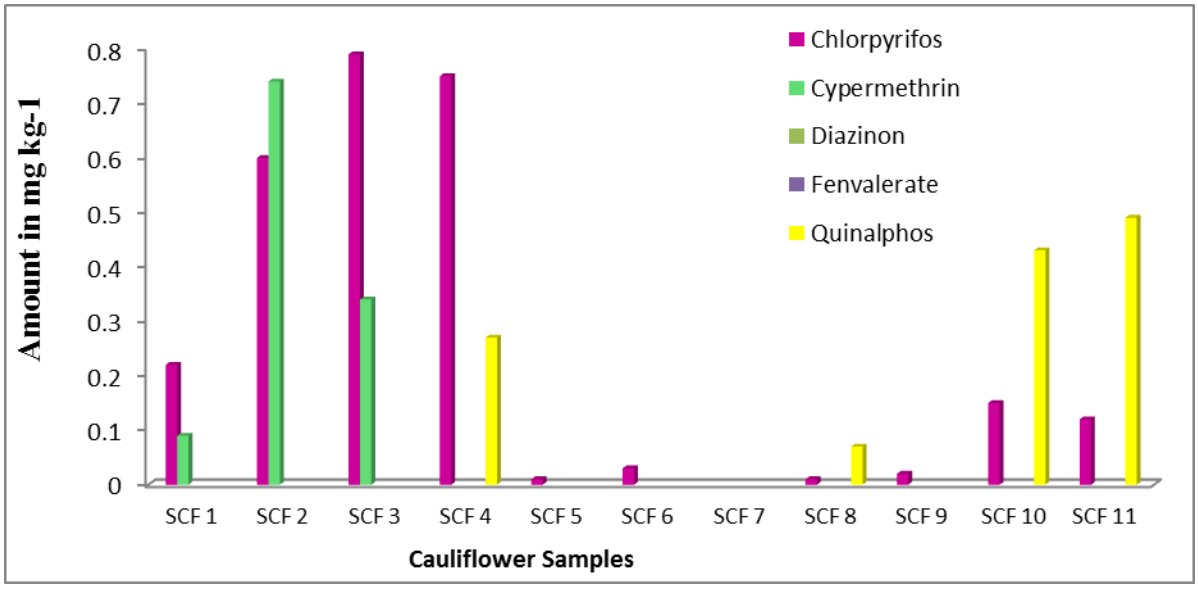


Figure 53: Pesticide Residues in Different Cauliflower Samples

A number of analytical methods were reported to determine multiple pesticide residues (Food and Drug Administration, 1999; Luke *et al.*, 1975; Specht and Tilkes, 1980; Lee *et al.*, 1991; Andersson and Pålsheden, 1991; Cook *et al.*, 1999; General Inspectorate for Health Protection, 1996; Fillion *et al.*, 2000; Sheridan and Meola, 1999; Lehotay, 2000). In 2003, the QuEChERS method for pesticide residue analysis was introduced by Anastassiades et al. (2003), which provides high quality results in a fast, easy, an inexpensive approach and the QuEChERS method was further validated for greater than 200 pesticides (Lehotay *et al.*, 2007). So, in the present study QuEChERS method was used successfully.

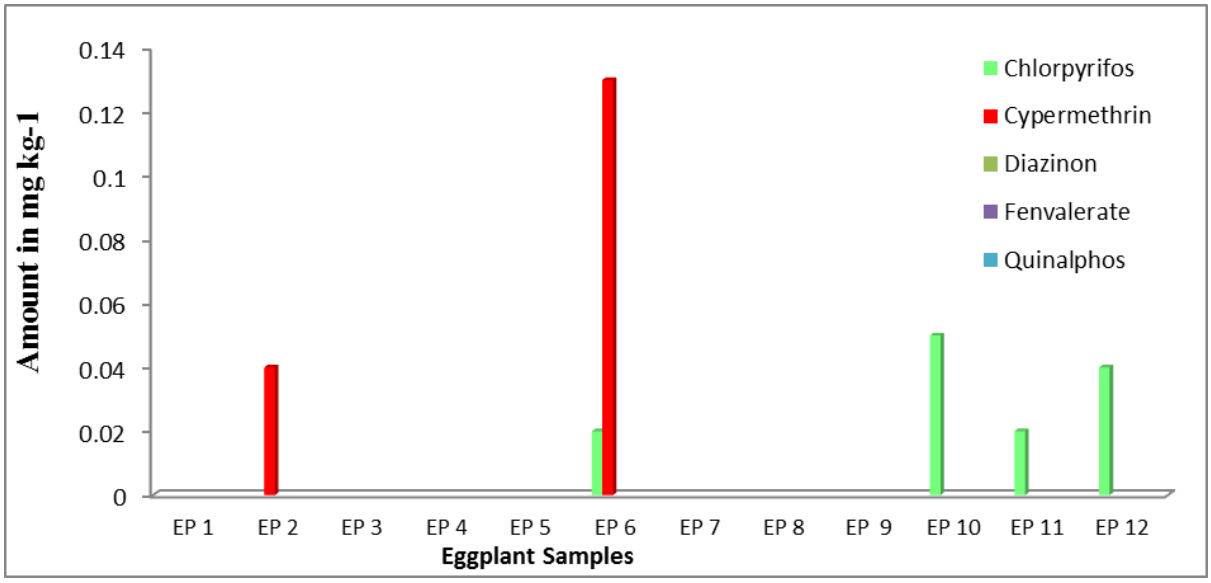


Figure 54: Pesticide Residues in Different Eggplant Samples

In the present study a total of 5 pesticide (chlorpyrifos, cypermethrin, diazinon , fenvalerate, and quinalphos) residues were detected in the tomato, eggplant, bean and cauliflower samples. This result is in agreement with Ahuja et al., (1998) who reported residues of hexachlorocyclohexane (HCH) and its isomers, endosulfan, dimethoate, monocrotophos, quinalphos, fenvalerate and cypermethrin were detected in cauliflowers, cabbages, tomatoes, eggplant, okra, field beans and cucumbers were monitored for in most of the samples. Cypermethrin residue was above the MRL value (0.05 mg kg^{-1}) in 4 bean samples (0.14 ± 0.01, 0.19 ± 0.01, 0.58 ± 0.07 and 0.74 ± 0.09 mg kg^{-1}) (**Table 29; Figure 55**). Chlorpyrifos residue was above the MRL value (0.05 mg kg^{-1}) in 6 cauliflower samples (0.12 ± 0.02 to 0.79 ± 0.02 mg kg^{-1}) (**Table 27; Figure 53**) and for tomato samples the residue was above the MRL (0.20 mg kg^{-1}) in 1 tomato sample (0.33 ± 0.02 mg kg^{-1}) (**Table 26; Figure 52**). Quinalphos residue was above the MRL (0.2 mg/kg) in 3 cauliflower sample (0.27 ± 0.05,

0.43 ± 0.08 and 0.49 ± 0.08 mg kg^{-1}) (**Table 27**). However, some pesticides were not detected in some samples analyzed from the different locations. This may be attributed to the fact that decomposition or degradation of pesticide before the analysis was carried out. This study confirms the work of Essumang *et al.* (2008) regarding the observation that some pesticide residues in vegetable were above the allowed MRLs. In Sweden (Pihlstrom *et al.*, 2007) fenvalerate and methamidophos have been detected in significant levels in the analysis of pesticide residues in fruits and vegetables. In Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur an experiment was conducted by Prodhan *et al.*, (2008) to detect and quantify the residue of fenitrothion in eggplant and comparison between the detected residue levels with a maximum residue limit (MRL) set by FAO and the left over residue of concerned pesticide was detected up to 7 days and the quantities were above MRL up to 3 days. Pesticide residues in Canada was reported that in vegetables and fruits the residue were far below the MRLs (Ripley *et al*., 2000). EL-Saeid (2003) used supercritical fluid extraction (SFE) in the analysis of pesticide residues in canned foods, fruits and vegetable but in the present study gas chromatography was used to detect and quantify pesticide residue in fresh vegetable. The approach to pesticide use should fit broadly in a framework in which certain criteria can be used for pesticide selection, specific instructions are followed for their application on crops and residue analysis should be used as one of the tools for enforcement (Damalas & Eleftherohorinos, 2011).

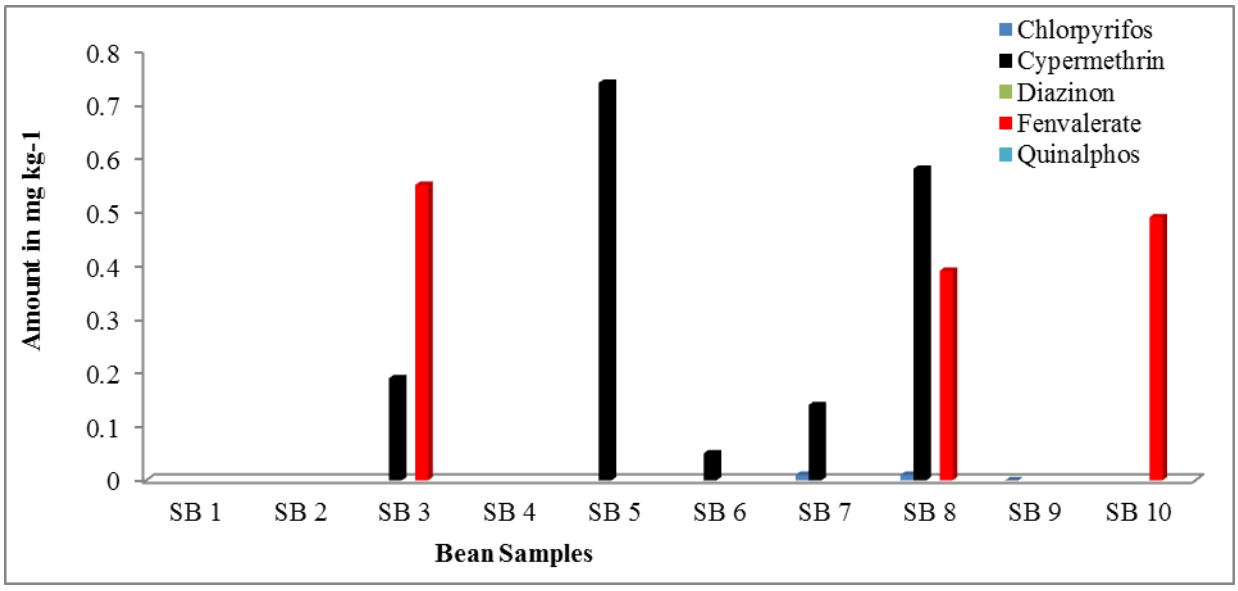


Figure 55: Pesticide Residues in Different Bean Samples

Table 31: Maximum Residue Limit (MRLs) of Pesticides in Tomato, Cauliflower, Eggplant and Bean (FAO, 2002)

Pesticide	MRLs value (mg kg^{-1})			
	Tomato	Eggplant	Bean	Cauliflower
Diazinon	0.50	0.50	0.50	0.50
Chlorpyrifos	0.20	0.50	0.01	0.05
Quinalphos	0.01	0.20	0.20	0.20
Cypermethrin	0.50	0.20	0.05	1.00
Fenvelarate	2.00	0.20	1.00	0.20

To meet the demand of increased food production, the use of pesticides are being increased probably because farmers assume that the only solution to pest problems is to spray more frequently and using different types of pesticides (Dinham, 2003). For application on vegetable farming the choice of pesticides should be biologically effective, user friendly and environmentally safe. From previous study (Ngowi & Partanen, 2002) it was revealed that farmers were not receiving agricultural extension service properly but highly influenced by manufacturers and pesticide dealers .The farmers are very interested in achieving large sales of their pesticides due to the lack of appropriate knowledge in pesticide use. In many developing countries the choice of pesticides to be used by farmers is influenced by the suppliers (Snoo *et al.*, 1997; Epstein and Bassein, 2003). This situation increases the risk on the health or safety of the environment.

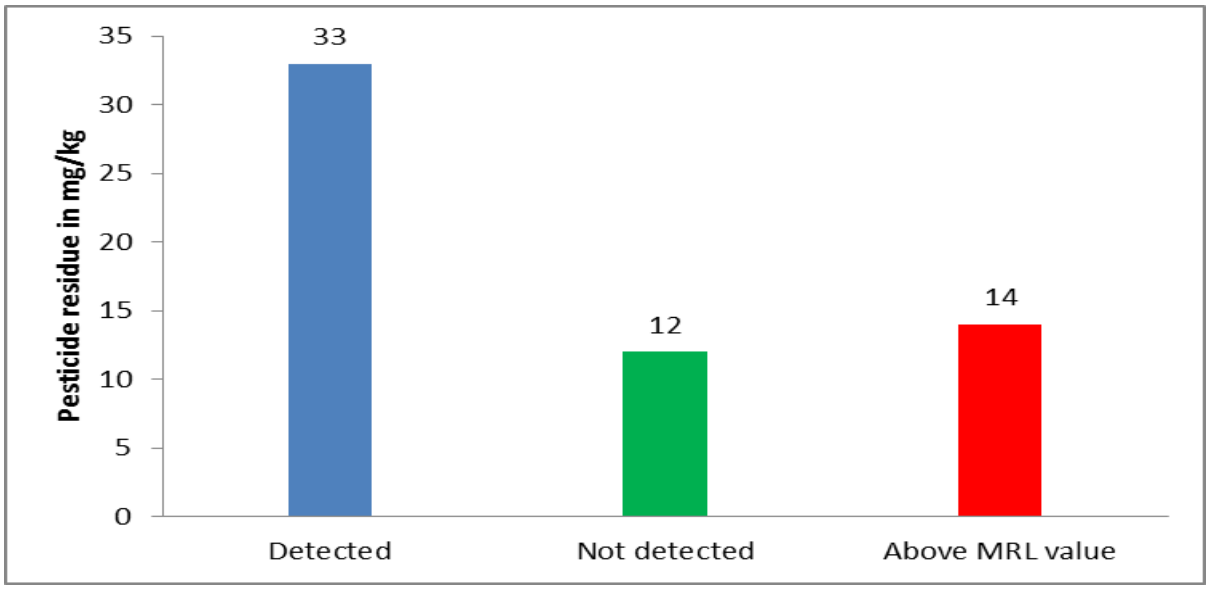


Figure 56: Pesticide residues in the analysed vegetable samples

From the present study it was found that in 14 vegetable samples out of 45, the residues of pesticides were above the MRL values (**Table 31; Figure 56**). The farmers may be used the pesticide more than one time. For the confidence of consumers in quality of food the monitoring studies are imperative to know the actual status of contamination due to toxic pesticide residues. It is, therefore, suggested that these studies may be extended to other vegetables and fruits grown in different agro-climatic regions of Bangladesh. To minimizing the risk posed to human health on exposure to pesticides in food these issues should be central.

4. QUALITY ASSURANCE (QA) OF THE THESIS

ISO 17025 was followed in doing research which is presented in the thesis. All calibrated equipment and apparatus were used for carrying experimental work. The Gas Chromatographs (Shimadzu 2010 GC-ECD & 2025 GC-FID); high performance liquid chromatograph (Shimadzu UFLC Prominence (LC-PDA/FLD)) and Tandem Mass Spectrometer (LC-MS/MS; 8050) were calibrated by the technical persons of local agent of the Company (AQC). Log books were maintained for each of the equipment. Results of all the experiment were recorded in the notebook and chromatograms were saved in the files. The volumetric flasks, pipettes, four digit analytical balance and standard weights used in the present studies were calibrated by BSTI (Bangladesh Standard Testing Institute). Micro pipettes used in the analysis were certified by the company (country of origin) according to ISO 17025. Experiments with toxic chemicals and reagents were done in the functional fume hood of the research laboratory and wastes were disposed in the waste bottle supplied by Department. Hazardous solvents and chemicals were handled carefully.

All equipment, GCs, LCs and LC-MS/MS were connected with a power generator (50 kVA) with online UPS. Freezers and refrigerators were connected to IPS. All the equipment rooms' has air condition facilities. Solvents were stored separately, away from the main working area. All the solvents waste were stored safely and disposed of safely in an environmentally friendly manner.

Certified standards reference used were with highest available purity (91-99 %) and were stored in the freezers and refrigerator according to their stability written by the producers of the respective standards. Personal safety measures *i.e.* wearing apron, taking hand (gloves; ordinary, acid & heat resistant), and face protection (goggles and masks), were taken according to the nature of experiments during working in the laboratory as well as during working with GCs and LCs. Amber coloured volumetric flasks and vials were used for UV sensitive compounds.

GCs and LCs were conditioned until smooth baseline was obtained. Before injecting the sample extracts in GC and LC triplicate solvent blank, matrix blank (control samples) were

injected to get smooth base line. For making calibration curves solvent and standards were injected from lower to higher concentration.

The limit of detection (LOD) of the targeted pesticides was determined using S/N of 3 with reference to the background noise obtained for the blank sample, whereas the limits of quantification (LOQ) were determined with as S/N of 10.

In all cases, the analysis time by GC temperature program was set 5 min. more than the last peak of the target compound was eluted whereas in the case of analysis by LC, solvent composition program was made so that the target compound was eluted after the void volume of the column.

Primary standard solutions of the reference certified samples were prepared ($\geq$10 mg) with four decimal values in the calibrated volumetric flasks, secondary standards was made from the primary standard by dilution, and working standards were prepared by serial dilution of the secondary standard solutions. All the prepared solutions were properly labeled with name, concentration and the date of preparation. The meniscuses of the solutions were marked with permanent black ink and stored in the freezer (-20 °C) away from the sample storing area.

All samples *i.e.* rice, turmeric power, vegetables either purchased and or collected were homogenized and kept in the freezers at -20^0C with proper label, name, date of collection and amount; control samples were also stored same way in a different freezer.

To avoid or minimize environmental hazards, less toxic solvents were used and their amount was also scale downed for extraction and clean-up process.

5. SUMMARY

Bangladesh is a rice producing country as the people consume rice everyday as the staple food. Post-harvest contamination can be produced by fungal attack on rice due to the delay in drying and moisture content (Asghar *et al*. 2013). There are six seasons of Bangladesh but the rice samples in the present study were collected in rainy season which can regulates fungal growth and mycotoxin expression. High grade and inferior quality rice samples were chosen for identification of aflatoxins (if any) present in the rice samples. The present study was aimed to evaluate the quantity of natural toxins in different rice samples.Twenty rice samples were purchased from different markets of Dhaka city, Kurigram and Noakhali districts. The determination was carried out by LC–FLD with Kobra cell. By following the established ISO 16050; 2003 method, the extraction, clean-up and analysis of aflatoxins in rice samples were done and validated at the laboratory. The immune affinity column (3 mL; polypropylene) containing a gel material loaded with monoclonal antibodies against aflatoxins B1, B2, G1 and G2 were used in the present study for sample clean up purposes. Validation was done in terms of selectivity, linearity, sensitivity and recovery experiment. The selectivity of the method was evaluated by injecting extracted blank samples. The absence of signals above a signal-to-noise ratio of at the retention times of the target compounds showed that the method is free of interferences. The analysis of aflatoxins in the spiked and market samples were done by using calibration curves. Calibration curves were prepared with different concentration levels and the linearity was excellent with correlation coefficients of $r^2 \geq$ 0.9998, 09997, 0.9956 and 0.9969 for B1, B2, G1 and G2, respectively. The limit of detection (LOD) and quantification (LOQ) were 0.009 and 0.025 ng mL^{-1} for B1, 0.006 and 0.018 ng mL^{-1} for B2, 0.039 and 0.116 ng mL^{-1} for G1 and 0.025 and 0.075 ng mL^{-1} for G2, respectively (**Figure 18)**. The lowest limit of detection was found for B2 than other. The LOD and LOQ were different for four different aflatoxins B1, B2, G1 and G2 due to their different sensitivity in LC-FLD with Kobra cell system.

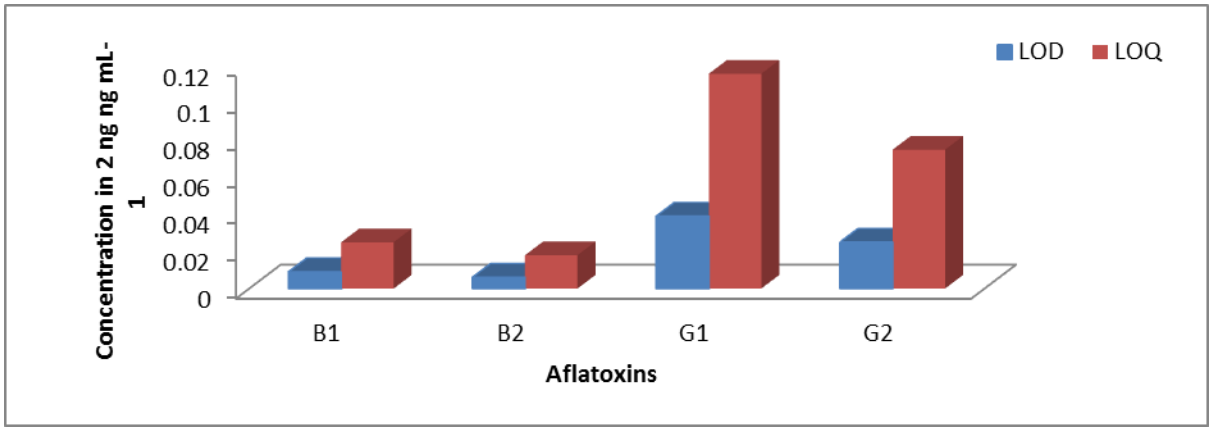


Figure 18: LOD and LOQ of Aflatoxins B1, B2, G1 and G2

The total aflatoxins (B1, B2, G1 and G2) in the rice samples were found to be in the range of trace to 3.54 µg kg^{-1}. Aflatoxin B1, B2, G1 and G2 were present in 70, 60, 40 and 10 % of rice samples, respectively. The results (**Figure 19**) revealed that 18 out of 20 samples contained detectable amount of aflatoxins. Aflatoxin B_1 (in the range of 0.04 to 0.70 µg kg^{-1}), B2 (in the range of trace to 0.20 µg kg^{-1}), G1 (in the range of 0.22 to 1.82 µg kg^{-1}) and G2 (in the range of 0.12 to 1.56 µg kg^{-1}) were quantified in 17, 16, 6 and 4 samples, respectively.

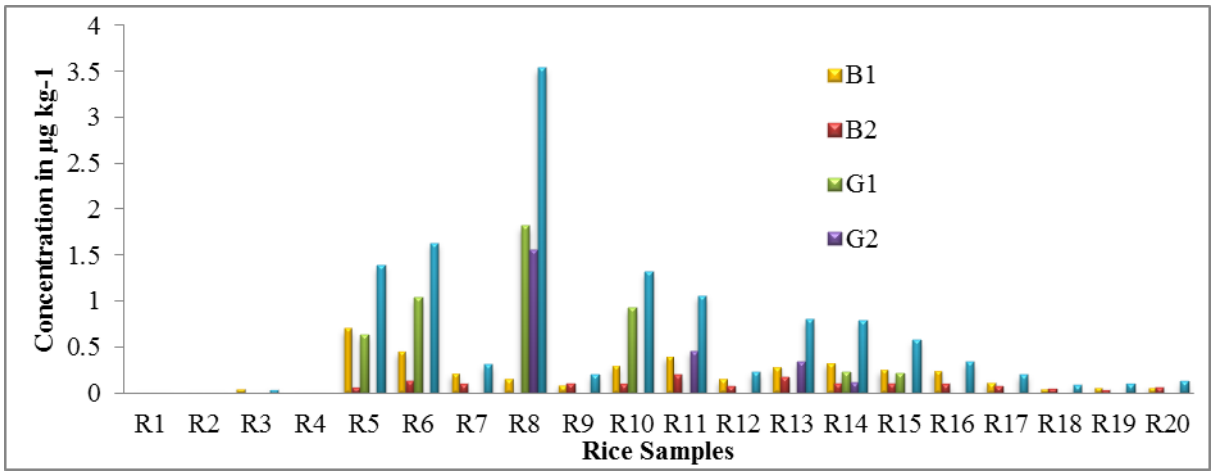


Figure 19: Amount of Aflatoxins (µg kg^{-1}) in rice samples

Recovery experiment was carried out by spiking control rice sample with known concentrations of certified aflatoxin standard working solutions for extraction efficiency of the analytical procedure. The recovery experiments were done at two spiking concentration levels by spiking of certified aflatoxin standard working solution of 10 (0.77, 0.27, 0.86 and

0.28 µg kg^{-1} concentrations of B1, B2, G1 and G2, respectively) and 20 µL (1.54 , 0.54 , 1.73 and 0.56 µg kg^{-1} concentrations of B1, B2, G1 and G2, respectively) in 4 (n=4) replicates analyses. The spiking concentration levels were chosen according to the amount of natural toxins found in rice samples. Both intra-day and inter-day recoveries were carried out to evaluate the efficiency of the method. The results for the intra-day recovery was 70.37 ± 5.59, 75.36 ± 6.77, 72.85 ± 5.93 and 99.20 ± 3.16 % of B1, B2, G1 and G2, respectively for spiking 10 µL mixture of certified aflatoxins solution and 65.08 ± 2.21, 63.39 ± 2.36, 71.85 ± 1.90 and 65.83 ± 2.92 % of B1, B2 G1 and G2, respectively for spiking 20 µL mixture of certified aflatoxins solution . The inter-day recoveries were 56.71 ± 1.60, 57.71 ± 0.58, 65.53 ± 0.73 and 76.34 ± 4.03 % of B1, B2, G1 and G2, respectively for spiking 10 µL mixture of certified aflatoxins solution and 63.40 ± 3.55, 61.39 ± 3.42, 69.57 ± 5.19 and 79.18 ± 7.10 % for B1, B2 G1 and G2, respectively for spiking 20 µL mixture of certified aflatoxins solution. The relative standard deviation (RSD) values of the recovery experiments were in the range of 1.01 to 8.99 % which showed the excellent performance of the method.

Different spices and condiments are used in preparing curry in Indian subcontinent. Among them turmeric powder is common. Carbofuran is being used in Bangladesh in the storage of spices specially turmeric powder in order to protect them from pest attack. The presence of residual amounts of carbofuran over the MRL value would be an important health concern considering its toxicity. Forty six ccommercial packet turmeric powder samples of different companies and different batch No. of the same companies and loose turmeric powder samples were purchased from different markets of Dhaka city for analysis of pesticides residue. The control turmeric powder sample was made by purchasing fresh turmeric from Dhaka city market, washed, boiled for 1 h, dried under sunlight for 15 days and powdered by grinding machine. The QuEChERS method was used for extraction of turmeric powder sample and was analyzed by LC-PDA. Method performance was assessed by evaluating quality parameters, such as selectivity, limits of detection (LOD) and quantification (LOQ), linearity, repeatability and recovery. Linearity test was made by plotting calibration curves with different standard concentrations (5.00, 4.00, 2.00, 1.00, 0.50, 0.20, 0.10, 0.05, 0.04, 0.02 and 0.01 mg L^{-1}) versus the respective area of the peaks obtained. The linearity was excellent with correlation coefficients of $r^2 > 0.99$. The limit of detection (LOD) was 0.01 mg kg^{-1}at a signal-to-noise ratio of 3 and the limit of quantification (LOQ) was 0.03 mg kg^{-1}at a signal to noise ratio of 10. Among 46 samples, carbofuran residue was found in 37 (**Figure 25**). The amount of carbofuran residues were found to be in the range of 2.5 ± 0.07 to 23.1 ± 0.30 mg

kg^{-1} in the packet samples and 2.06 ± 0.14 to 7.8 ± 0.32 mg kg^{-1} in loose samples. The standard deviation in all the analysis was in the range of 0.06 to 1.53 % (< 4.00 %) which were proved the excellent repeatability of the study. For confirmation, two of the extracted samples were analyzed using liquid chromatography (LC-MS/MS) with Turbo Ion Spray tandem mass spectrometry in positive ionization mode. The m/z ratio for carbofuran was at 223 → 166, and the major fragment ions observed in product spectrum were at m/z 207 and 166 and 123.8, respectively.

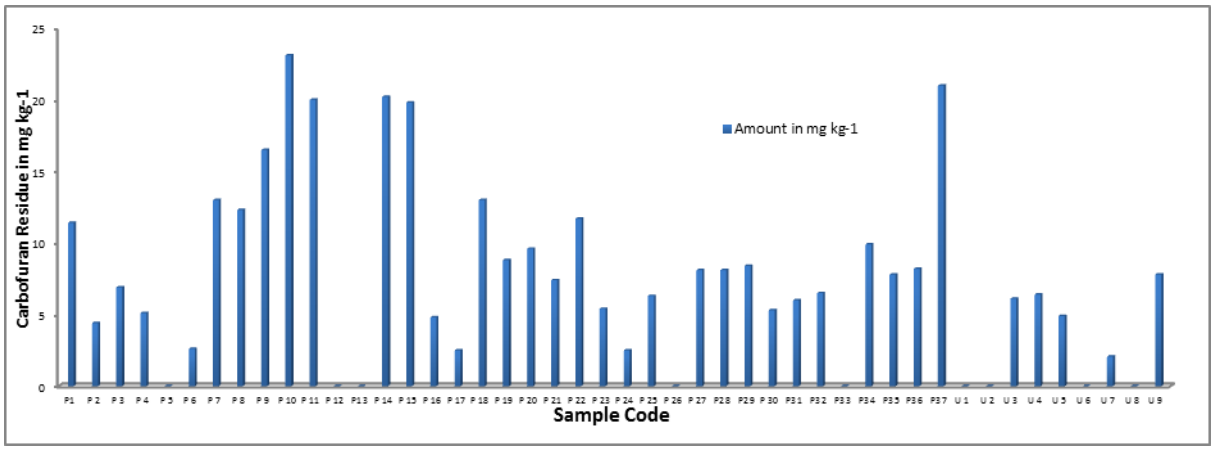


Figure 25: Amount of carbofuran (mg kg^{-1}) in turmeric samples

The efficiency of the method has been evaluated by spiking control turmeric powder samples with known concentrations of certified standard carbofuran working solutions (2, 4, 5 and 100 mg L^{-1}). The results of recovery experiments with seven (n=7) replications at four different spiking concentrations (0.4, 0.8, 1.0 and 20 mg kg^{-1}) were ranged from 92.52 ± 0.01 to 103.14 ± 2.41 %. The relative standard deviation (RSD) was < 12%. All values indicated good accuracy and repeatability.

The common vegetable of Bangladesh are cauliflower, cabbage, tomato, potato, beans, reddish, peas, carrot, radish, pumpkin, eggplant, drumstick, bitter-ground, arum-root, bind-weed and many others. The large amount of chemical fertilizers and pesticides are required to get higher yield. Residual pesticide higher than acceptable limit is harmful for health and environment. Diazinon (Shabion 60 EC) was applied to the tomato, cauliflower, eggplant and bean grown in BARI experimental fields and dissipation patterns in vegetable were studied. Four experimental fields were made for each vegetable sample (three for diazinon application and one control field where no pesticide was applied). In this study, samples were

extracted by using QuEChERS method (Anastassiades *et al*., 2003) and analysed by GC-ECD. Samples were extracted in three replicates and standard deviations were calculated. Linearity test was made by plotting calibration curves with different standard concentrations (0.5, 0.2, 0.1, 0.05, 0.02, 0.01, 0.005, 0.002 and 0.001 µg mL^{-1}) versus the respective area of the peaks obtained. The linear regression equations were obtained with r^2 0.9220, 0.9967, 0.9905 and 0.9976. The limit of detection and the limit of quantification were found to be 0.001 and 0.003 mg L^{-1}, respectively. The study revealed that under open field conditions, the initial deposits (2 hours after spraying) of diazinon (2 mL /L) were 4.02 ± 0.37, 1.66 ± 0.24, 1.29 ± 0.09 and 0.152 ± 0.007 mg kg^{-1} for cauliflower, tomato, eggplant and bean, respectively. The residues of diazinon in tomato, bean, cauliflower and eggplant were found in the range of 0.02 ± 0.01 to 1.66 ± 0.24, 0.005 ± 0.001 to 0.152 ± 0.007, 0.03 ± 0.01 to 4.02 ± 0.37, and 0.02 ± 0.01 to 1.29 ± 0.09 mg kg^{-1}, respectively. The mean recovery percentage was found to be 99 to 105 % for tomato at 0.02, 0.50 and 1.50 mg kg^{-1} spiking concentration levels. For cauliflower the mean recovery at 0.05, 2.00 and 4.00 mg kg^{-1} spiking levels was 97 to 104%. The recovery at 0.005, 0.01 and 0.15 mg kg^{-1} spiking levels was 89 to 108 % for bean. For eggplant the mean recovery was 93 to 104 % at 0.02, 0.50 and 1.00 mg kg^{-1} spiking levels. The standard deviations of recovery percentages for four vegetable were in the range of 0.01 to 0.17 % which showed the repeatability of the method was excellent. The maximum residue limit (MRL) of diazinon on cauliflower, tomato, eggplant and bean has been fixed by CODEX is 0.5 mg kg^{-1}. The diazinon residues declined to a level below the maximum residue limits within 3, 3 and 10 days for eggplant, tomato and cauliflower, respectively. The residue of diazinon was below the maximum residue limit even at 0 day (two hours after spraying) for bean. The waiting periods for safe consumption of cauliflower, tomato, bean and eggplant are 10, 3, 0 and 3 days, respectively. The degradation kinetics of diazinon in tomato, cauliflower, eggplant and bean were determined by plotting residue concentration against time. The dissipation of diazinon residues was followed first-order kinetics. Half-life ($t_{1/2}$) of the total diazinon residues was observed to be 2.23, 2.63, 0.90 and 1.12 days on tomato, cauliflower, eggplant and bean, respectively (**Figure 57**).

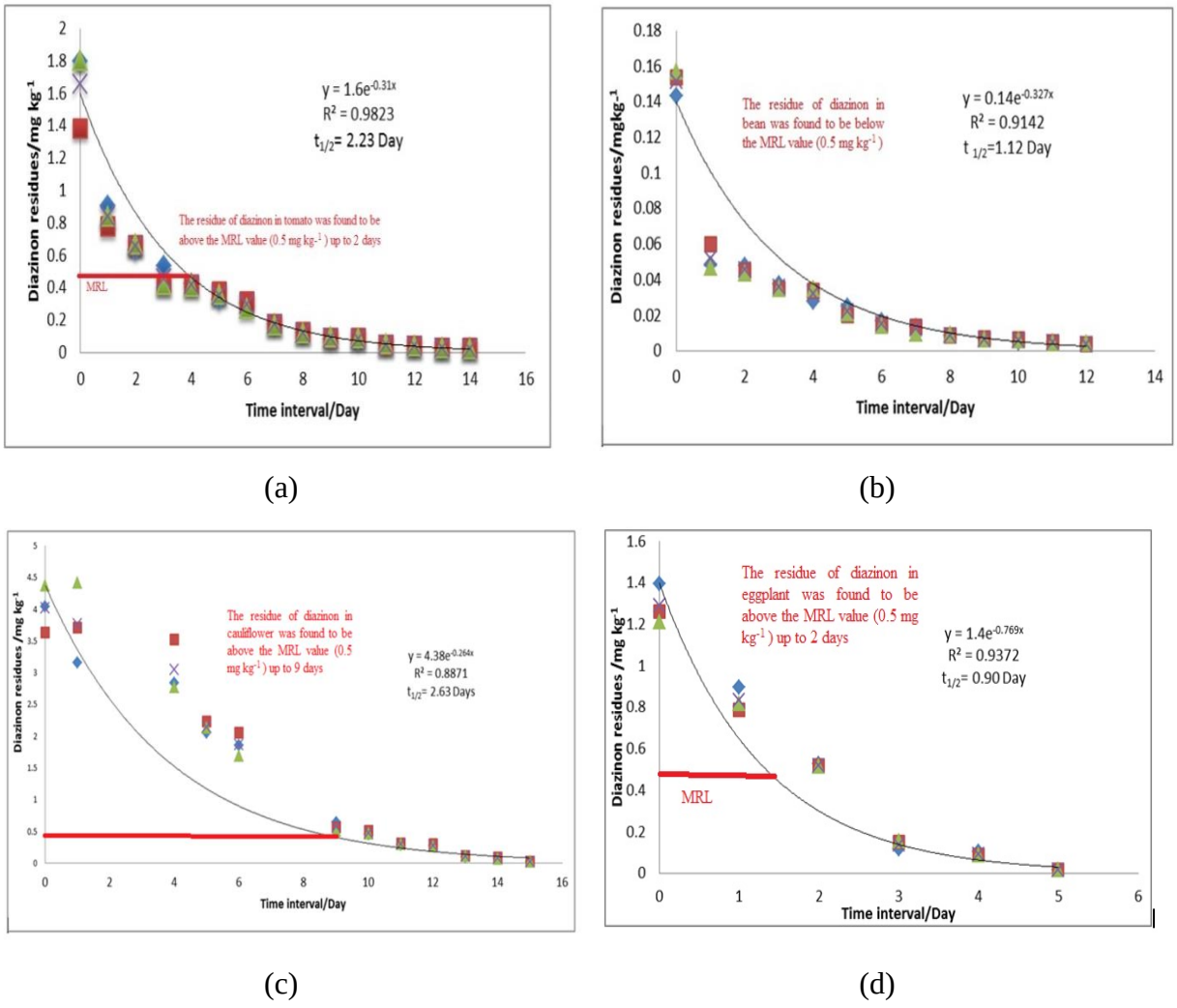


Figure 57: Dissipation curves of diazinon in tomato (a), bean (b), cauliflower (c) and eggplant (d) samples

The residue of diazinon at 0 day (**Figure 42)** was found to be followed the following order:

Cauliflower (4.02 ± 0.37 mg kg^{-1}) > Tomato (1.66 ± 0.24 mg kg^{-1}) > Eggplant(1.29 ± 0.09 mg kg^{-1}) > Bean (0.152 ± 0.007mg kg^{-1})

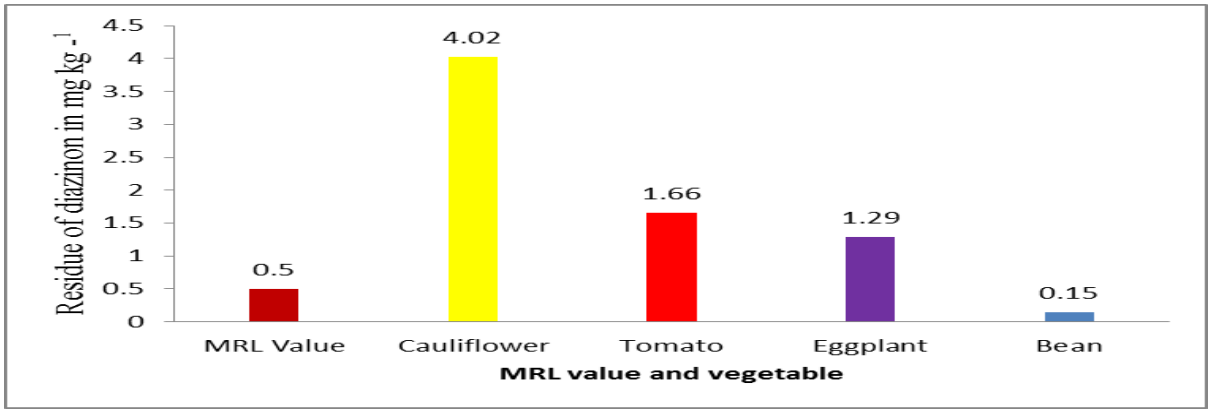


Figure 42: The MRL value and the residue of diazinon in cauliflower, tomato, eggplant and bean samples at 0 day (2 hours after spraying**)**

The post-harvest intervals of carbosulfan (Marshal 20 EC) were studied in tomato grown in BARI experimental fields. Four experimental fields were made for tomato sample (three for carbosulfan application and one control field). In this study, samples were extracted by using QuEChERS method (Anastassiades *et al*., 2003) and analysed by GC-FID. The study of dissipation of carbosulfan residues on tomato was carried out in open field condition. Calibration curves were linear over the calibration ranges with coefficient of determinants 0.9978 and 0.9967 for carbosulfan. The LOD and LOQ were 0.10 and 0.30 mg kg^{-1}, respectively. The extraction efficiency was evaluated by recovery experiment. The mean recovery of carbosulfan in tomato was 98 to 102 % at 2.50 and 4.00 mg kg^{-1} spiking levels with five replications. The standard deviations of recovery percentages were 0.25 to 0.35 %; thus the repeatability of the method was excellent. The relative standard deviation was 8.64 and 10.15 % which showed excellent efficiency of the method.

The initial deposits of carbosulfan were found to be 9.43 ± 0.16 mg kg^{-1} at 2 hours after spraying, which degraded to BDL by 15th day. The initial deposits dissipated to 6.17 ± 0.07, 5.75 ± 0.37, 4.90 ± 0.29, 4.45 ± 0.02, 4.33 ± 0.41, 4.05 ± 0.20, 3.86 ± 0.24, 2.96 ± 0.47, 2.25 ± 0.29, 2.04 ± 0.24, 1.95 ± 0.26, 1.68 ± 0.25 and 1.19 ± 0.06 mg kg-1, at 1, 2,3 ,4, 5,6, 7, 8, 9, 10, 11, 12, 13 and 14 days after spraying, respectively. The dissipation pattern showed constant decrease of residues from first day to 14th day (**Figure 43**).

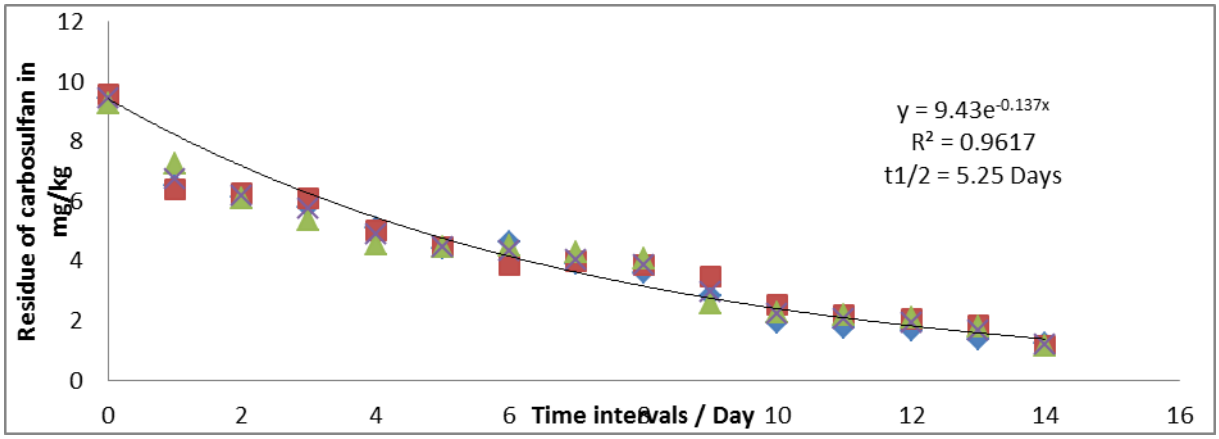


Figure 43: Dissipation curve of carbosulfan in tomato

According to Europion Union, the MRL value of carbosulfan in tomato is 0.05 mg kg-^{1}. The residue of carbosulfan in tomato was found to be above the MRL value (0.05 mg kg^{-1}) up to 14 days (9.43 ± 0.16 to 1.19 ± 0.06 mg kg^{-1}). The degradation kinetics of carbosulfan in tomato was determined by plotting residue concentration against time (**Figure 43**). The dissipation of carbosulfan residues on tomato was followed first-order kinetics. Half-life ($t_{1/2}$) of the total carbosulfan residues was observed to be 5.25 days.

The four vegetable samples cauliflower, eggplant, tomato and bean are very popular winter vegetable in Bangladesh. Pesticide residue in food has become a consumer's safety issue and the consumers have the right to know how much pesticide get incorporated in the food they eat. The detection, identification and quantification of pesticide in the food are becoming the public interest. However, very little references are available on the presence of pesticides in vegetable in Bangladesh (J. A. Khatoon *et al*, 2004).Therefore, this study was undertaken to assess the quantity of residue of five frequently used pesticides in cauliflower, eggplant, tomato and bean. Forty five vegetable samples namely bean, eggplant, cauliflower and tomato were purchased from different markets of Dhaka, Noakhali and Kurigram districts. Cypermethrin, chlorpyrifos, diazinon, fenvalerate and quinalphos were detected in some of them. Quantification of residues was done on a gas chromatograph (GC) with an electron capture detector (ECD). The QuEChERS method (Anastassiades *et al*., 2003) with some modifications was used for analysis of pesticide residue in market vegetable samples. The analytical method was validated in terms of selectivity, linearity, sensitivity and recovery. Selectivity was assessed by injecting standard mixture of pesticides, blank matrices and blank

matrices spiked with the mixture of pesticides simultaneously and by checking their retention times. The chromatograms of the standard, blank sample and spiked samples were checked and no interference peak was observed at the retention time of chlorpyrifos, cypermethrin, diazinon, fenvalerate and quinalphos. The target peaks were obtained at 7.7, 9.2, 9.9, 17.5 and 19.5 min for diazinon, chlorpyrifos, quinalphos, cypermethrin and fenvalerate, respectively. The amount of chlorpyrifos, cypermethrin, diazinon, fenvalerate and quinalphos in the spiked and market vegetable samples were determined via calibration curve. Calibration curve at different ranges were made for each five pesticide in order to quantify them in the spiked and market vegetable samples in the proper range. The linearity was excellent for each pesticide with coefficient of determinant $r^2 \geq 0.9912$, 09962, 0.9929, 0.9947 and 0.9907 for chlorpyrifos, cypermethrin, diazinon, fenvalerate and quinalphos, respectively. The LOD was found to be determined 0.50 µg L^{-1} for chlorpyrifos, 2.50 µg L^{-1} for diazinon and quinalphos and 5.0 µg L^{-1} for cypermethrin and fenvalerate, respectively. LOQ was found to be determined 1.65 µg L^{-1} for chlorpyrifos, 8.25 µg L^{-1} for diazinon and quinalphos and 16.5 µg L^{-1} for cypermethrin and fenvalerate, respectively. The different values of LOD and LOQ (**Figure 51**) for five different pesticides were due to their different sensitivities in GC–ECD.

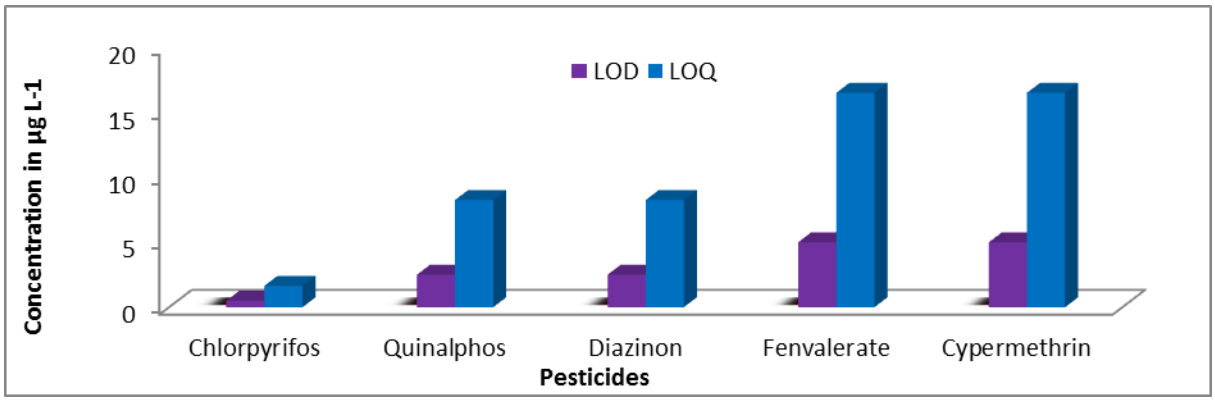
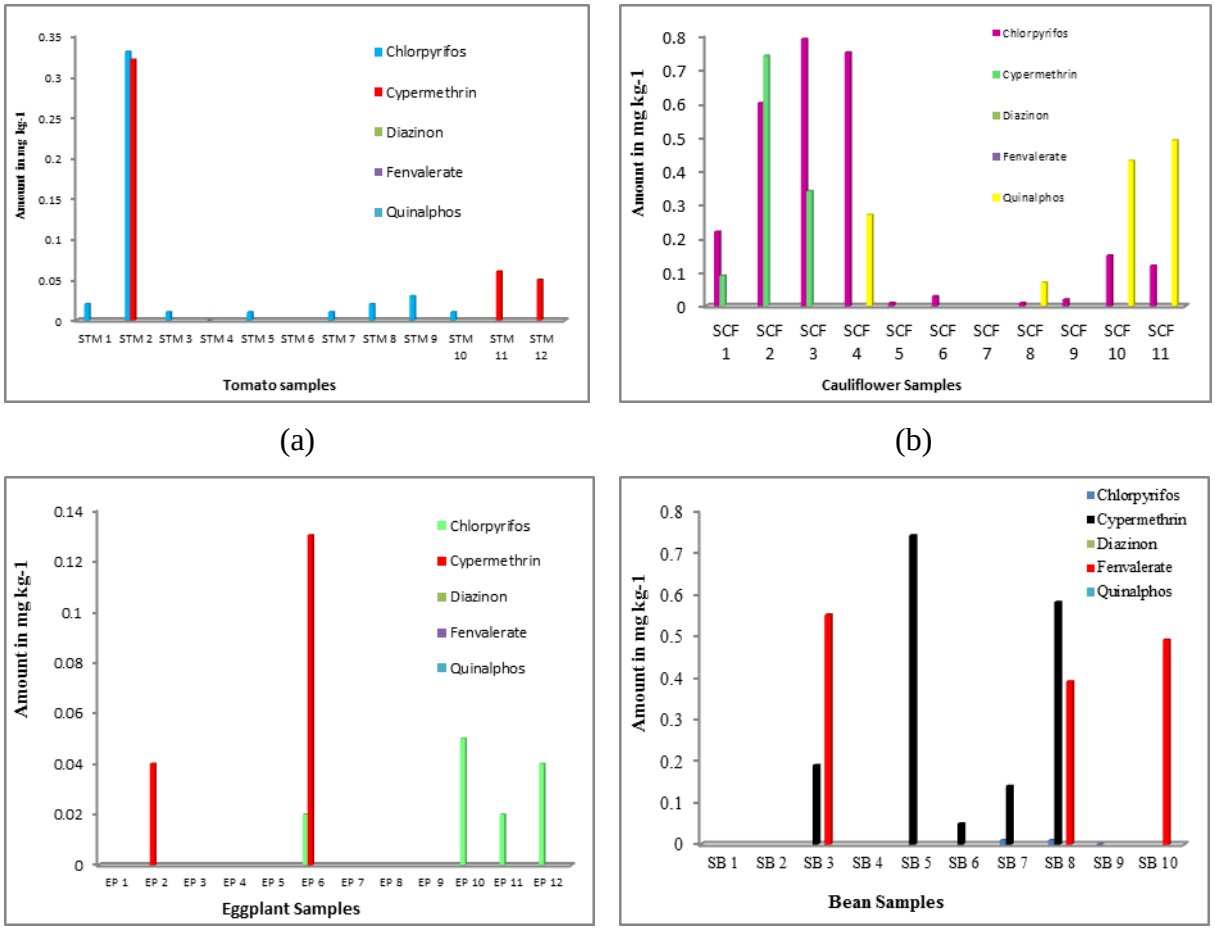


Figure 51: LOD and LOQ of Chlorpyrifos, Quinalphos, Diazinon, Fenvalerate and Cypermethrin

The extraction efficiency of the analytical procedure was evaluated via recovery experiments. The average recovery of chlorpyrifos in tomato (n=5) was 98.48 ± 2.73% and in eggplant (n=6) was 99.57 ± 6.98 % at spiking level of 0.05 mg kg^{-1}. In bean (n=3), the average recovery of chlorpyrifos was 88.51 ± 2.64 % at spiking level of 0.15 mg kg^{-1}. The average recovery of cypermethrin in tomato (n=5) was 79.65 ± 5.56 %, in eggplant (n=6) was 86.29 ±

7.33 % and in bean (n=3) was 97.43 ± 8.52 % at spiking level of 0.10 mg kg^{-1}. The average recovery of diazinon in tomato (n=5) was 109.92 ± 2.33 % and in eggplant (n=6) was 101.41 ± 4.72 % at spiking level of 0.10 mg kg^{-1}. In bean (n=3), the average recovery of chlorpyrifos was 106.78 ± 3.55 % at spiking level of 0.15 mg kg^{-1}. For fenvelarate, the average recovery in tomato (n=5) was 90.88 ± 2.15 % and in eggplant (n=6) was 84.10 ± 8.91 % at spiking level of 0.10 mg kg^{-1}. In bean (n=3), the average recovery of fenvelarate was 90.04 ± 9.29 % at spiking level of 0.15 mg kg^{-1}. For quinalphos the average recovery in tomato (n=5), eggplant (n=6) and bean (n=3) were 78.28 ± 4.85 , 85.15 ± 7.72 and 85.28 ± 2.32 % at the spiking level of 0.10,0.15 and 0.20 mg kg^{-1}, respectively.

In 12 market tomato samples (**Figure 58a**), the residue of chlorpyriphos was detected in 9 in the range of 0.01 ± 0.01 to 0.33 ± 0.02 mg kg^{-1} (MRL 0.2 mg kg^{-1}) and cypermethrin was detected in 3 samples in the range of 0.05 ± 0.01 to 0.32 ± 0.04 mg kg^{-1} (MRL 0.5 mg kg^{-1}).



(a) (b)

(c) (d)

Figure 58: Quantity of Pesticide Residues in Different Tomato (a), Cauliflower (b), Eggplant (c) and Bean (d) Samples

In 11 market cauliflower samples (**Figure 58b**), the residue of chlorpyriphos was detected in 10 in the range of 0.01 ± 0.01 to 0.79 ± 0.02 mg kg^{-1} (MRL 0.05 mg kg^{-1}), cypermethrin was detected in 3 samples in the range of 0.09 ± 0.01 to 0.74 ± 0.16 mg kg^{-1} (MRL 1.0 mg kg^{-1}) and quinalphos was detected in 4 samples in the range of 0.07 ± 0.01 to 0.49 ± 0.08 mg kg^{-1} (MRL 0.2 mg kg^{-1}).

In the 12 market eggplant samples (**Figure 58c**), the residue of chlorpyriphos was detected in 4 samples in the range of 0.02 ± 0.01 to 0.05 ± 0.01 mg kg^{-1} (MRL 0.5 mg kg^{-1}) and cypermethrin was detected in 2 samples in the range of 0.04 ± 0.01 to 0.13 ± 0.01 mg kg^{-1} (MRL 0.2 mg kg^{-1}).

In 10 market bean samples (**Table 58d**), the residue of chlorpyriphos was detected in 3 samples (0.01 ± 0.01 mg kg^{-1}; MRL 0.01 mg kg^{-1}), cypermethrin was detected in 5 samples in the range of 0.05 ± 0.01 to 0.74 ± 0.09 mg kg^{-1} (MRL 0.05 mg kg^{-1}) and fenvalerate was detected in 3 samples in the range of 0.39 ± 0.05 to 0.55 ± 0.04 mg kg^{-1} (MRL 1.0 mg kg^{-1}).

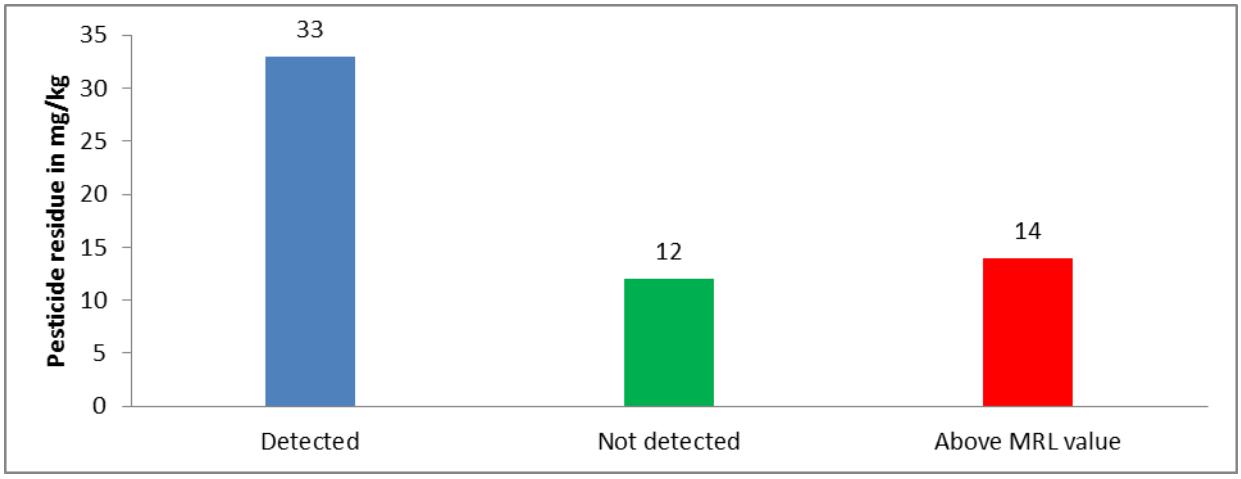


Figure56: Pesticide residues in the analysed vegetable samples

Out of 45 market vegetable samples, single or multiple pesticide residue/residues were found to be present in 33 samples (**Figure 56**). In 14 vegetable samples, the residues of pesticides were above the MRL values.

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