Spatial non-targeted screening and risk assessment of organic pollutants in honey samples collected from Bhakkar District

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This work is submitted as an MS thesis in partial fulfillment of the requirement for the degree of

(MS in Chemistry)

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MS THESIS WORK

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Dedicated to

My Father

His love, encouragement, hard work, prays and faith

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ABSTRACT

Environmental pollution is reaching at alarming proportions globally. Due to increased urbanization, vast industrialization, energy consumption, and waste production, there has been an increasing trend of pollution worldwide. To keep environmental parameters in balance proper monitoring of environmental pollutants is important. Biomonitoring is the quality check of the environment with the help of living organisms and their products. This study was performed for the first time in Pakistan in the Bhakkar district of Punjab. The presence or absence of organic airborne pollutants was determined with the help of honey as a biomonitor. 34 honey samples were collected from different nearby locations of the Bhakkar district, out of which 30 were nominated as polluted samples based on the NIST library match. QuEChERS a robust method of extraction was performed for the extraction of the analyte of interest (organic component) from the sample. After that, the analyte was referred to GC/MS analysis for identifying pollutants in samples. The presence or absence of these identified pollutants was further supported with the help of FTIR and UV-Visible spectroscopy. Almost 36 types of pollutants were identified in 30 different samples, including systemic toxins, carcinogenic, mutagenic, ecotoxic, cytotoxic, and neurotoxins based on literature. HRI (health risk index) values were assumed by using a relative concentration of identified pollutants. For risk factor assessment specific software technique (EPI-suite software) was used. This study has furnished a clear picture of the environment confirming the presence of different organic pollutants in the targeted area. This work also proposed honey as a good bioindicator of the environment because of its ease of availability in rural areas, mobility, intense foraging activity, and morphological features of the honeybee.

Chapter 01

1-Introduction

1.1 Background and Motivation

Great efforts have been made to eradicate pollution but it's still an alarming issue.[1] Due to increased urbanization and vast industrialization the energy consumption and waste production have been increased. International public health problems, global environmental issues including climate change, acid depositions, green-house gases effects, water and soil pollution, waste management, and other leading environmental issues should be investigated through many ways including chemical, physical, physiochemical, socio-economical, legislation, and environmental monitoring and engineering systems to minimize environmental pollution.

This issue is very common in under-developed countries where classical methods of waste management are used. Due to poor management of industrial and domestic waste, contaminated water supplies, biomass fuel causing indoor air pollution are the major reasons affecting human health in these regions. Environmental pollution also exists in poor sectors of developed countries reflecting it a global issue of concern existing everywhere.

During the recent few years, the usage of modern chemicals has been increased. Pollutants Emerging from road traffic, food ingredients, pesticides, and water treatment plants are due to the use of modern chemicals in the composition of all things. During product formation, these chemicals are used in safer limits or threshold limits depicting no risk for the surrounding and human health, but its long-term exposure and aftereffects risk are not negligible posing a serious concern regarding human health even a small increase in relative risk can cause major public health concerns. Some novel types of pollutants have been added to the list of environmental pollutants due to new sources of exposure and risk factor. Some of them may include endocrine disrupters which can disrupt human health for the living being. There is a need to address this issue on a serious note. This issue not only requires assessment but also there needs to understand the magnitude of the problem, its causes, and underlying processes. In this way, we can take an action to control the spread of diseases and the effects of environmental pollution on our lives.

These days due to the increase in the food demand the modern method to increase the quantity of food like the use of agrochemicals has been increased. These agrochemicals include

fertilizers and pesticides .[2] During the last five decades, due to pesticides usage, the quality and quantity of food have been enhanced but their excess usage has drastic effects on side targets or non-targeted organisms which include human beings too.[3] Misuse of pesticides is also a problem especially in Pakistan due to the lack of awareness in farmers regarding pesticides usage. A large portion of pesticides applications hit the nontargeted sectors due to nontechnical staff and defective instruments.[4]

1.2 Environmental pollution

Any Potential damage to the environment or human health due to the presence of an agent in that environment is called Environmental pollution.[1] Any substance or energy added to the environment has undesirable and drastic effects on the environment. This damage could be long-term or short-term resulting in a change in the growth rate of targeted species like plants and animals. Pollutants may interfere with human convenience, health, comfort, and possessions.[5] Every year about 400 million metric tons of hazardous wastes are produced [6] There is a threshold and allowed limit for every chemical to be used but If this concentration exceeds the threshold limit it becomes a pollutant.[7]

Figure 1: Diagram of Environmental pollution

1.3 Major types of Environmental Pollution

- Air pollution
- Soil or land pollution
- Water pollution [8]

1.3.1 Air pollution

Air pollution is the presence of one or more unfavorable content in the composition of air. Its duration would be longer and would not be safer limits. Air pollution is the contamination of air. It may have drastic effects on biotic as well as abiotic factors in the ecosystem. Air pollution is the major contributor among all types of pollution and a serious topic of concern in recent decades due to its drastic toxicological effects.[9] As far as non-communicable diseases are concerned these are linked to the 70 percent air pollution death rate. So this is a major cause for non-communicable deaths.[10]

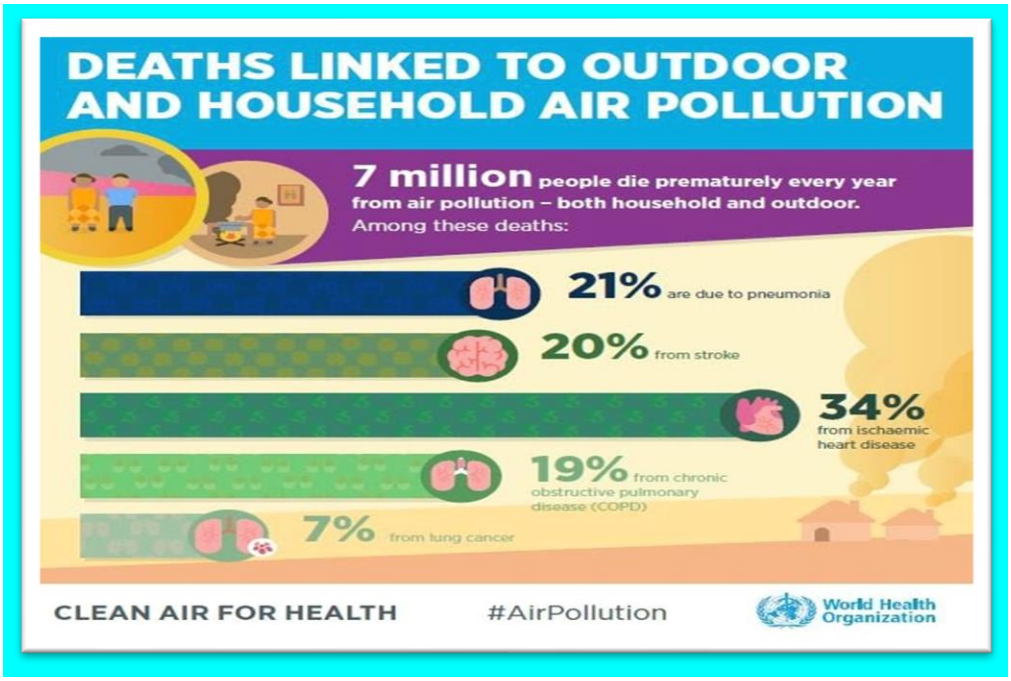


Figure 2: Risk factor Assessment

1.3.2 Water pollution

Water pollution is the contamination of water bodies like rivers, oceans, lakes, and underground water supplies.

Water gets contaminated via two types of sources:

1. **Point sources**
    - Directly pointed towards the water bodies from the source of origin of pollution
    - Easy to manage
2. **Non-point sources:**

- These sources are related to many diffuse sources
- Difficult to regulate.
- Some of the sources are
- Industrial and community wastewater
- Agricultural sources, thermal pollution, and underground water pollution

Marine pollution, river discharge, manmade pollution, and oil spills, etc. [11] Fresh and clean water supply is not only a basic need but also an important thing for the safety of poor and children.[12] Any organic, inorganic, or biological charge affects the quality of water when present in a larger amount and leads toward water pollution.[13]

1.3.4 SOIL POLLUTION

The addition of any undesired substituent which alters the composition of soil and makes it less fertile and productive is called soil pollution. The pollutant may change the physical, chemical, and biological features of the soil.

- **Soil pollutants**

  Any substance which affects the pH, composition, texture, and quality of soil is called a soil pollutant.

  Examples:

  The use of pesticides for pests' control, fertilizers, and other spray products to enhance the growth of plants have drastic effects on soil ultimately.
- **Industrial waste:**

  Heavy metals include Hg, Cr, Pb, alkali products, and organic solvents and chemicals.
- **Agricultural waste:**

  Fertilizers, pesticides, insecticides, and manures.

  Discarded stuff, plastic sheets, bags, and radioactive elements.

1.3.5 Effects

Soil pollution results in infertility of soil, soil erosion, and salinity of the soil. This results in the reduction of crop yield and quality. Which ultimately affects the quality of the soil. It may also disturb ecological balance by disturbing flora and fauna.

1.4 Categories of pollutants

The materials that cause pollution are of two types:

1.4.1 Persistent pollutants:

This is a type of pollutant that remains persistent in the environment for a longer period without converting into a less harmful form. This is non-biodegradable by nature.

Examples: Organic pollutants i.e., pesticides, nuclear waste products, and plastic products.

1.4.2 non-persistent pollutants:

These pollutants are the opposite of persistent pollutants and break down in a simple form. If this process of breaking down is done by living organisms, then such pollutants are referred to as biodegradable pollutants. These types of pollutants are less harmful, to the environment because these pollutants can be converted into less harmful forms. These are biodegradable by nature.[14]

1.5 Pollutant classification

1.5.1 Primary Pollutants:

Those pollutants retain their original form when added to the environment. Their emission occurs directly into the environment.

Example: DDT, Plastic

1.5.2 Secondary Pollutants:

Secondary pollutants are not emitted directly into the environment. These pollutants are formed due to the interaction of primary pollutants among themselves. For example

Peroxyacyl nitrates are formed by the interaction of NOx and hydrocarbons.[8]

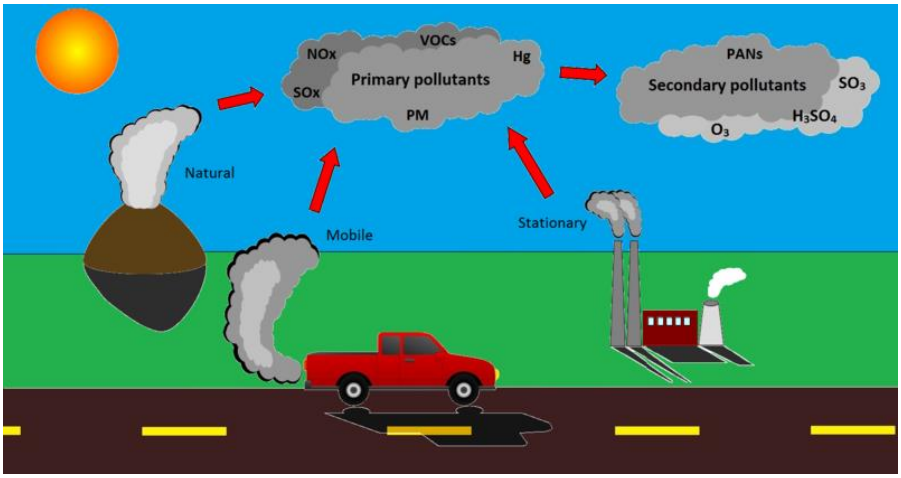


Figure 3: Sources of Pollutants

1.6 According to the origin

1. Man-made Pollutants
2. Natural Pollutants

1.7 Health effects of pollutants

Air pollution is the biggest contributor towards total pollution this not only destroys our climate sustainability but also contributes towards public health illness and deaths there are different types of pollutants causing multiple types of diseases. These pollutants include particulate matter, ozone, volatile organic compounds, dioxins, and polycyclic aromatic hydrocarbons, oxides of sulfur and nitrogen are major pollutants causing health issues in human beings. Ozone causes cardiovascular issues in human beings. Carbon mono oxide leads to direct poisoning when inhaled in larger amounts. Lead also causes poisoning or chronic intoxication depending on exposure rate., all the above-mentioned pollutants cause neurological issues, respiratory problems, chronic obstructive pulmonary disease, asthma, bronchitis, lung cancer, gene mutations, and cutaneous diseases. As a result of climate change, many infectious diseases' geographical distribution is being affected.[15]

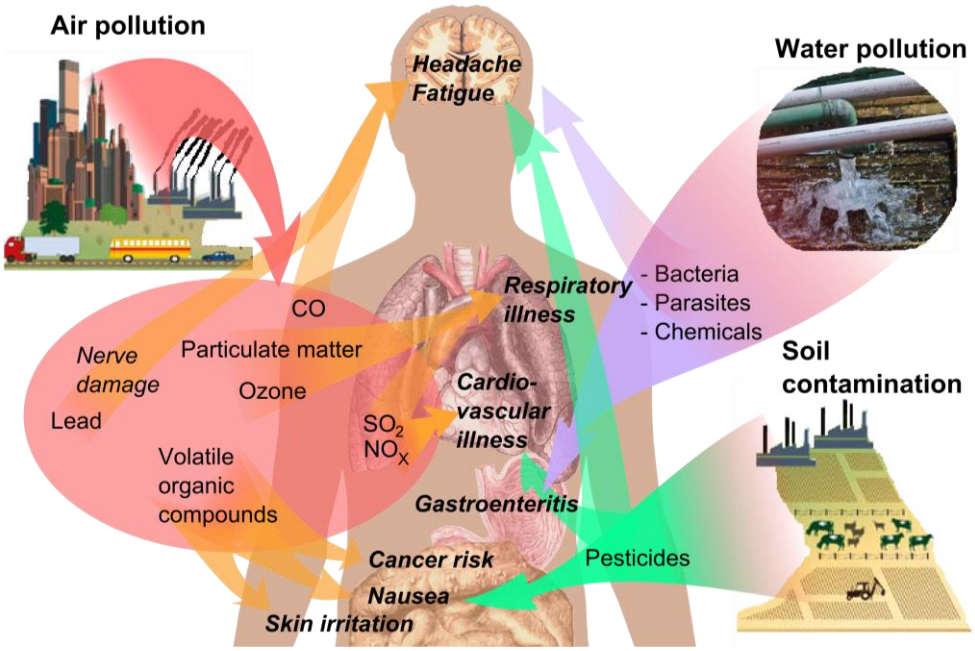


Figure 4 : Effects of pollution on human health

1.8 Monitoring Environmental Pollution

Due to immense changes in biodiversity, climate change, water quality, and land conditions cause fluctuations in the environment and its quality is a serious topic of concern these days. As we know the environment has a direct influence on our lives.

According to P. Gisbert, "Environment is anything immediately surrounding an object and exerting a direct influence on it. So environmental safety is very important to keep humans and wildlife in the safe zone. To keep the environment and environmental factors in balance and harmony proper monitoring or quality check Is important which is called environmental monitoring.

1.8.1 Environment monitoring

The process which checks the quality of the environment is called environmental monitoring Environmental monitoring can be done.

- Chemical methods
- Physiochemical methods
- Physical methods
- Biological methods or biomonitoring[16]

1.8 Biomonitoring

This phenomenon involves the usage of natural monitors or organisms to check environmental contamination of surrounding air or water.

- **Biomonitor/bioindicator;** A group of organisms whose presence and behavioral traits can be easily linked with some environmental conditions can be used as a marker or quantitative test. These are those organisms that absorb certain toxins in the environment to their body or tissues over some time.

Bioindication is actually "breakdown of the information content regarding biosystems to make to evaluate complete areas".[17]

1.8.1 Types of Biomonitoring

- Qualitative biomonitoring

  Observing and noting changes in organisms in the presence or absence of contaminants in the environment are assessed.

- Quantitative biomonitoring

  The amount and quantity of pollutants present in the organism is measured[16]

  Examples:

  Lichens, microalgae, Honeybees, or their products i-e honey

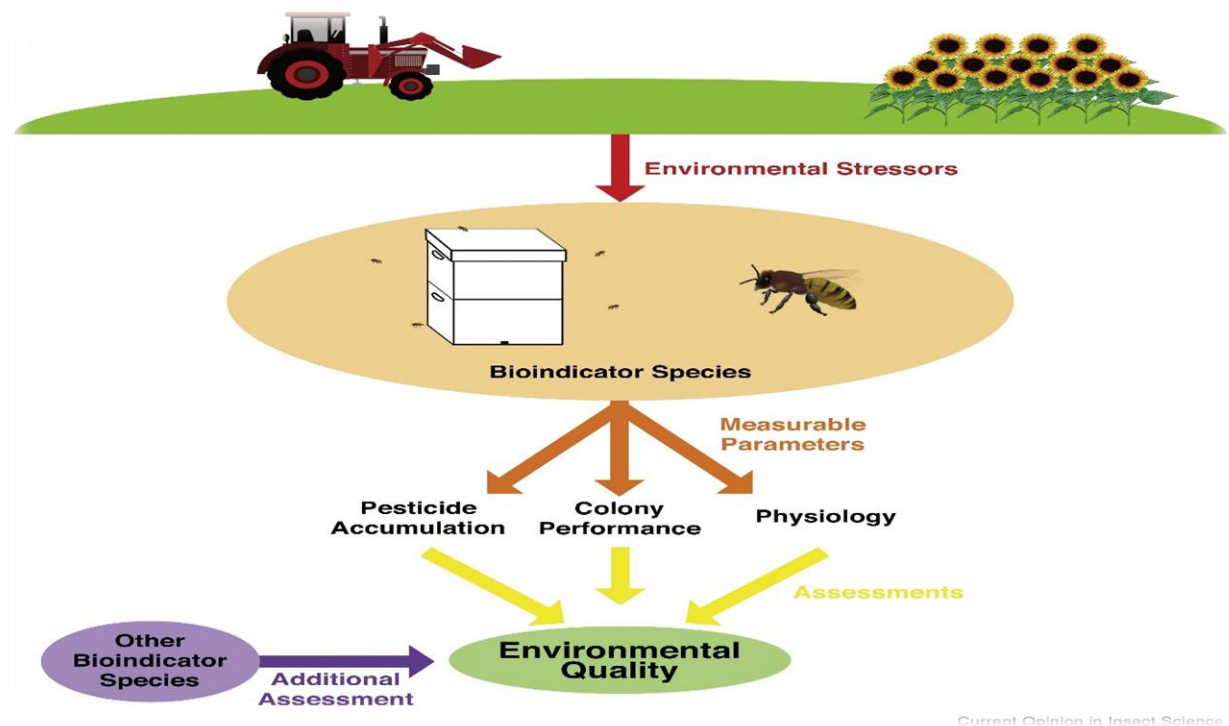


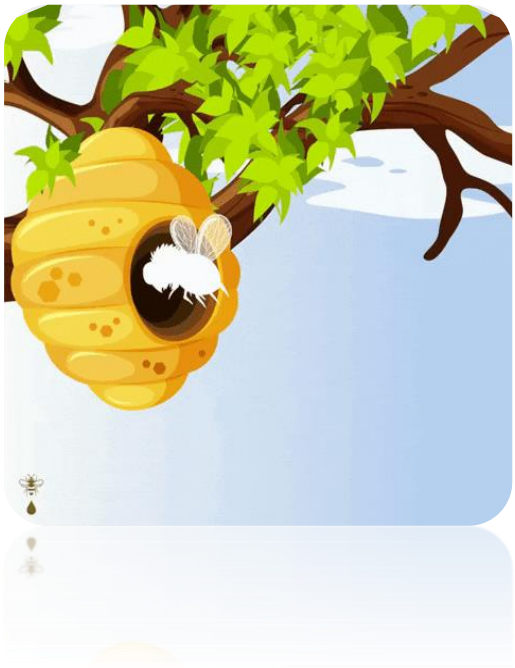
Figure 5 : Scheme of biomonitoring

1.9 Honey

Honey a natural sweetener produced by honeybees is a magical food item having multiple uses and applications. Honey is a complex organic compound made up of 300 different chemical substances majorly including carbohydrates, proteins, dyes, organic acids, enzymes, hormones, vitamins, fatty acids, minerals, etc. The amount and nature of compounds depend upon the type of plant from which honey was made and deposited. Honey may absorb different external substances or pollutants inside it. As honey is a natural product it should be free from any outside irreverent chemical or pollutant due to its medicinal uses for almost every kind of diseases. [18]

1.9.1 Why honey as Biomonitor?

Honeybee and its products like honey, pollen, waxes, etc. are good environmental biomonitor due to the following reasons.



1.9.1.1 Mobility

The honeybee is a moving insect that is delocalized and covers the maximum distance in its territory. So, we can get maximum information from its products regarding targeted sites due to its moving ability.

1.9.1.2 Intense foraging activity

Honeybees can transfer or transport pollens grains at maximum distances and help in pollination. In this way honey bee can also carry other side products including pollutants and chemicals along with these required products which become ultimately part of their bodies and body products.

1.9.1.3 Morphological features

Honeybees have specific furry body composition which increases the chances of pollutants attachments to their bodies.

1.10 Sources of Pollutants

A honeybee can get pollutants from almost all the sources which are deposited in air, plants surfaces, flowers, from inside soil and water dissolved content. Due to all these reasons, honeybees, and their products (pollen, nectar, and honey) are good indicators of environmental pollutants. These pollutants could be persistent organic pollutants i.e, pesticides, heavy metals, radioactive elements, etc.[19]

1.11 Bhakkar District

1.11.1 Why Bhakkar District?

Bhakkar is among the districts of the province of Punjab. Bhakkar was made district out of parts of Mianwali in 1952. If we see Bhakkar as an area-wise its mostly area consists of riverine along with the Indus named as" kaccha". The district area is mostly deserted which is named as Thal desert.[20] Location-wise Bhakkar is present on the west side of Punjab province. This city is bordered by Layyah to its south Jhang in southeast D. I Khan on the west side and Khushab in its northeast.

According to the census in 2017 , Bhakkar is administratively divided into four tehsils and sixty-four union councils. The total population of the Bhakkar district was 1,650,000 in 2017. Based on the 1998 census, the major languages are Saraiki by 73 percent, Punjabi 18 percent, and Urdu 7 percent.[20]

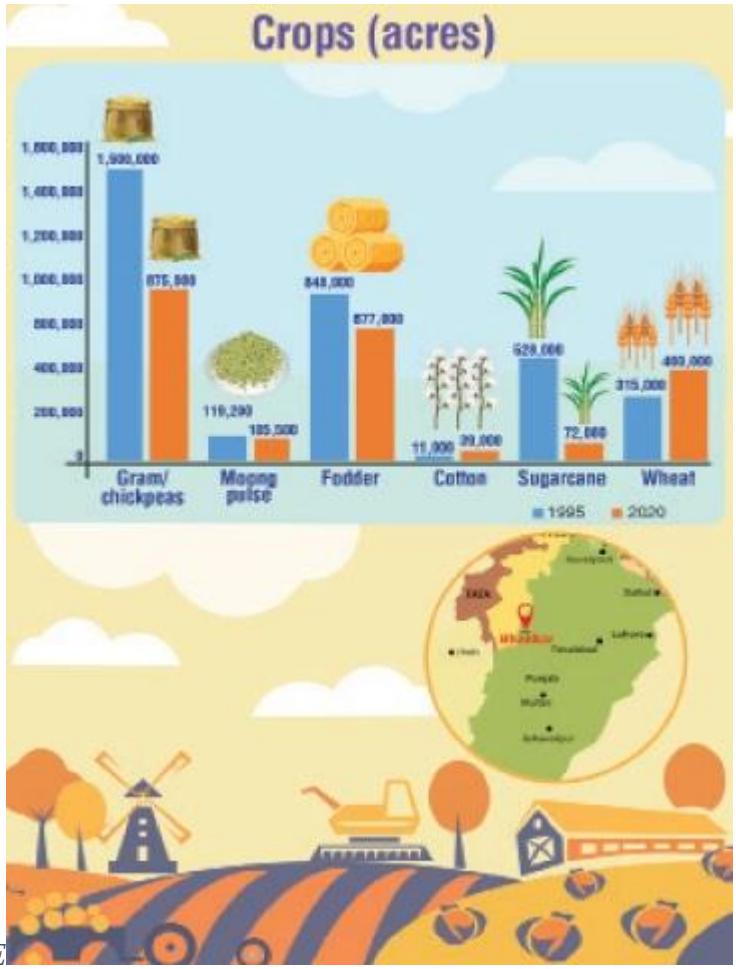


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Figure 6: Major production in Bhakkar District

1.12 QuEChERS Method of Extraction

QUECHERS stands for a quick, easy, cheap, rugged, and safe method of extraction. This method is a very effective technique of sample preparation for pesticide multi-residue analysis in different complex sample matrices i.e. honey or plant and food extracts. [21]This method, we use polar solvents to contribute to extracting water-containing matrices. Buffering salts are being added to get phase separation. It's a two-step process. One is extraction while the other is clean up. To remove potential interferences, different sorbents are used. Sorbents include PSA (primary secondary amine, C18, etc. Interferences are removed by a quick shock followed by centrifugation. This method is proved to be successful for different samples i.e., soil, vegetables, oil, olives, honey, eggs, and grains.[22]

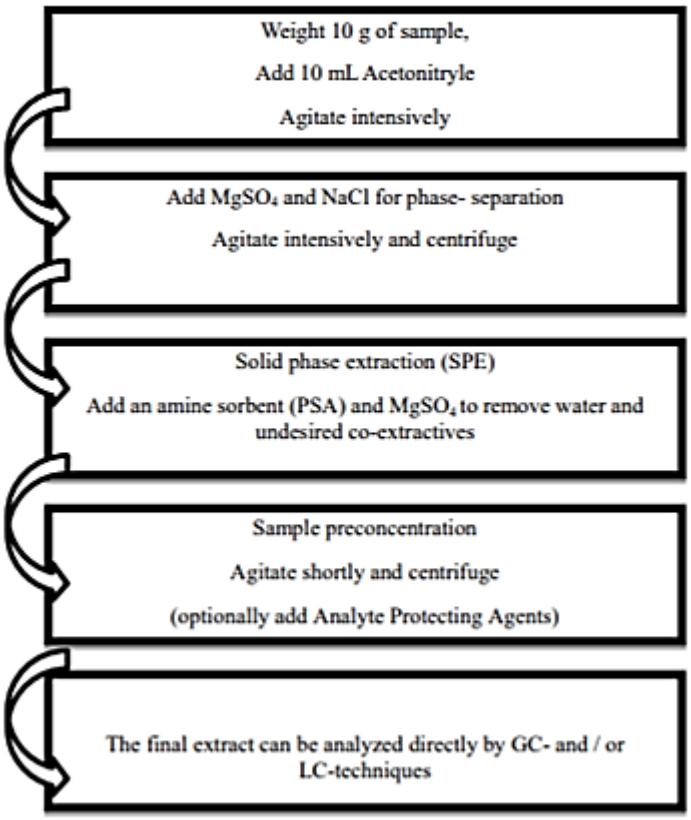

Fig. 1. Steps in the QuEChERS procedure of sample preparation for the determination of pesticide residue in fruit and vegetables

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Figure 7 : Steps performed during the preparation of the sample for QuEChERS procedure

1.13 Techniques used for Characterization

1.13.1 Gas Chromatography

1.13.1.1 Definition

Gas chromatography is a type of separation technique that is used for the analysis of volatile compounds.

1.13.1.2 Principle

The compounds of the mixture are distributed between two phases stationary and mobile phase. The mobile phase is carrier gas that carries the sample components which then meet the stationary phase while passing. Due to the difference in properties and chemical composition

of each component they will show different affinities towards the stationary phase. As a result, they can travel and elute through the column at different rates and get separated based on their affinities.[24]

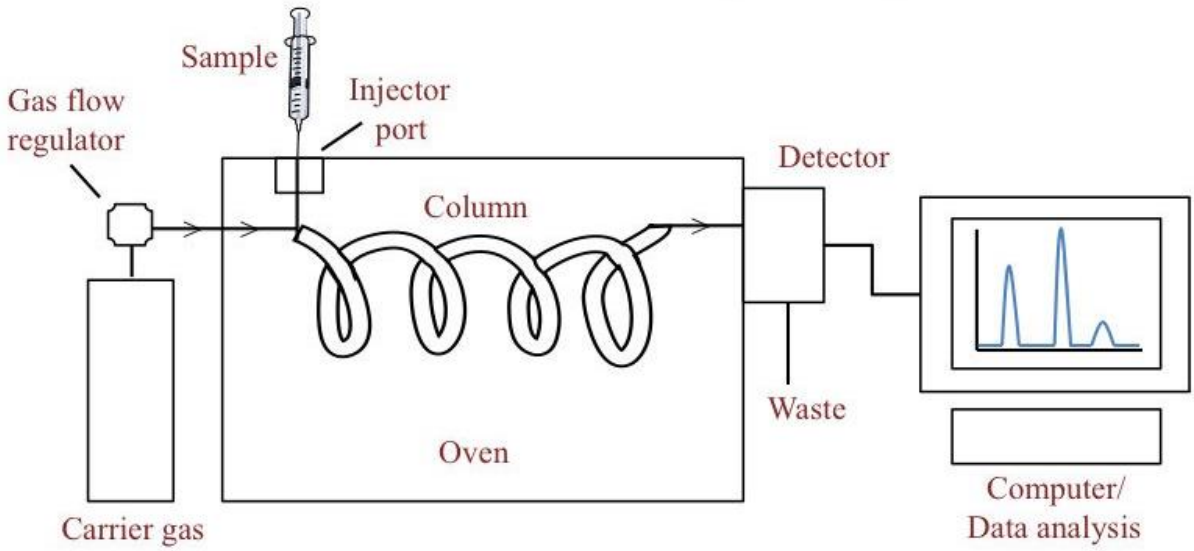


Figure 8: Representation of working principle of GC

1.13.1.3 non-target screening using GCMS

Gas chromatography/mass spectrometry GC/MS is the most abundant analytical technique which is used for the identification and quantification of organic substances present in complex matrices. (GC-MS) is essential in different fields including, forensics, health care department, environmental science, medical and biological research, health and safety, the food, flavor, and fragrances industry, food safety, packaging, and many others. The analytical methods which are applied for the detection and quantification of contaminants are based on gas chromatography (GC) or liquid chromatography (LC) further coupled with mass spectrometry (MS).

The selection of GC and LC is based on the physical and chemical properties of the substances.

1.13.2 FT-IR SPECTROSCOPY

1.13.2.1 1DEFINITION

in Fourier transform infrared spectroscopy we obtain an infrared spectrum of absorption or emission of solids, liquids, and gases. For this purpose, an FTIR spectrometer is used through which light resolution spectral data is obtained over a wide spectra range.

1.13.2.2 Principle

In FTIR analysis Fourier transform measures electromagnetic radiations in the form of an infra-red region of light which has a longer wavelength and low energy values. The sample is exposed to infra-red radiations when need to measure or analyze. An infra-red spectrometer measure out the light produced and shows its output in the IR spectrum we get a graph between infra-red light absorbed by the sample vs frequency (wavelength) on the x-axis.[25]

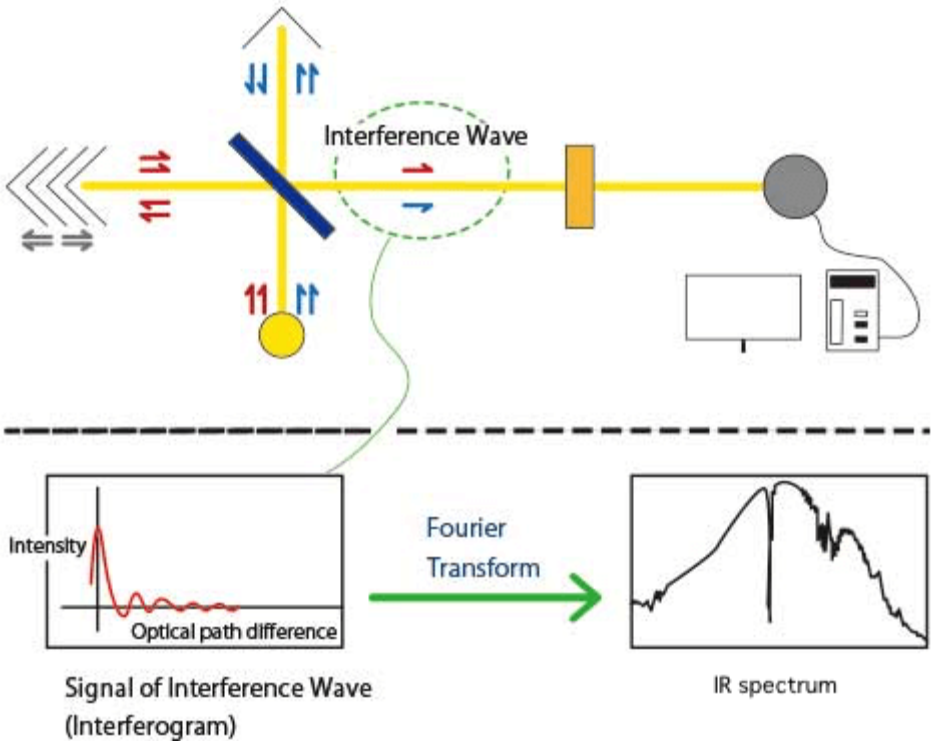


Figure 9: Working of Fourier transform spectrometer

1.13.3 UV-VISIBLE SPECTROSCOPY

1.13.3.1 DEFINITION

UV-Visible spectroscopy is a quantitative technique that indicates the amount of light absorbed by a chemical substance. The light is absorbed by the reference sample or blank. This technique applies to liquids solids, gases, glass, and thin films. [26]

1.13.3.2 PRINCIPLE

UV-VIS spectroscopy is an interaction of light with matter. The compound absorbs light in the visible and ultraviolet regions of light which then gives a discrete spectrum. UV- spectrometer work based on beer -lambert law.

A= log ((IO/I) = ECl (Beer lambert law)

A= absorbance

IO= intensity of light upon a sample cell

I= intensity of light departing the sample cell

E= molar absorptivity

C= concentration of the sample

l= length of the sample cell

As a result of UV absorption electrons of the sample, atoms excite from the ground state to the excited state. The energy of absorbed radiations will be equal to the energy difference between two energy states .[27]

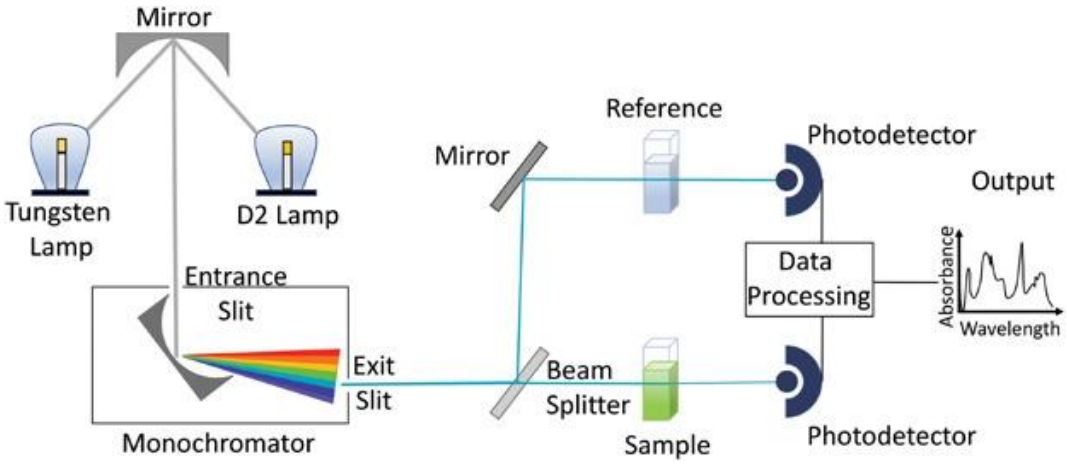


Figure 10: UV-VIS spectrophotometer

1.14 Risk Assessment using EPI suite Software Technique

OPPT designed a window-based software technique.The EPI (Estimation Programs Interface) suite to screen new chemicals identified from any experimental data. Different physical and chemical properties like melting point, vapor pressure, solubility, biodegradability, bioaccumulation, etc. are identified by this program. Environmental fate of chemical is also identified, either it absorbs in the atmosphere, water, or soil etc. This is a very important software technique for the risk assessment of chemicals.[28]

1.14.1 EPI-Suite Modelling for Risk Assessment

Structure of the data that was being retrieved from GCMS, FTIR and UV-Visible spectroscopy. For risk assessment, different attributes were observed which involve toxicity, solubility, bioaccumulation, and biodegradation.

1.14.1.1 Toxicity

According to US Environmental Protection Agency defined toxic substance as "a substance whose concentration value is greater than or equal to 0.1 mg / L is considered as toxic.

1.14.1.2 Bioaccumulation

By EU REACH regulations, chemicals with a factor (BCF) ≥ 2000 (log BCF) 3 (BCF) ≥ 5000 (log BCF) greater than or equal to 3.7 are classified as bio accumulation (B) and very bioaccumulation (vB), respectively (REACH 2007). BCF is defined as the equilibrium distribution in between the lipid pool and water of organisms (i.e., membrane plus storage lipids).

According to EU REACH regulations, those chemicals whose factor (BCF) ≥ 2000 and (log BCF) 3 (BCF) ≥ 5000 (log BCF) greater than or equal to 3.7 are bioaccumulative (B) and very bio accumulative (vB) respectively (REACH 2007) BCF stands for bioconcentration factor is actually "equilibrium distribution between the lipid poll and water content of organism".

1.14.1.3 Biodegradation

According to ECHA guidelines for persistent assessment, A substance is said to be very persistent (P, VP) when the values of BIOWINN2 or BIOWINN7<0.5 while BIOWINN3 <2.2. If BIOWINN3 value is in between 2.2 and 2.7 (ECHA Guidelines, European Chemicals Agency R.7.9.4, R.7.9.5 and European Chemicals Agency R.11.1.3), then the substance is said to be very critical. In this regard chemicals were detected using different sets of parameters of Epi-suite modeling.

1.15 Health risk index assessment

A health risk index (HRI) greater than one indicates the consumption of such honey sample may provoke health risks to the people using this honey in their daily diet.

The health risk index for pollutants was calculated by this formula.

$HRI = DI / R_fD$

DI = Daily intake

R_fD = Reference (oral permissible) dose

$DI = C_{pollutant} \times D_{food\ intake} / B_{average\ weight}$

1.16 Objectives of the study

- To generate qualitative and quantitative data about airborne environmental pollutants in rural areas of Punjab Pakistan.
- Use of modern techniques and robust methods to enhance the authenticity of generated data.
- Study of environmental health hazards by using some statistical tools.
- A health risk assessment by using scientific tools and a specific software technique.

CHAPTER NO 2

2-Literature review

Environmental pollution is a major issue of concern and a huge contributor in health issues throughout the world. Risk is greater in all the developing countries where there is poverty, lack of awareness, facilities, and modern technology. Various studies are performed to know the reasons behind the health risks associated with pollution. In recent years many attempts have been made to monitor the death ratio linked with environmental pollution. At about 8 to 9 percent of the total disease, burden was linked to pollution especially in developing countries. Along with indoor air pollution, poor sanitation, poor hygiene are considered major contributors in pollution. [29]

In 2007 Rissat et.al, determined 48 pesticides within major groups of pesticides (organophosphates, organochlorines, pyrethroids in local samples of Sau Paulo, Bauru, and Brazil. A high concentration of malathion was detected in all the samples due to its usage in mosquito control. The recovery range was proved to be around 76% to 95% with a limit of detection lower than 0.01mg/kg. it was for(GC-MS-SIM) electron impact mass spectrometric detection in selected ion monitoring Mode .[30]

In 2014 Eissa et.al studied 46 pesticides including organochlorines, organophosphorus, pyrethroids, and organonitrogen were detected in honey samples which were collected from different apiaries located in 9 centers of Egypt. Samples analysis was performed through the Richerson method followed by gas chromatography. Results showed that estimate daily intaking (EDI) of different pesticides was much lower than acceptable daily intaking values (ADIs). This thing showed the honey consumption contributes a minimum of toxicological risks. This study also suggested a proper monitoring program should be established to check pesticides residues levels in honey. 55.6 percent of the collected samples contain residues mostly consisting of organochlorine and organophosphorus pesticides groups. Decifol was detected in most of the samples almost 38.9 percent of the samples were analyzed which is used to control Varroa destructor. Some other acaricides which are used by beekeepers were also detected. Some chemicals which were used by the apiculturists to control diseases detected like bromopropylate, tetradifon, and malathion were also present in honey.[31]

In 2015, Malahat et. al studied the residues of organochlorine and pyrethroid pesticides. Their major purpose was to check the quality of honey along with the assessment of its usage in environmental biomonitoring. Residues levels were determined via (GC-ECD) In this finding only one sample was exceeding the maximum residue limit of honey (001). Although organochlorines were banned their presence in honey samples raises questions about their source. Most of the samples contain a wide spectrum of organochlorine and pyrethroids(synthetic) pesticides. Among organochlorines, hexachlorobenzene (HCB) was on the top followed by permethrin and heptachlor epoxide. [32]

In 2018, Alam et al collected 18 samples from north Lebanon in which pesticides level of 84 types were monitored. Varying trends were seen in different pesticides level proving honey a good biomonitor for the environment. In this study, the QUECHERS method of multi-residue extraction was used. Chromatographic analysis was performed via LC-MS/MS and GC - MS/MS. For analytes pre-treatment (concentration) to make it ready for chromatographic analysis. The concentration of honey samples collected from Akkar, and Byblos were almost 1753.92 and 695.13 respectively. in this study, it was also discussed target honeybees and their products are proved to be a good indicator of envir0nment health. the major reasons are due to mobility, furry body composition, and sensitivity towards environmental and physiological changes. That's why these products prove to be a good indicator of the environment.[33]

In 2021, Jurak et.al determined pesticides residues in raw honey samples of different floral regions. With the help of this work, it was confirmed that beehive products i-e honey could be used as biomonitor for the environment. Toxicological risk link to honey consumption was proved to be very low but this work emphasized the usage of honey must be in monitored form to make it safe for the consumption of infants.[34] 40 samples from 4 regions of the variation country were analyzed. 78 active substances were. This work significantly proved that honey bees could be excellent bioindicators of environmental pollutants and pesticides. [34]

In 2018 Kumar et.al, collected 100 raw honey samples from different floral regions of India. Results showed that levels of estimated daily intake of the contaminant were at low levels as compared to accepted daily intake values. But this work also emphasized the safe usage of honey especially in the case of infants due to its contamination capacity due to chemicals. [35]

Panseri et.al collected 72 kinds of honey from different areas of Italy. From these samples, 28 pesticides were determined. The sample was measured through SPE clean-up and GC/MS

detection majority of the pesticides contained on of the pesticide, despite having their concentrations below MRL DD, DDT, DDE values. Pesticides residues were absent in the honey sample from the mountains area. This study proved honeybee and honey hive matrices a good indicator of environmental pollution. This work also provides direction to the beekeepers in selecting the proper location for organic honey production or cultivatio.[36]

Nadaf et.al collected 30 samples from the hsar region of India. 22 pesticides were separated from organochlorine, organophosphate, and pyrethroid groups. Techniques of analysis were the quenchers method of extraction and GC-ECD. [18]

Ruiz-tokar et.al performed a study in which two species were selected for pollutant analysis i-e honeybees and a stinging bee, *S. Mexicana*. Each species was moved to two sites in three colonies. During the first collection at the beginning of the study, three samples of polluted honey and three samples of polluted pollen were collected from each species. The process was repeated every six months. So, a total of 36 samples were collected for honey and pollen samples it was found that honey samples with a percentage of 88.44% and 93.33%, and pollen samples with 22.22% and 100% of Apis mellifera were proved to be positive for one organochlorine the most abundant among all of them was heptachlor which was 4 4 percent in the sample by percentage. The remaining were) 36 percent γ-HCH,19 percent DDT,18 percent Endrin and 11 percent DDE.[37]

Kazazic et.al performed a study to determine the degree of polycyclic aromatic hydrocarbons bees are proved to be a good indicator of the environment either through their bodies or body products. In these 10 samples were collected from Herzegovina region (including polluted and unpolluted sites) extraction was performed through ultrasonic bath and for chromatographic analysis, HPLC technique was performed along with UV-vis detector. Results revealed that 6 of them were non-polluted while four of them contain low levels of (PAHs) with a maximum value never exceeds 7ug/kg. a strong carcinogen benzo(a)pyrene was present in a sample. Moreover, the count. Of PAHs in samples were in safer limits to use. This study also proved to honey a good bioindicator of environmental health.[38]

Hungerfod et.al studied 26 minerals, trace elements, and elemental differences between honey samples of different sources. For sample analysis, the techniques used were ICP- MS and ICP-OES. the results showed that the concentration levels of toxic heavy metals were at low levels in honey products. This study proved that Australian honey is a good source of K and Zn trace

elements. Based on this study, honey was classified into four major groups i-e urban, rural, peri-urban, and blend honey.[39]

Lesharaz et.al determined 399 pesticides residues in 99 raw honey samples of northeastern Spain. Quechers method of extraction along with some modification was applied for analyte separation. Liquid and gas chromatography techniques were applied for determining pesticides residues in honey samples. Two age groups including adults and infants were suspected to be at risk according to dietary risk assessment for pesticides in honey above their lowest calibrated level. Results indicated that honey possesses the least impact on consumer health due to its contributing value which is very low as compared to acceptable daily intake value. But proper monitoring of pesticides levels in honey is very important due to their toxic nature.[40]

Luca maria et.al in 2017 performed a study in which 53 pesticides were detected in honey samples. The effectiveness of the simple extraction method (accelerated solvent extraction was checked with QUECHERS accelerated method for extraction for the detection of pesticides in honey samples along with gas chromatography linked with quadrapole mass spectrometry. method validation was done according to European union guidelines. The results showed that quencher along with ASE having PSA as sorbent shows better repeatability. The accelerated hex.et acetate and florosil. good recovery rate was observed in case of quechers along with ASE-PSA sorbent for pesticides. This work also revealed that pesticides contaminants affect the colony growth rate and life span of honeybees. So these pesticides may affect the consumer health as well.[41]

In 2021, For better analysis of pesticides and pollutants exposure for wildlife was monitored. Sample of quechers method was prepared for 5mg liver samples which w the, were then extended for 100mg liver samples. QUECHERS method was modified (micro QuECHERs) by using 1 percent acetic acid and acidified ACN with buffering salts. Almost 209 pesticides or organic pollutants can be identified for each sample with the help of the micro QuECHERs method. This newly modified micro QUECHERs method requires only one minute of biomaterial and give but prove to be a good technique for multi-residue analysis of pesticides.[42]

Pau et, al in 2016 did a comparison from both matrices between simple extraction methods and QuECHERs method of extraction for honey and honeybees matrix analysis. Matrix was obtained by solvent-based method while for honey matrix solid-phase extraction protocol was used. Acidified quechers method was used for the extraction of pesticides from both matrices.

Results revealed that quechers is the cheapest and likes time-consuming procedure. Solid-phase extraction and solvent-based extraction always show equal recoveries to quechers.[43]

Pau calatayud et.al in 2018 studied pesticides residues distribution in a sample of worker bees, beekeeping matrices, beeswax, and pollen grains from 45 different apiaries of Spain. hazard quotient (HQ) was studied in 133 samples with 63 different pesticides. Results revealed that pollen samples contain the largest pesticides level than others having (hazard quotient) greater than 50. The most important contributor was acrinathrin in wax and pollen samples. [44]

MS Jovetić et. al in 2018 contributed to the development of urban beekeeping by this study. This work was designed to check the pesticides and toxic heavy metals polycyclic aromatic hydrocarbons in urban honey. Samples of honey, pollen, and floral, nectar were collected from the university of Belgrade University of faculty of agriculture situated in zaman. Heavy metals (Cd, As, Pb, Cu, Zn, Cr, Fe, Mn, Ni, and Hg were determined through (ICP-QMS) inductively coupled plasma coupled with quadrupole mass spectrometry. Poly cyclic aromatic hydrocarbons were determined through (HPLC- FLD) pesticides were determined through (GC-MS). The levels of all the analyzed pesticides (123) were below the limit of quantification. Our results suggest that the investigated urban honey meets the regulatory requirements for metals, PAHs, and pesticides and is, therefore, safe for consumption. Results revealed that urban honey contains safer amounts of heavy metals, PAHs, and pesticides. [45]

Grenier et .al, in 2021 suggested that honeybees could be used as environmental biomonitor for heavy metals and polycyclic aromatic hydrocarbons (PAHs) in Europe. Urban residents and stake holders are interested to monitor the quality of air. The aim of the study was to evaluate honey as biomonitor for the assessment of heavy metals and PAHs in Québec City, Canada, in multiple socioeconomic districts of Quebec City. Results of this study depicted that honeybees are sensitive enough to predict differential urban environments in a city having similar population levels.[46]

Sheldon et.al, in 2019 studied urban honey bees and its related aspects. Honey contaminated with pesticides residues is a major issue of concern due to its drastic effects on human health and beehives. Urban beehives are suspected to be at risk from chemicals due to maximum usage of sprays in residential areas, parks, and commercial sites. Honey and bees wax was tested which were collected from urban hives in Central Kentucky for organochlorine, pyrethroid pesticides, and heavy metals. (endosulfan) in 2010.Results indicated that 72 percent of the

tested honey samples exceeds the allowed daily intake levels of U.S. Environmental Protection Agency. These samples contain lead levels upto 5ppm. [23]

CHAPTER NO 3

3.Material and Method

This chapter has complete detail of the scheme of study followed. This involved sample collection, storage, extraction, and clean-up methods. In the end, all the characterization techniques have also been discussed.

3.1 Chemicals

- Acetonitrile,
- n-hexane,
- acetone
- acetic acid (glacial)
- de-ionized water
- 2 ml quechers clean-up tube with 50 mg PSA, and 150 mg $MgSO_4$
- Anhydrous magnesium sulfate
- sodium chloride,
- sodium citrate and
- sodium hydrogen citrate sesquihydrate were of analytical grade).
- acetonitrile acidified with acetic acid (1%):
- (10 ml glacial acetic acid was dissolved in 100 ml acetonitrile)

  APPARATUS USED
- Glass vials
- Sample holder
- 10 ml measuring flask
- 10 ml measuring cylinder
- Pipettes
- 50 ml centrifuge tubes
- Spatula
- Watch glass
- Aluminum foil

3.2 Sample collection

34 samples were collected from different locations of the Bhakkar district. The samples were kept in glass vials in a laboratory at room temperature until analysis.

3.3 Location sites

1. Anar shah	2. Rakh dala
2. Notak	4. Dary khan
5. Mithu pindu	6. Khansar
7. Chak no 4	8. Ali khel
9. Dajjal	10. Chak no 1
11. Bhattityan wala	12. Janju shrif
13. Dagar rahtaaas	14. Chak no209
15. Mehraab wala	16. Kiraari kot
17. Js dulay wala	18. Chak no 45
19. Darya khan 2	20. Panj giraen

21. Kotla jam	22. Shahaani
23. Mankera	24. Nawa gassu
25. Naurang wala	26. Bakhtawar wala
27. Bhakkar city	28. Chak no 183
29. Chak no 185	30. Jafar wala
31. basti panja	32 . lakha khooh
33.Behal	34. Mahoota

3.4 Extraction procedure

Extraction of the analyte of interest was performed through a robust method of extraction named QUECHERS method of extraction, which is quick, easy, cheap, effective, rugged, and safe. This is a two-step process that involves

- Extraction
- Clean-up

3.5 Methodology

First, the honey sample was homogenized. 5 g of homogenate was weighed in 50 ml PFTE (poly tetra fluoro ethylene tube. The tube was already filled with 10ml deionized water and 10 ml acetonitrile (acidified with acetic acid). The content was shaken manually for one minute. After that, buffering salts including 4.0 g anhydrous magnesium sulfate, 1.0 g sodium chloride, 1.0 g sodium citrate, and 0.5 g sodium hydrogen citrate sesquihydrate were added into the solution. The mixture was again shaken for one minute and shifted towards the centrifuge machine for subsequent centrifugation at 4000 rpm for 3 minutes.

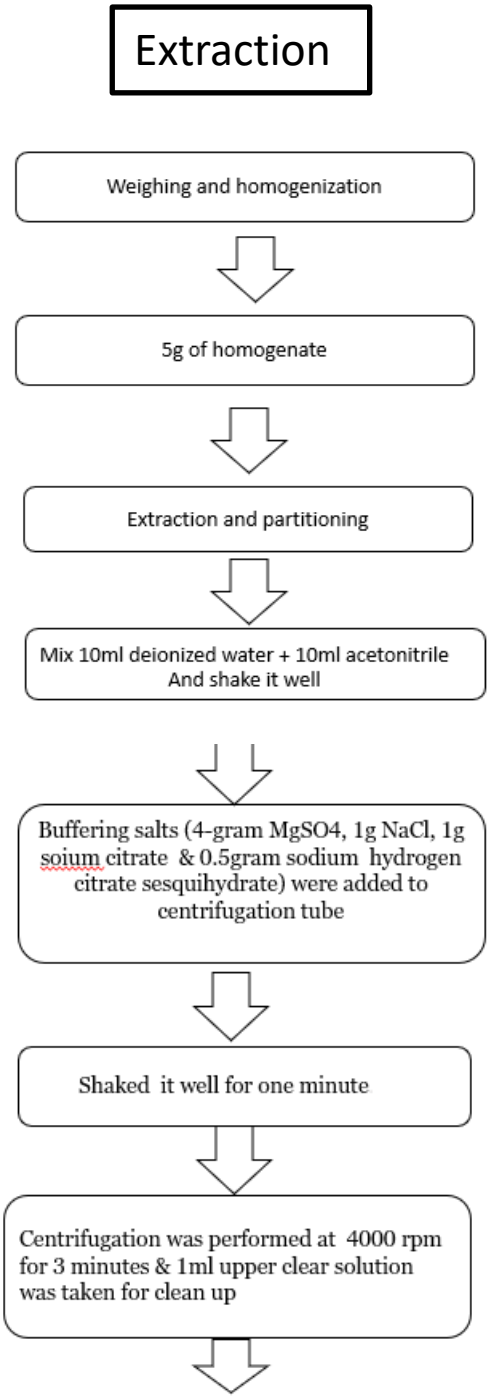
Clean-up

For clean-up ,1 ml upper clear organic layer was transferred into a 2 ml quenchers cleanup kit containing 50 mg PSA, and 150 mg $MgSO_4$ sorbents. This tube was again shaken vigorously for one minute and centrifuged at 5000 rpm for one minute. Finally, 0.5 ml of the upper clear

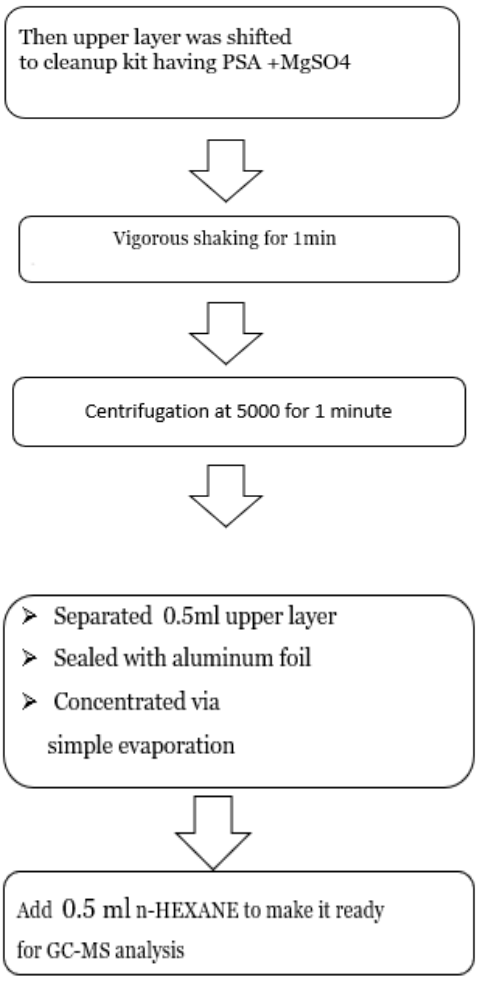
solution was transferred into the glass vessel. The analyte was concentrated via simple evaporation covering it through aluminum foil.

In the end, 0.5 ml n-hexane was added to make the sample ready for GC-MS analysis.

3.6 FLOW CHART OF QUECHHERS METHOD OF EXTRACTION



CLEAN-UP


Then upper layer was shifted
to cleanup kit having PSA +MgSO4
Vigorous shaking for 1min
Centrifugation at 5000 for 1 minute
Separated 0.5ml upper layer
Sealed with aluminum foil
Concentrated via
simple evaporation
Add 0.5 ml n-HEXANE to make it ready
for GC-MS analysis

CHAPTER NO 4

4-RESULTS AND DISCUSSION

Gas chromatographic analysis

GC-MS analysis of the analyte was performed. Helium was used as carrier gas while detector was mass spectrometer detector. Initial temperature (T_o) of the column was 80 ^{O}C which was kept for 1 minute. Temperature was increased up to 160^{O}C(T1) for two minutes. Temperature was again increased up to 260 ^{O}C(T2), which was maintained for 12 minutes. During this temperature programming the temperature of the injector was 300 ^{O}C while temperature of the detector was 320 ^{O}C.

4.1 GC MS RESULTS

GC-MS results were generated based on the NIST chemical library match. Based on the NIST library match, 30 0ut of 34 samples were identified as polluted while the remaining 4 samples were un-polluted. So, the further study was applied to only 30 samples. All 30 samples were contaminated with multiple types of pollutants listed below in Table 1.

Table 1: List of pollutants identified during GC-MS analysis

Sample number	**Location**	**Pollutant Identified**
Sample no 1	Annar shah	1,7 Dimethylxanthine
		CynamideDI-2-propenyl-
		3-ACETYL THYMINE
Sample no 2	Rakh daala	ORCINOL
		1,3-DIMETHYL-2,4,5 TRIOXOIMIDAZOLIDINE
		CYANAMIDE, DI-2-PROPENYL-
Sample n 3	Notak	CYANAMIDE, DI-2-PROPENYL-

		3H-N-TRIAZOLO(4,5-D) PYRIMIDINE-5,7(4H,6H)-DIONE, 3,6- DIMETHYL-
		P-DIOXANE, 2,5-DIVINYL-
Sample no 4	Darya khan	1,5-HEXADIENE, 3,4-DIMETHYL-
		CYCLOPROPANE, 1,1'-METHYLENE BIS
		2-METHYLTETRACOSANE
Sample no 5	Mithu pindu	BUTANEDINITRILE,2,3-DIMETHYL-
		5-AMINO-3 –AZIDO-1,2,4-TRIZINE-6-CARBONITRILE
		CYCLOPROPANE, 1,1'-METHYLENE BIS
Sample no 6	Khansar	CYCLOPROPANE, 1,1'-METHYLENE BIS
		2-PROPENOIC ACID
		5-Amino-3-azido-1,2,4-triazine-6-carbonitrile
		CYANAMIDE, DI-2-PROPENYL-
Sample no 7	Chak no 4	CYCLOPROPANE, 1,1'-METHYLENE BIS
		3,3’SULPHONYLDIPROPIONITRLE
		5-Amino-3-azido-1,2,4-triazine-6-carbonitrile
		CYANAMIDE, DI-2-PROPENYL-

Sample no 8	Ali khel	3,3-sulphonylpropionitrile
		3,3-sulphonylpropionitrile
		1,2 benzene diols, o- (cyclobutane carbonyl)
		p-dioxane, 2,5, Divinyl
		Furazan –amino,4-(hydroximino)(triazirinyl)methyl
		5-AMINO-3-AZIDO-1,2,4-TRIAZINE-6-CARBONITRILE
		P-DIOXANE, 2,5-DIVINYL-
		CROTYL ALCOHOL, TRIFLUOROACETATE
Sample no 9	Dajjal	CYCLOPROPANE, 1,1'-METHYLENE BIS
		3H-N-TRIAZAOLO(4,5-D) PYRAMIDINE -5,7(4H,6H0-dione,3,6-dimethyl
		6-AZABICYCLO [3,2,0] HEPTAN-7-ONE

		5-AMINO-3-AZIDO-1,2,4-TRIAZINE-6-CARBONITRILE
		3,3’SULPHONYLDIPROPIONITRLE
		P-DIOXANE, 2,5-DIVINYL
Sample no 10	Chak no 1	5-AMINO-3-AZIDO-1,2,4-TRIAZINE-6-CARBONITRILE
		CYCLOPROPANE, 1,1'-METHYLENE BIS
		N-(1-CYNOCYCLOPROPYL) FORMIDE
		CYANAMIDE, DI-2-PROPENYL
Sample no 11	Bhattiyan wala	1,2-DIOXOLAN-3-ONE, 5,5-DIETHYL-4-METHYLENE
		3,3’SULPHONYLDIPROPIONITRLE
		(S)-(-)-1,2,4-BUTANETRIOL,4-TRIFLUOROACETATE

		CYCLOPROPANE, 1,1'-METHYLENE BIS
		1H-1,2,4-TRIAZOLE,1-(2-PROPENYL)-
		Heptenyl angelate

		CROTYL ALCOHOL, TRIFLUOROACETATE
		p-dioxane, 2,5, Divinyl
		N-(1-CYNOCYCLIOPROPYL) FORMIDE
Sample no 12	Janju sharif	5-AMINO-3-AZIDO-1,2,4-TRIAZINE-6-CARBONITRILE
		CYANAMIDE, DI-2-PROPENYL
Sample no 13	Dagar rehtaas	PIPERAZINE,2,5-DIMETHYL
		CYANAMIDE, DI-2-PROPENYL

		CYCLOPROPANE, 1,1'-METHYLENE BIS
		5-AMINO-3-AZID-1,2,4-TRIAZINE-6-CARBONITRILE
		BUTANENITRILE,3 METHYL-2-METHYLENE-
		CYCLOBUTANEACETONITRILE, -1-METHYL-2-(1-METHYLETHENYL)-
SAMPLE NO 14	Chak no 209	CROTYL ALCOHOL
		CYCLOPROPANE, 1,1'-METHYLENE BIS

		5-AMINO-3-AZID-1,2,4-TRIAZINE-6-CARBONITRILE
		PENTANEDINITRILE, -2-METHYL
		2-propenyl-3-vinyloxirane
		CYANAMIDE, DI-2-PROPENYL
		3,3-'SULPHONYL DIPROPINONITRILE
		BUTENYL TIGLATE,3-METHYL-3-
SAMPLE NO 15	Mehraab wala	P-DIOXANE,2,5-DIVINYL
		3,3'-SULPHONYLPROPIONITRILE
		CYCLOPROPANE 1,1'-METHYLENE BIS
		2,3-DIAMINO-2-CYNOSUCCINONITRILE
SAMPLE NO 16	Kirarri kot	1,2-DIOXOLANE-3-ONE,5-5-DIETHYL-METHYLENE
		3,3'-SULPHONYLPROPIONITRILE
		5-AMINO-3-AZID-1,2,4-TRIAZINE-6-CARBONITRILE
SAMPLE NO 17	Js dulay wala	CROTYLALCOHOL, TRIFLUOROACETATE

		5-HEXANENITRILe
		2-METHYL HEPTANE NITRILE
		5-AMINO-3-AZID-1,2,4-TRIAZINE-6-CARBONITRILE
		3,3’-SULPHONYLPROPIONITRILE
		CYCLOPROPANE 1,1’-METHYLENE BIS
		Cyanamide DI-2-PROPENYL
Sample no 18	Chak no 45	CROTYLALCOHOL, TRIFLUOROACETATE
		5-AMINO-3-AZID-1,2,4-TRIAZINE-6-CARBONITRILE
		3,3’-SULPHONYLPROPIONITRILE
		P-DIOXANE-2,5-DIVINYL-
Sample no 19	Darya khan 2	CYANAMIDE, DI-2-PROPENYL
		3,3’-SULPHONYLPROPIONITRILE
		P-DIOXANE-2,5-DIVINYL-
		CROTYL ALCOHOL, TRIFLUOROACETATE

		1,2-DIOXALAN-3-ONE,5,5, DIETHYTL-4-METHYLENE-
Sample no 20	Panj giraen	-2,3-DIAMINO-2-CYNOSUCCINO NITRILE
		5-AMINO-3-AZID-1,2,4-TRIAZINE-6-CARBONITRILE
		3,3'-SULPHONYLPROPIONITRILE
		N-(1-CYNOCYCLOPROPYL) FORMIDE

Sample no 21	Kotla jaam	5-AMINO-3-AZID-1,2,4-TRIAZINE-6-CARBONITRILE
		3,3'-SULPHONYLPROPIONITRILE
		N-(1-CYNOCYCLOPROPYL) FORMIDE
		CYCLOPROPANE 1,1'-METHYLENEBIS
		5-HEXENENITRILE,2-METHYL-
Sample no 22	Shaaahani	-2,3-DIAMINO-2-CYNOSUCCINO NITRILE
		3,3'-SULPHONYLDIPROPIONITRILE
		5-AMINO-3-AZID-1,2,4-TRIAZINE-6-CARBONITRILE
		CYCLOPROPANE 1,1'-METHYLENEBIS
		HEPTANENITRILE

Sample no 23	Mankera	1,2-DIOXALAN-3-ONE,5,5, DIETHYTL-4-METHYLENE-

		5-AMINO-3-AZID-1,2,4-TRIAZINE-6-CARBONITRILE
		3,3’-SULPHONYLDIPROPIONITRILE
Sample no 24	Nawa gassu	CYANAMIDE, DI-2-PROPENYL
		2,3 DI AMINOBUT-2-ENEDINITRILE
		3,3’-SULPHONYLDIPROPIONITRILE
		• 5-AMINO-3-AZID-1,2,4-TRIAZINE-6-CARBONITRIL
		N-(1-CYNOCYCLOPROPYL) FORMIDE
Sample no 25	Naurang wala	TETRAHYDROCYNOCYLOPENTA (1,3) DIOXIN 4-ONE
		CYCLOPROPANE 1,1’-METHYLENEBIS
		CYANAMIDE, DI-2-PROPENYL
		P_DIOXANE ,2,5_DIVINYL
		3,3’-SULPHONYLDIPROPIONITRILE
Sample no 26	Bhaktawar wala	CYANAMIDE, DI-2-PROPENYL
		5-AMINO-3-AZID-1,2,4-TRIAZINE-6-CARBONITRILE
		3,3’-SULPHONYLDIPROPIONITRILE
		5 HEXENE NITRILE, 2-METHYL-
		P-DIOXANE,2,5-DIVINYL-

Sample no 27	Bhakkar city	CYANAMIDE, DI-2-PROPENYL

		TETRAHYDROCYNOCYLOPENTA (1,3) DIOXIN 4-ONE
		5-AMINO-3-AZID-1,2,4-TRIAZINE-6-CARBONITRILE
Sample no 28	Chak no 183	3,3'-SULPHONYLDIPROPIONITRILE
		CYANAMIDE, DI-2-PROPENYL
		CYCLOPROPANE 1,1'-METHYLENEBIS
		TETRAHYDROCYNOCYLOPENTA (1,3) DIOXIN 4-ONE
		5-AMINO-3-AZID-1,2,4-TRIAZINE-6-CARBONITRILE

Sample no 29	Chak no 185	CYANAMIDE, DI-2-PROPENYL
		1,2-DIOXOLOAN-3-ONE,5,5-DIETHYL-4-METHYLENE
		5-AMINO-3-AZID-1,2,4-TRIAZINE-6-CARBONITRILE
		CYCLOPROPANE 1,1'-METHYLENEBIS
Sample no 30	Jafar wala	5-AMINO-3-AZID-1,2,4-TRIAZINE-6-CARBONITRILE
		3,3'-SULPHONYLDIPROPIONITRILE
		5_HEXENE NITRILE,2-METHYL-
		PENTANEDINITRILE,2-METHYL-

4.2 FTIR analysis

4.2.1 Sample no 1

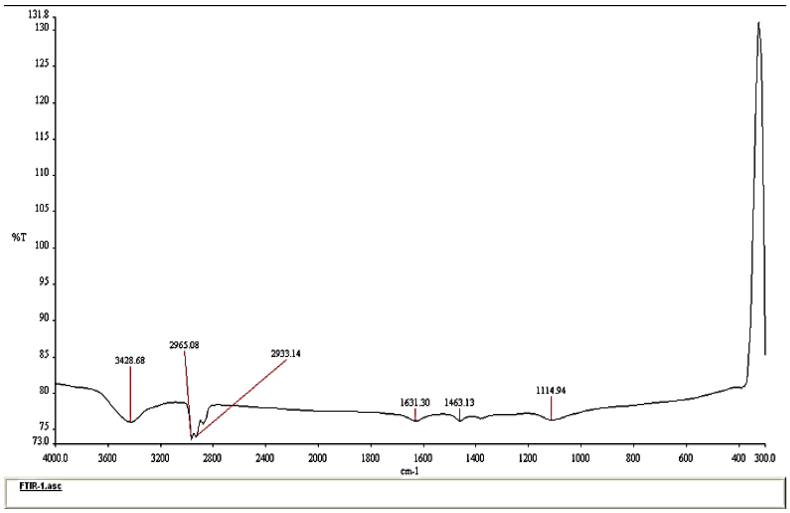


Figure 11 :FTIR Spectrum of Sample # 1

FTIR spectrum of sample no 1 shows the presence of band at 1613cm^{-1} which is a clear indication of the carbonyl functional group in any pollutant detected in our samples. Carbonyl band generally appears at 1600 cm^{-1}. [47]According to GC-MS spectra the sample contains 1,7 dimethylxanthine and 3-Acethythymine which both contain carbonyl functional group. cm^{-1} [48]. The band at 3428.68 cm^{-1} is due to OH stretching.[49]

4.2.2 Sample no 2

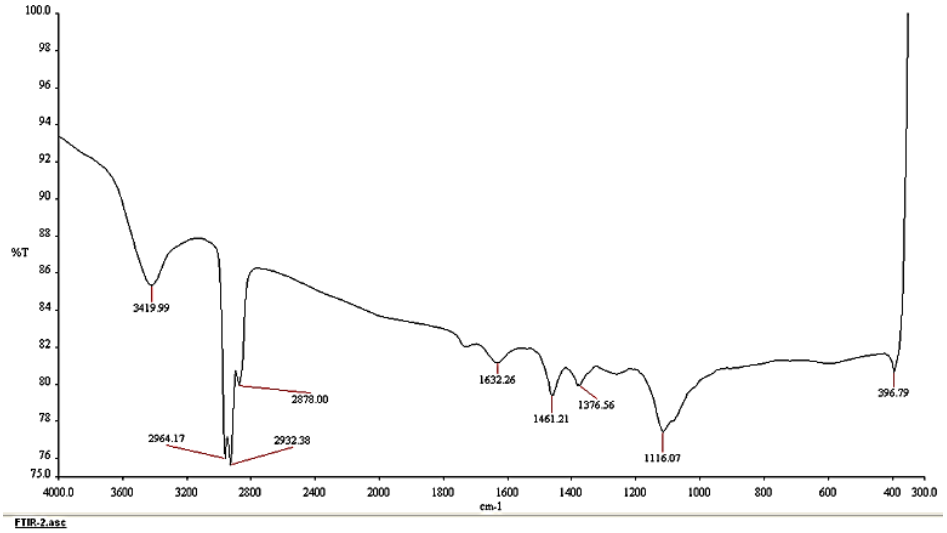


Figure 12: FTIR Spectrum of Sample # 2

The band at 1632 cm^{-1} is may be due to sp^2 carbonyl carbon present in 1,3 DIMETHYL-2,4,5-TRIOXOIMIDAZOLIDINE or CYANAMIDE, DI-2-PROPENYL. [47]

Alkyl stretches at 2964 cm^{-1} and 2932 cm^{-1} may be confirming the presence of alkyl substituents in respective compounds. The band at 1376 cm^{-1} may be due to C-O stretching. [50]

4.2.3 Sample no 3

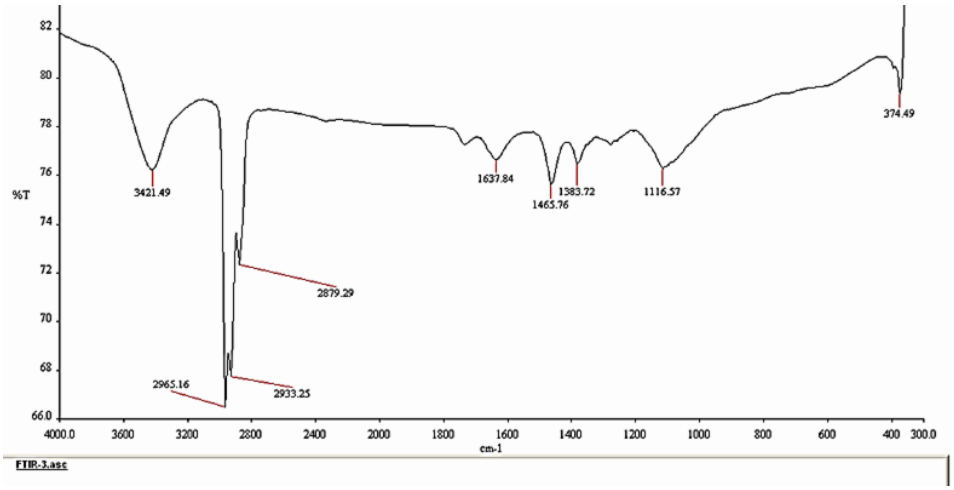


Figure 13: FT-IR Spectrum of Sample # 3

Band a 1637.84 cm^{-1} may be indicating the presence of the carbonyl group in cyanamide and 3H-N-TRIAZOLO(4,5-D)PYRIMIDINE-5,7(4H,6H)-DIONE, 3,6-DIMETHYL. [47]Bands at 2965.16cm^{-1} and 2933.25 cm^{-1} may be due to alkyl stretches. . [48]

4.2.4 Sample # 4

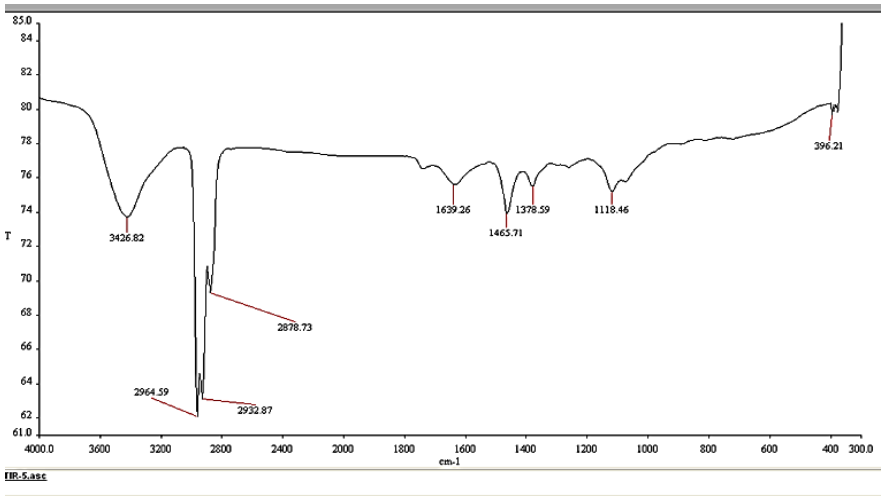


Figure 14: FY-IR spectrum of sample # 4

Bands indicating alkyl stretches at 2932.87 cm^{-1} and 2964.59 cm^{-1} may be due to alkyl stretches present in methyltetracosane and cyclopropane methylenebis. . [48] Band at 1639.26 cm^{-1} may indicate sp2 hybridized carbon present in 1,5-HEXADIENE ,3,4-DIMETHYL- [47]

4.2.5 Sample no 5

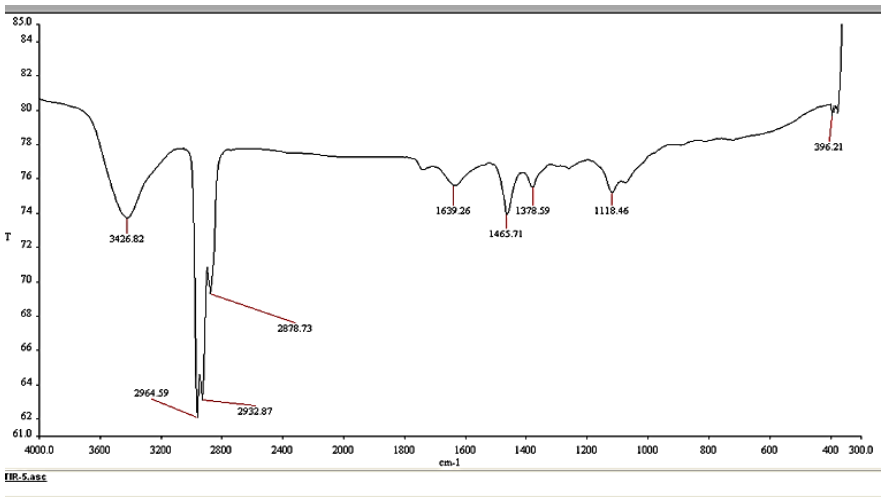


Figure 15: FT-IR Spectrum of Sample # 5

Band at 1639 cm^{-1} is due to carbonyl group present in 5-AMINO-3 –AZIDO-1,2,4-TRIZINE-6-CARBONITRILE. [47] Alkyl stretches reflecting cyclopropane ,1,1'methylenebis and butane,2,3- Dimethyl- are present which are confirmed by two bands at 2964.59 cm^{-1} and 2932.37 cm^{-1}.[48]

4.2.6 Sample # 6

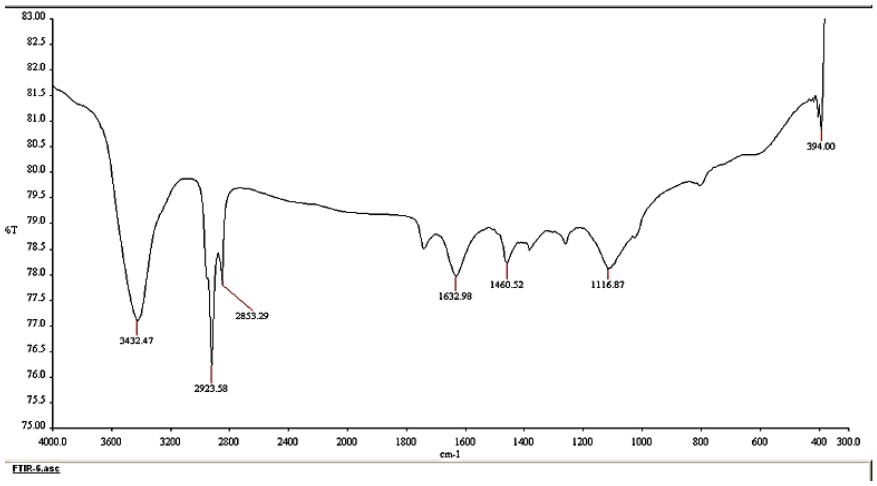


Figure 16: FT-IR Spectrum of Sample # 6

Bands at 3432.47 cm^{-1} are due to the OH- group present in 2-propenoic acid. [50]

IR band at 1632.98 cm^{-1} region is due to carbonyl group which may be due to 5-Amino-3-azido-1,2,4-triazine-6-carbonitrile and CYANAMIDE, DI-2-PROPENYL. [47]

4.2.7 Sample no 7

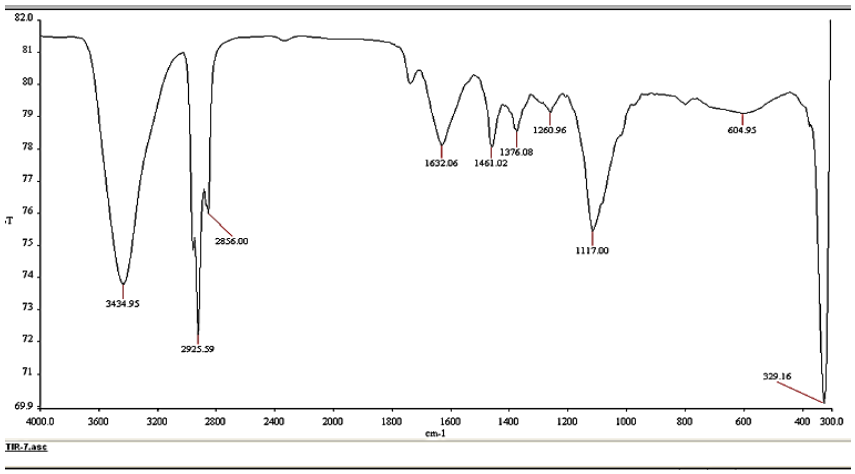


Figure 17: FT-IR Spectrum of Sample # 7

The band at 1632.06 cm^{-1} may be due to the carbonyl group present in 5-Amino-3-azido-1,2,4-triazine-6-carbonitrile, CYANAMIDE, DI-2-PROPENYL. [47] Absorption at 1260 cm^{-1} is due to the sulphonyl group present in 3,3'SULPHONYLDIPROPIONITRLE.[48, 51]

4.2.8 Sample no 8

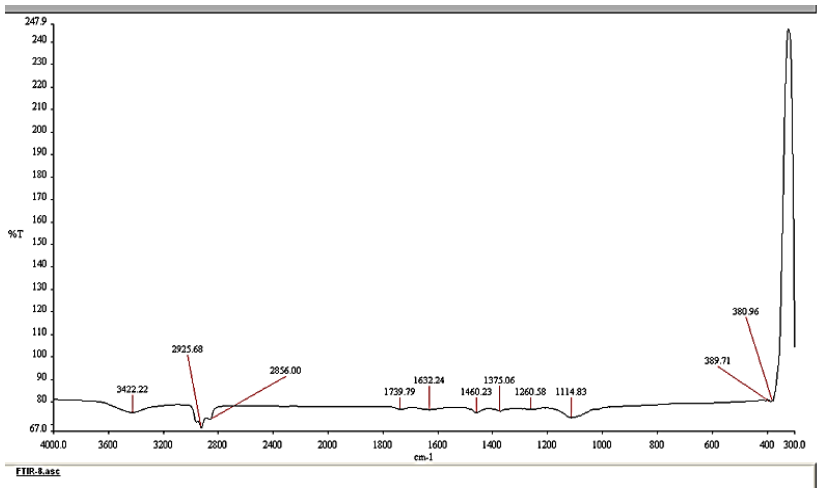


Figure 18: FT-IR Spectrum of Sample # 8

Absorption at 1260 cm^{-1} may be due to the sulphonyl group present in 3,3'SULPHONYLDIPROPIONITRLE. The band at 1632.06 cm^{-1} may be due to the carbonyl group present in 5-Amino-3-azido-1,2,4-triazine-6-carbonitrile. [47] The bands at 1739 cm^{-1} are indicative of the hydrogen bonding effect clearly in the carbonyl stretching region. [52]

4.2.9 Sample no 9

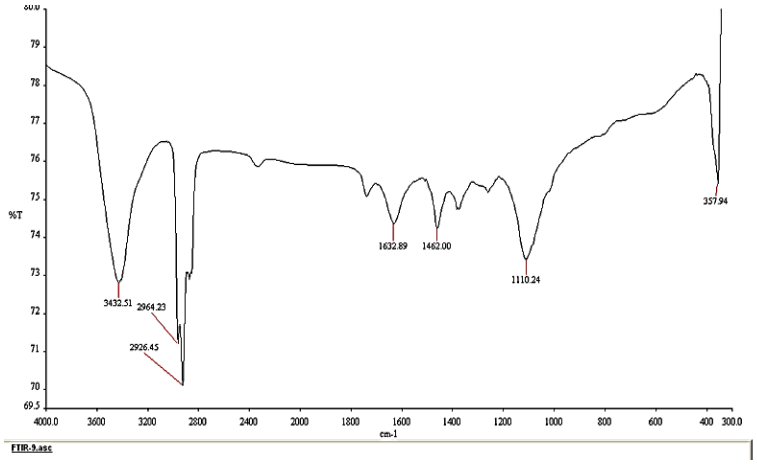


Figure 19: FT-IR Spectrum of Sample # 9

IR bands at 2926.45 cm^{-1} are due to 6-AZABICYCLO[3,2,0]HEPTAN-7-ONE having alkyl stretches inside. [48] Peak at 1632.89 cm^{-1} may be due to carbonyl group present in 5-AMINO-3-AZIDO-1,2,4-TRIAZINE-6-CARBONITRILE. [47]

4.2.10 Sample no 10

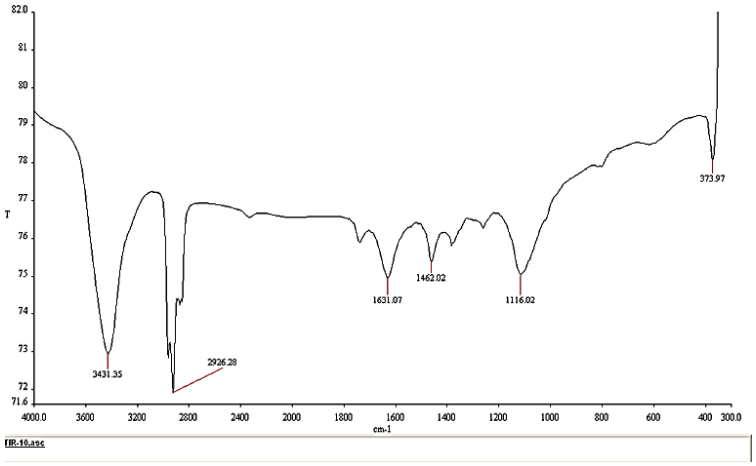


Figure 20: FT-IR Spectrum of Sample # 10

A prominent band at 1631.07 cm^{-1} may be due to the carbonyl group present in N-(1-CYNOCYCLOPROPYL)FORMIDE. [47]

4.2.11 Sample #11 to 20

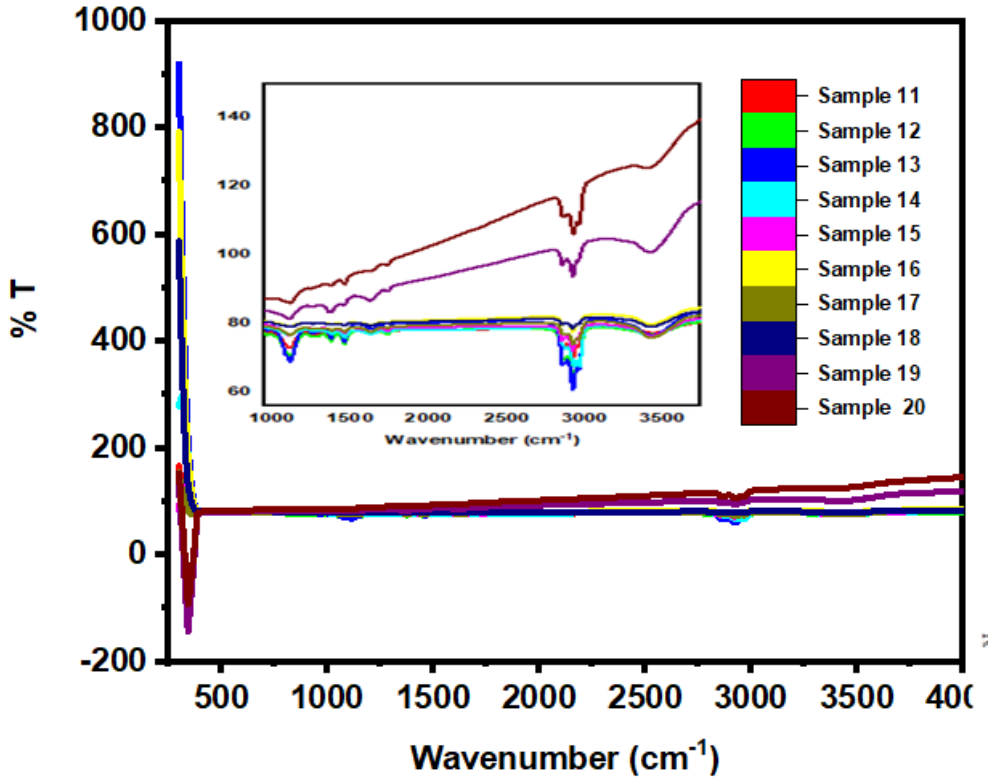


Figure 21: FT-IR Spectrum of Sample # 11 to 20

4.2.12 Sample 21 to 30

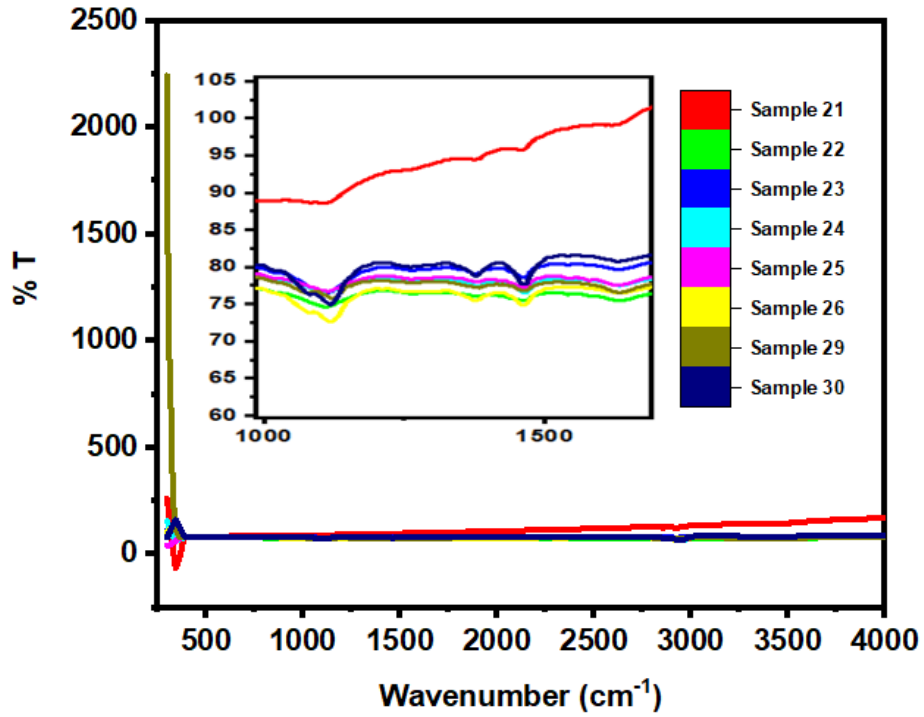


Figure 22 :FT-IR Spectrum of Sample # 21 to 30

These accumulative graphs of FTIR show bands of the remaining 20 samples. The majority of the compounds contain a carbonyl group which is confirmed by the presence of IR bands in the range of 1600 cm^{-1} in almost every spectrum. Alkyl stretches are also present which is confirming the organic nature of all compounds.

4.3 UV-Vis-RESULTS

UV-VIS results show the presence of a doubly bonded carbon atom or sp^2 hybridized carbon atom which is the part of the carbonyl functional group present in almost every compound being analyzed. Majority of the bands are observed in the ultraviolet region which confirm the pi to pi transitions that are present in the sp^2 system. [20]

4.3.1 UV vis spectra 1-10

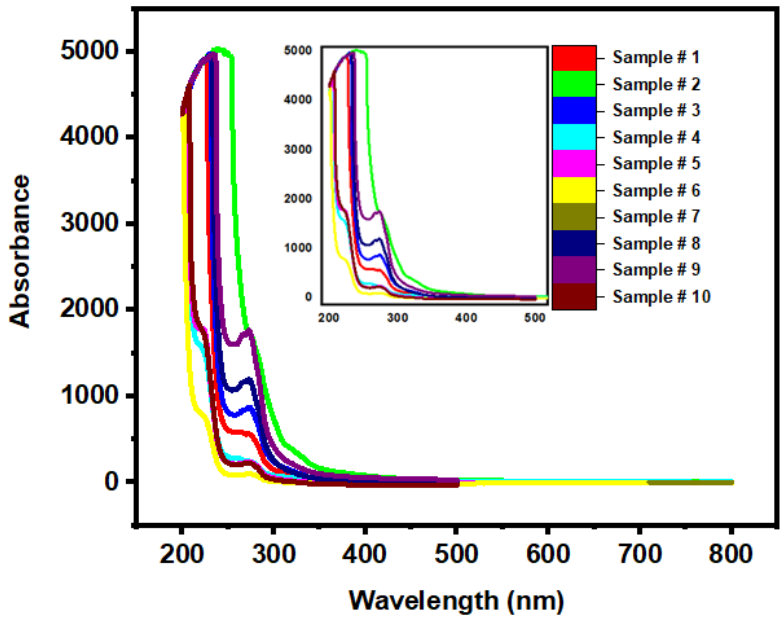


Figure 23 :UV-VIS spectrum of samples (1 to 10)

These are UV-Visible results for sample no 1 to sample no 10. All bands are present in ultraviolet range which may be a clear indication of the presence of pi to pi transitions in sp2 system. [20]

4.3.2 UV-VIS SPECTRA 11-20

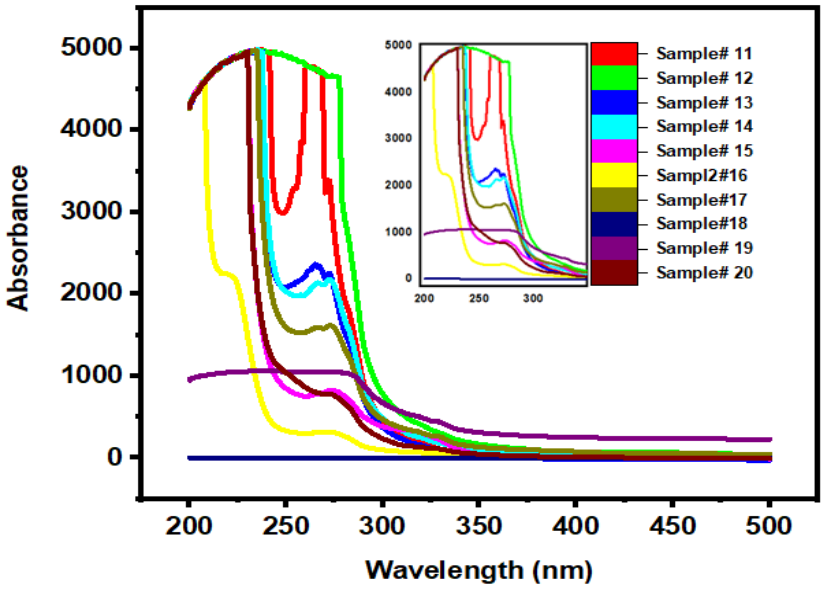


Figure 24: UV-VIS spectrum of samples (11 to 20)

These are UV-Visible results for sample no 11to sample no 20. All bands are present in ultraviolet range which may be a clear indication of the presence of pi to pi transitions in sp^2 system present in almost every compound. [20]

4.3.3 UV-VIS SPECTRA 21-30

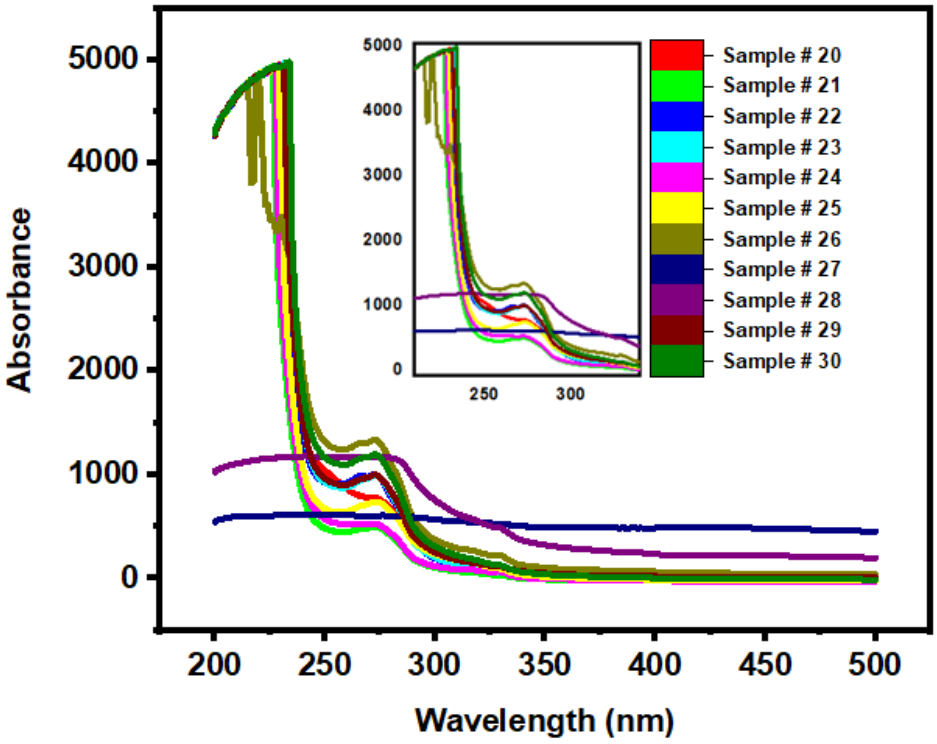


Figure 25: UV-VIS spectrum of samples (21 to 30)

These are UV-Visible results for sample no 1 to sample no 10. All bands are present in ultraviolet range which may be a clear indication of the presence of pi, pi transitions in sp^2 system present in almost every compound. [20]

4.4 Statistical results

Statistical analysis was performed for all the results. In statistical data analysis, few parameters like pollutant counts, their chemical nature, and HRI (Health risk index) values were applied to provide a complete picture of the environment of targeted area.

A map representation of all the location sites from where honey samples were collected are mentioned in (Figure no 27). Samples were collected from agricultural district of Punjab Pakistan named Bhakkar, which is divided into two" portions. One is named "kacha'' while the other is named "Thal**"** which consist of desolate plains. Out of 30 locations few locations are part of thal side while other are part of kacha region. Although both regions are agricultural, but crops production is different there. In thal area mostly wheat, chickpea, Moong, and cotton are cultivated while in kaccha the major crop is sugar cane and moong. Thal area includes Anar shah**(1)** Rakh dala**(2),** khansar**(6),** chak no 4**(6)**, dajjal**(9)**, ali khel**(8)** bhattiyan wala **(11)**, bhaktwar wala **(26)**, naurang wala**(25)**, dagar rehtas**(13)**, janu sharif **(12)**, mehraab wala **(15)**, kirari kot **(16)**, dulay wala **(17)**, chak no 45 **(18)**, chak no 209**(14)**, panj giraen**(20)**, nawa gassu**(24)**, chak no 1**(10)** , Basti panja **(31)**,Lakha khoo**(32)**,Mahoota**(34)**,which are all agricultural sites. Rest of the sites including notak **(1)**, darya khan, **(19)** mithu pindu **(5),** shahaani **(22)**, mankera **(23)**, Bhakkar city **(27)**, kotla jam **(21)** Behal **(33)** are part of kaccha which is a reverine tract along the indus. Among all these Bhakkar city and darya khan is an urban area where chances of traffic pollutants also exist. Few industries including food industry, textile industry, metal industry, sugar cane and flour mills are present in Bhakkar city, Darya khan, and dajjal. But these industries are not very much active so there are less chances of pollution due to this industrial area but still may have some role in pollutants accumulation in environment.

Bhakkar district located in Punjab, Pakistan was selected as targeted area for this Study. Figure 26 shows a complete map of Pakistan along with all its 36 districts including Bhakkar.

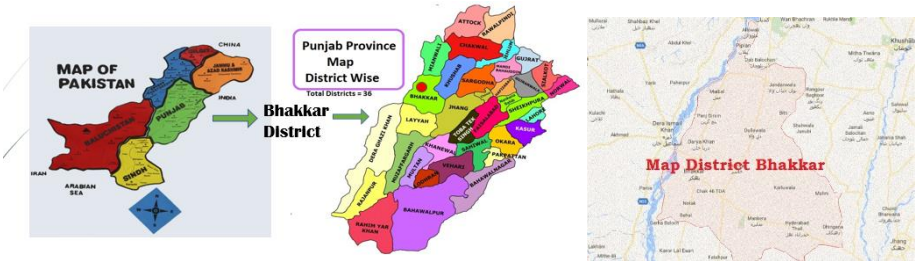


Figure 26: Map Representation of Pakistan,Districts of Province Punjab and Bhakkar District.

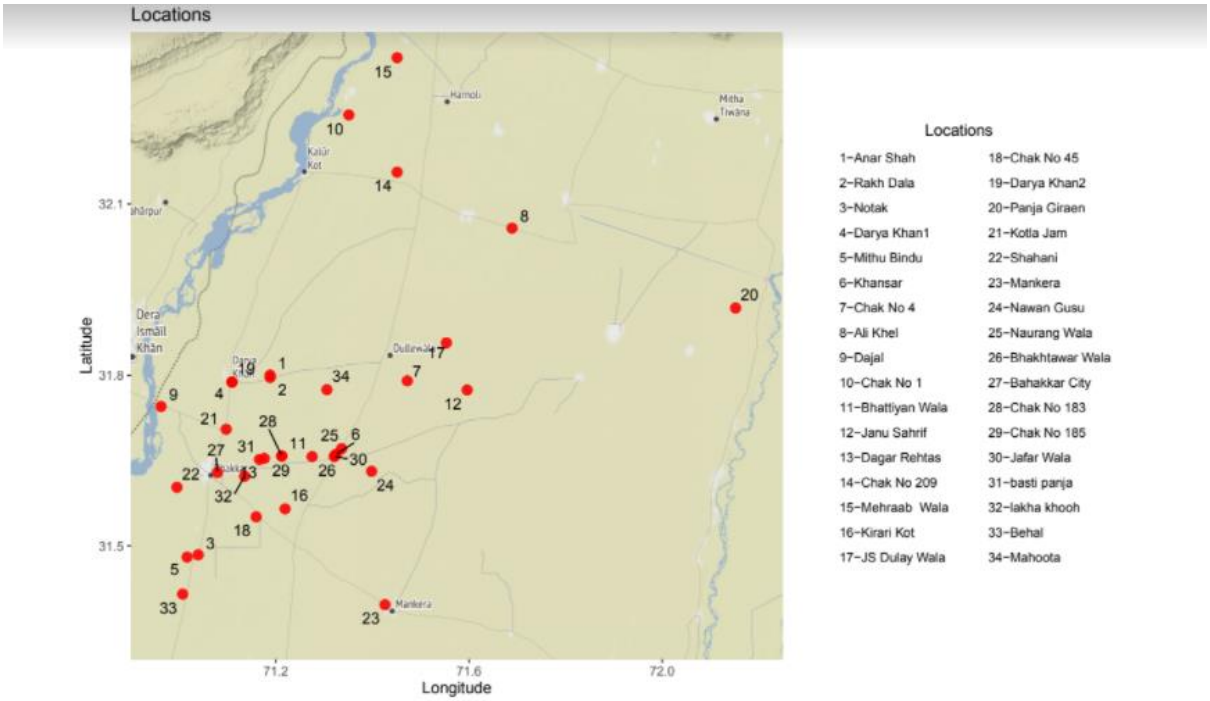


Figure 26: Map of samples location in Bhakkar District

In (Figure 27) there is a map representation of all the location points from where samples were collected. 1 to 34 numbers are assigned to all the locations from where samples were collected. Based on Gas chromatography -Mass spectrometry data only 30 samples were identified as polluted. That's why only 30 samples were proceeded for further analysis and statistical data development.

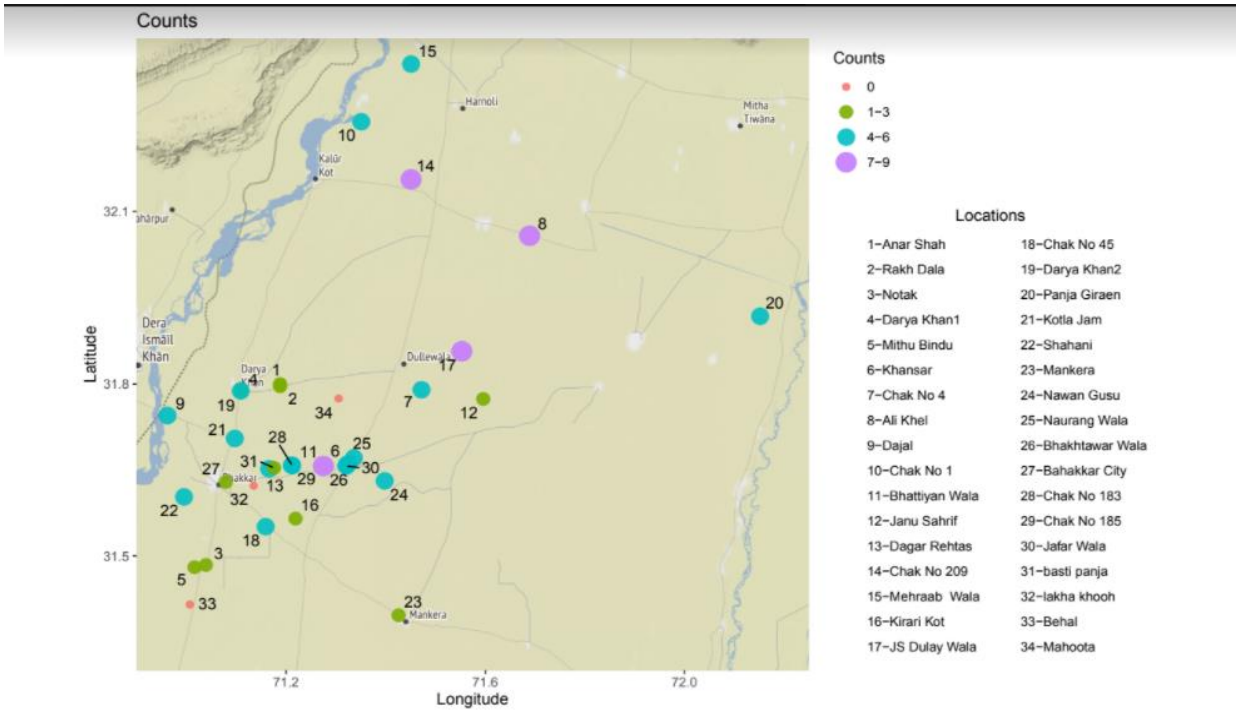


Figure 27: pollutant count wise representation of locations in Bhakkar District

Map in (Figure 28) provides information regarding pollutant count location wise. The color scheme was used to mention category based on pollutant count. e.g., pink color circles were used for labelling the locations where 1 to 3 pollutants were present in their corresponding samples. While green color circles represent pollutant count from a number 4-6 and blue circles represent those pollutants which were found 7 to 9 in numbers inside their corresponding samples.

Results depicted that sites Ali Khel **(8)**, Bhattiyan Wala **(11)**, Chak No. 209 **(14)** and JS Dulay Wala (**17**) were more polluted in terms of pollutant count. Moderte pollutant count were present at Darya khan 1 (**4),** chak no 4, (**7**) chak no 1(**10**) kirari kot (**16)**, khansar (**6),** naurang wala **(25)** Bhakhtawar wala **(26),** jafar wala **(30)** shahaani **(22**) dajjal **(9),** chak no 183***(28),*** chak no 185**(29).** Few numbers of pollutants were identified in the samples of Anaar shah **(1),** Rakh dala **(2),** janu sharif **(12),** kirari kot (**16),** Bhakkar city **(27),** Notak **(3),** mithu pindu **(5),** mankera **(23).** No pollutants were characterized at Basti panja **(31)**, Lakha khooh, **(32)**, Behal **(33)**, Mahoota **(34)**.

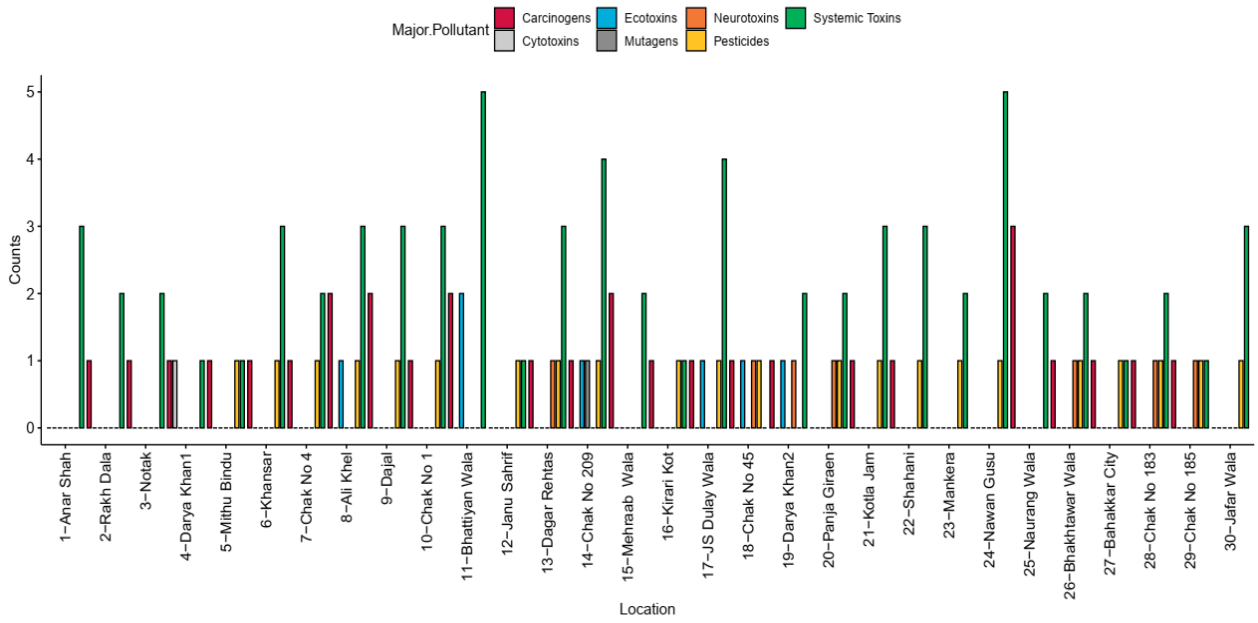


Figure 28:Graphical representation of pollutant count vs locations.

Graph in (Figure 29) is a representation of pollutant count vs locations. On the y-axis, numbers are showing pollutants count while on the x-axis their respective locations are listed. The-color scheme gives information regarding pollutant categories based on their toxicity i.e carcinogenic, mutagenic, systemic toxin etc. Results showed that sites Ali Khel **(8)**, Bhattiyan Wala **(11)**, Chak No. 209 **(14)** and JS Dulay Wala (**17**) were more polluted in terms of pollutant count. Site Ali Khel **(8)** has 7 number of pollutants in its sample. These are systemic toxins, pesticides, carcinogen and ecotoxic in nature distributed in four bars. Chak No. 209 **(14)** has 8 number of total pollutants inside its sample. These include ecotoxic, mutagen, pesticide, systemic toxins and carcinogens pollutants inside. JS dullay wala **(17)** contains 4 bars.Yellow bar shows the presence of one pesticide, green bar shows the presence of 4 systemic toxins, sky blue for one ecotoxic pollutant, and red bar shows the presence of a carcinogenic pollutant in its sample.

Moderate pollutant count was present at Darya khan 1 (**4)** which contain**s** one systemic toxin, one cytoxin and one carcinogen shown by three separate bars. Chak no 4, **(7**) has 5 number of pollutants which are carcinogenic, systemic toxin and pesticides in nature. chak no 1(**10**) which has 6 number of total pollutans which include systemic toxins, carcinogens, and pesticide. kirari kot (**16)** has three number of total pollutants which include systemic toxin, carcinogen,

and pesticide. khansar (**6**) has 6 number of pollutants in its sample which include systemic toxins, pesticide, and carcinogen**,** naurang wala **(25**) has total three pollutants in its sample, one is carcinogen while two are systemic toxin in nature**.** Bhakhtawar wala **(26)** has total 5 polluatnts in its sample which include 2 systemic toxins, one neurotoxin, one pestcides and one carcinogen**.** Jafar wala **(30)** has four number of pollutants which include three systemic toxins and one pesticide in its sample.Shahaani **(22**) has four number of polluatnts which include three systemic toxins and one pesticide in its sample. dajjal **(9)** has total 5 number of pollutants which include 3 systemic toxins, one carcinogen and one pesticide in its sample.chak no 183***(28)*** total five number of poluatnts which include teo systemic toxins, one carcinogen, on pesticide and one neurotoxin inside its sample. chak no 185**(29).** Few numbers of pollutants were identified in the samples of Anaar shah **(1),** Rakh dala **(2),** janu sharif **(12),** kirari kot (**6),** Bhakkar city **(27),** Notak **(3),** mithu pindu **(5),** mankera **(23).** Anar shah (**1)** depicts 4 pollutants distributed in two bars. Green bar shows three pollutants which are systemic toxins while red bar shows a carcinogenic pollutant. Rakh dala **(2)** shows two bars including total of 3 pollutants (systemic toxin and carcinogen) in its sample notak **(3)** contains 4 pollutants distributed in 3 bars. Red one shows carcinogenic pollutant, green shows two pollutants which are systemic toxin while grey shows cytotoxic pollutant in its sample. janu sharif **(12)** has three number of pollutants which include one carcinogen, one pesticide and one pesticide, kirari kot (**6**). Bhakkar city **(27)** has three number of pollutants which include one carcinogen, one pesticide and one systemic toxin. Site Jafar wala **(30**) depicts 4 pollutants distributed in two bars. Yellow colored bar shows the presence of a pesticide in its sample while green color bar shows the presence of three systemic toxins in its sample.

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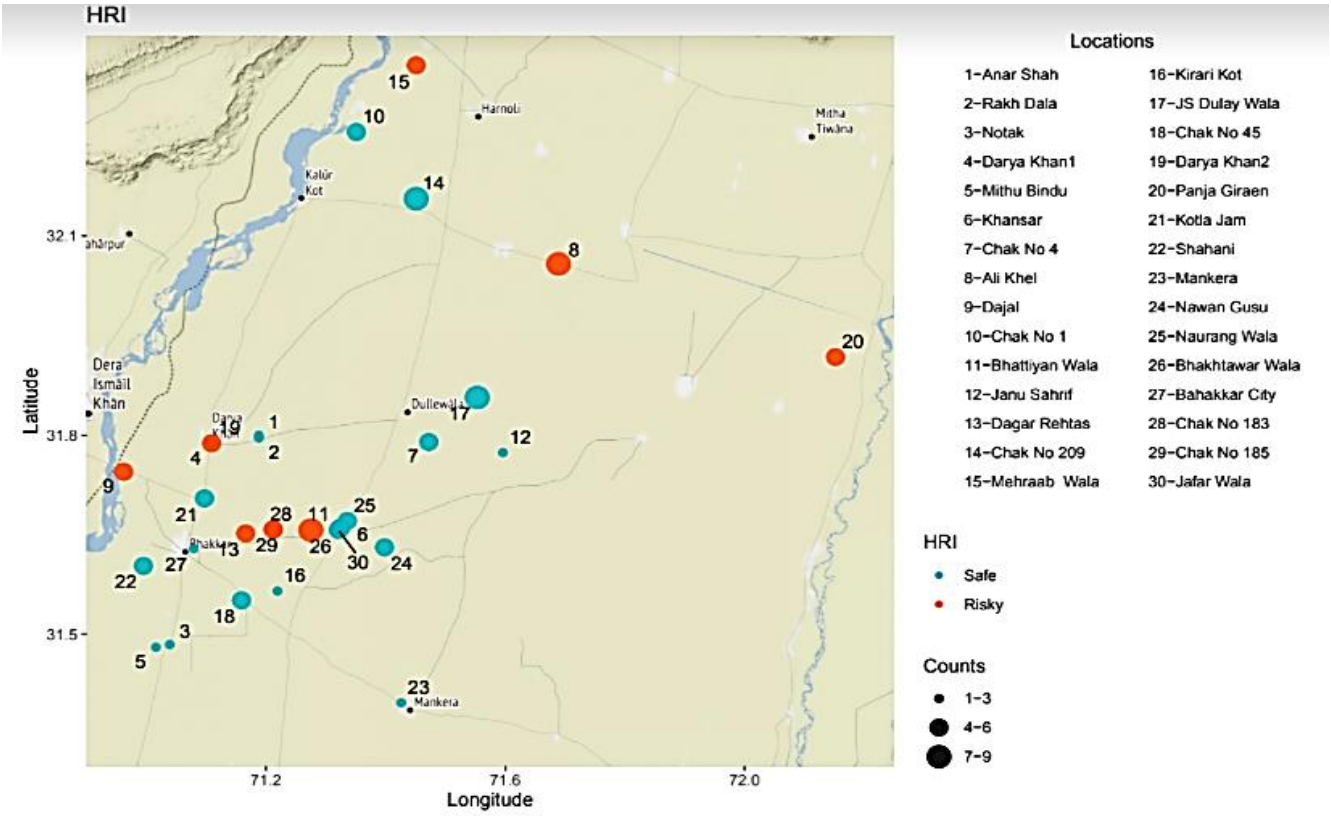

Figure 29: Map representing risky and safe locations based on their HRI values.

Map in (Figure 30) is represents all the safe and risky locations based on their HRI values. HRI values were determined by using a formula based on literature. A health risk index (HRI) greater than one indicates that the consumption of such honey sample may provoke health risks to the people using this honey in their daily diet.

The health risk index for pollutants was calculated by this formula.

$HRI = DI / R_fD$

DI = Daily intake

R_fD = Reference (oral permissible) dose

$DI = C_{pollutant} \times D_{food\ intake} / B_{average\ weigh}$

The color scheme is used to differentiate safe and risky sites while the circle size is indicating pollutant count. Safe locations are those whose HRI values are less than 1 while risky sites will have HRI values greater than 1. Resulted depicted that locations sites darya khan 1 **(4),** Ali khel

(8), darya khan 2 **(19),** panj giraen **(20),** bhattiyan wala **(11),** chak no 183 **(28),** chak no 185 **(29), dagar** rehtas **(13)** had maximum number of pollutant count in terms of **HRI** values greater than 1(risky). On the other hand, Chak No 01 **(10)** chak no 209(**14)** and JS dulay wala **(17)** had maximum number of pollutant count in terms of HRI values less than 1. Sites including Anar Shah **(1)**,Khansar **(6)**, Chak No 4 **(7)**, Dajal **(9)**, Dagar Rehtas **(13)**, Shahani **(22)**, Kotla Jam **(21)**, Nawan Gusu **(24)**, Bahakkar city **(27)**, Chak No 183 **(28)**, Chak No 185 **(29)**, Panja Giraen **(20)**, Jafar Wala **(30)**, Naurang Wala **(25)**, Chak No 45 **(18),** Mehraab Wala **(15)** and Chak No 01 **(10)** were observed to have moderate number of pollutants in terms of safer limit of HRI values(less than 1). Few counts of pollutants were observed at Kirari Kot **(16)**, Janu Sahrif **(12)**, Mithu pindu **(5),** Notak **(3),** Mankera **(23)** in terms of safer limit of HRI values (less than 1).

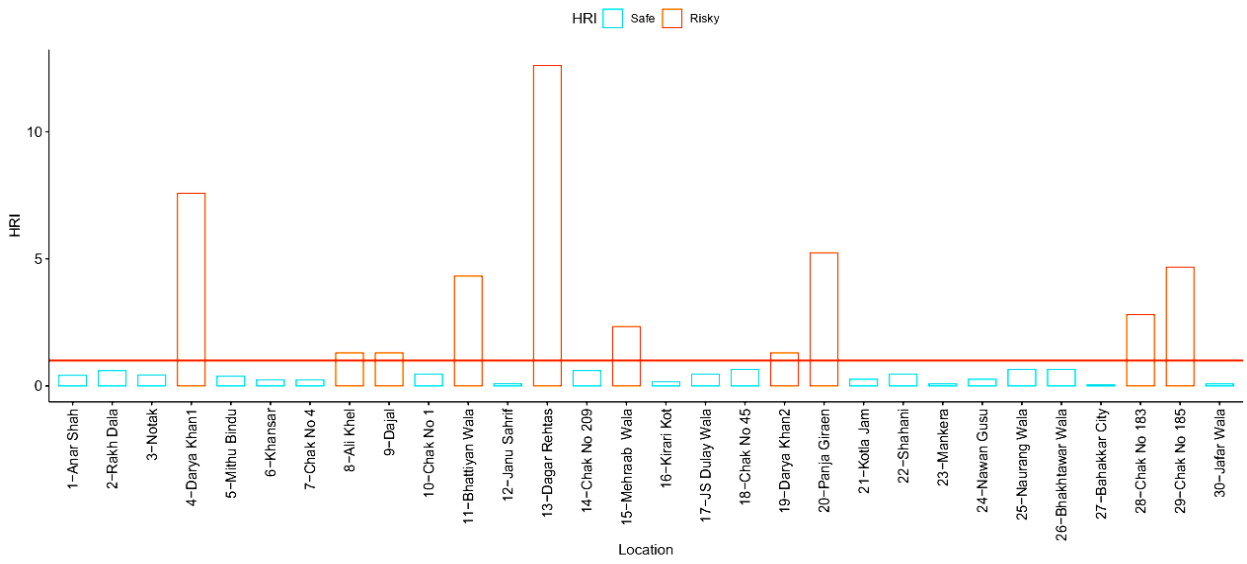

Figure 30: Graphical representation of HRI vs locations

The graph in (Figure 31) is HRI vs Locations. HRI value of any pollutant below threshold limit (1) is considered as safer while above threshold limit is considered as toxic. Orange colored bars indicate those locations which are declared as risky based on HRI values which exceed the threshold limit shown by orange line. The sky-blue colored bars indicate safer locations based on HRI data of their pollutants. Figure 31 shows a graph of location vs HRI values. Orange colored bars which exceed the threshold limit of one depict the risky locations based on HRI data. Sites Darya khan 1**(4),** Ali khel **(8),** darya khan 2 **(19),** panj giraen **(20),** bhattiyan wala **(11),** chak no 183 **(28),** chak no 185 **(29**), mehrab wala **(13)**, Dajal **(9)**, Dagar Rehtas **(13)** shown risky zones based on HRI values. **(17)**. Sites including Anar Shah **(1**) rakh dala**(2)**,Khansar **(6)**, Chak No 4 **(7)**, Dajal **(9)**, Dagar Rehtas **(13)**, Shahani **(22)**, Kotla Jam **(21)**, Nawan Gusu **(24)**, Bhakkar city **(27)**, Chak No 183 **(28)**, Chak No 185 **(29)**, Panja Giraen **(20)**, Jafar Wala **(30)**, Naurang Wala **(25)**, Chak No 45 **(18),**Chak No 01 **(10),** janu

sharif **(12),** kirari kot (**16),** Bhakkar city **(27),** Notak **(3),** mithu pindu **(5),** mankera **(23**) are shown by sky blue bars which are safe zones based on HRI values.

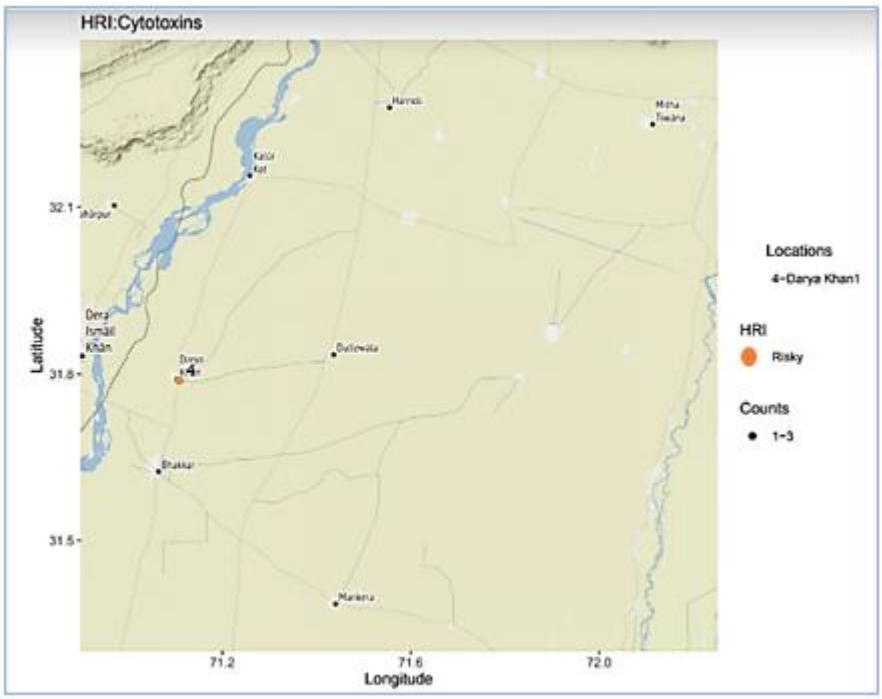

Figure 31: Indication of Cytotoxin based on location in Bhakkar District

The individual map in (Figure 32) represents those specific locations that contain cytotoxic pollutants. Color is indicating the risk factor while the size of the pollutant refers to pollutant count. The numbers indicate those locations which contain cytotoxins pollutant in their samples. The orange color circle showed Darya khan **(4)** was observed as polluted and risky in terms of cytotoxin count. No cytoxins were observed at Ali khel **(8),** darya khan 2 **(19),** panj giraen **(20),** bhattiyan wala **(11),** chak no 183 **(28),** chak no 185 **(29),** dagar rehtas**(13),** Shah **(1)**, Notak **(3)**, Mithu Bindu **(5)**, Janu Sahrif **(12)**, Kirari Kot **(16)**, Mankera **(23)** ,Khansar **(6)**, Chak No 4 **(7)**, Dajal **(9)**, Dagar Rehtas **(13)**, Shahani **(22)**, Kotla Jam **(21)**, Nawan Gusu **(24)**, Bahakkar city **(27)**, Chak No 183 **(28)**, Chak No 185 **(29)**, Panja Giraen **(20)**, Jafar Wala **(30)**, Naurang Wala **(25)**, Chak No 45 **(18)**, Kirari Kot **(16)**, Mehraab Wala **(15)** and Chak No 01 **(10)**

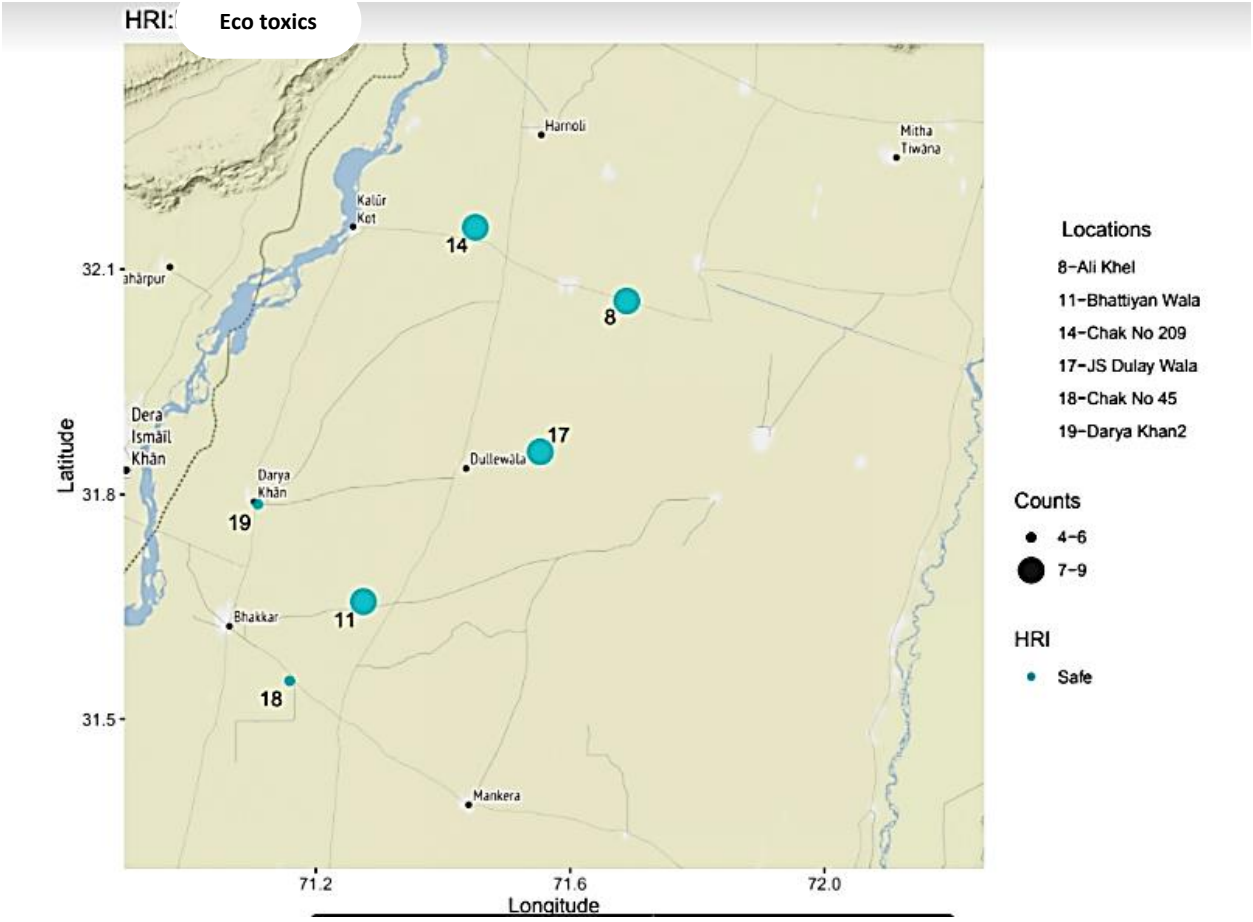

Figure 32: Location wise representation of presence of Eco toxics in Bhakkar District

The map in (Figure 33) represents ecotoxic compounds on their respective locations denoted by numbers. Safe and risk zones are assigned based on their HRI values. Blue color circles indicate the safe zones having ecotoxic pollutants in safer limits. The size of the circle shows the pollutant count like the number of specific pollutants at corresponding location. Map shows that sites, Ali khel **(8),** bhattiyan wala **(26**), chak no 209**(14),** JS dulay wala **(17)** were observed more polluted in terms of ecotoxic count. But based on supposed HRI values, these sites were considered as safe sites. Moderate ecotoxic compounds were observed at present at chak no 45**(18)**, and darya khan 2 **(19)** samples. No ecotoxic compounds were observed at **,** chak no 183 **(28),** chak no 185 **(29),** dagar rehtas**(13),** Anar Shah **(1)**, Notak **(3)**, Mithu Bindu **(5)**, Janu Sahrif **(12)**, Kirari Kot **(16)**, Mankera **(23)** ,Khansar **(6)**, Chak No 4 **(7)**, Dajal **(9)**, Dagar Rehtas **(13)**, Shahani **(22)**, Kotia Jam **(21)**, Nawan Gusu **(24)**, Bhakkar city **(27)**, Chak No 183 **(28)**, Chak No 185 **(29)**, Panja Giraen **(20)**, Jafar Wala **(30)**, Naurang Wala **(25)**, Chak No 45 **(18)**, Kirari Kot **(16)**, Mehraab Wala **(15)** and Chak No 01 **(10)**

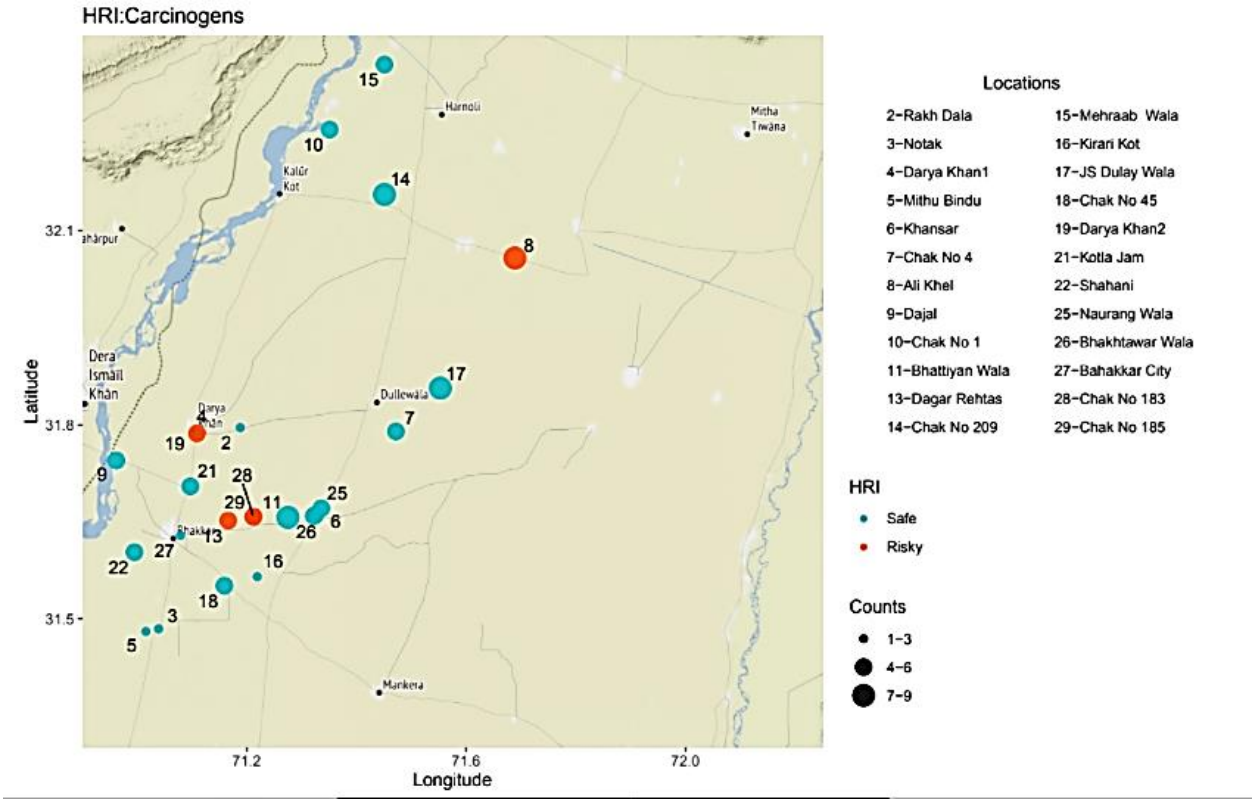

Figure 33 :Representation of Carcinogens in Bhakkar district

The map in (Figure 34) represents the carcinogens on their respective locations denoted by numbers. Safe and risk zones are assigned based on their HRI values. Color indicates the risk factor while the size of the pollutant is showing the pollutant count. Blue color circles indicate the safe zones having carcinogen in safer limits while orange color circles indicate those locations which may have carcinogens above safer limits so considered as risky. The size of the circle is owing us the pollutant count like the number of specific pollutants at corresponding location. The smallest sized circle represents pollutants which are 1 to 3 in number. While medium sized circle represents 4 to 6 and larger sized circles represents those pollutants which are 7 to 9 in number. Sites including chak no 209**(14),** bhattiyan walan **(11)**, JS dulay wala **(17)**, Ali khel **(8))** were observed more polluted in terms of carcinogens. Darya khan 1**(4),** chak no 183**(28),** chak no 185**(29),** darya khan 2**(19),** dagar rehtaas **(13),** chak no 1(**10),** mehrab wala **(15**), shahhni (**22**) were suspected to be moderately polluted with carcinogens. Rakh dala (**2),**

notak (**3),** mithu pindu (**5),** Bhakkar city (**27),** kirrari kot (**16)** have few numbers of carcinogens in their samples. Among all polluted sites, only darya khan 1**(4),** chak no 183(**28),** ali khel (**8),** darya khan 2(**19),** chak no 185**(29),** dagar rehtas **(13**) were supposed to be risky site based on supposed HRI values.

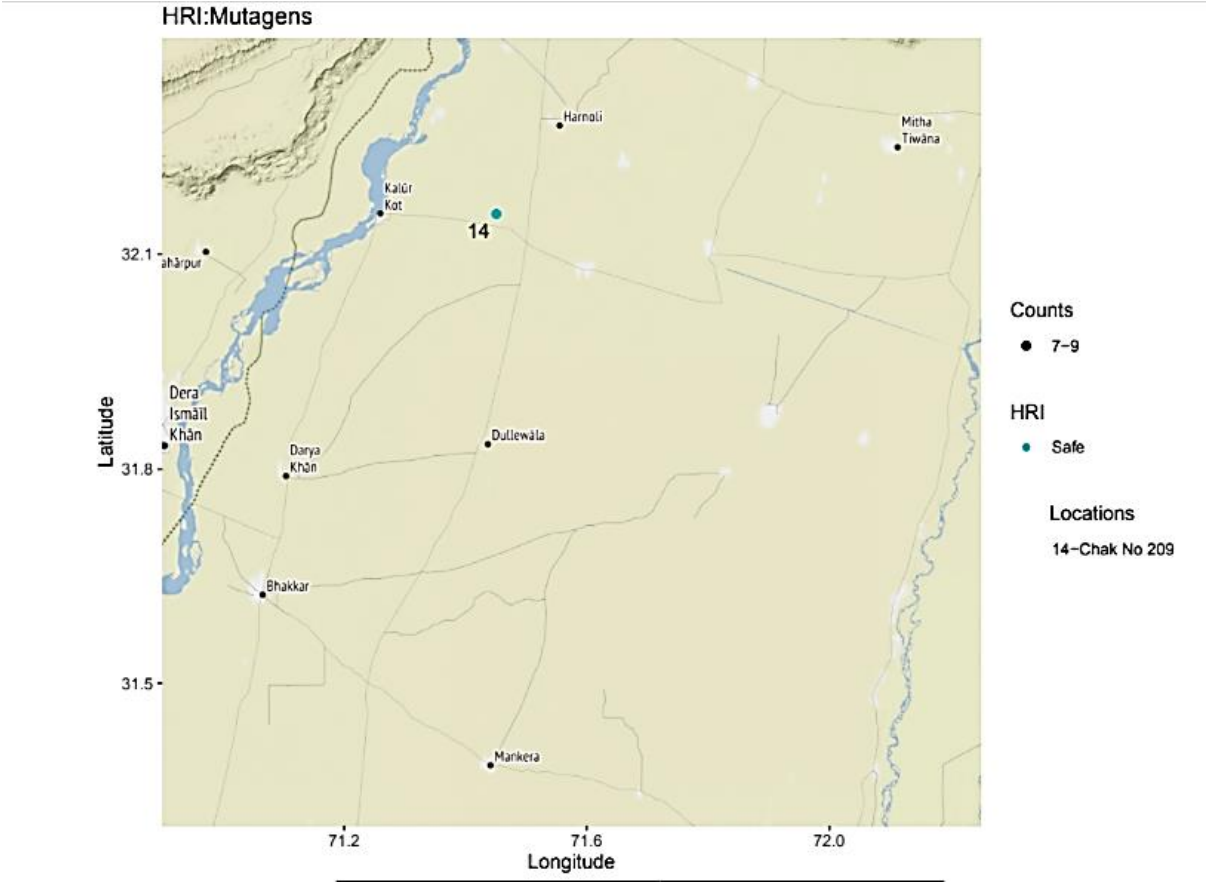

Figure 34: Location base representation of mutagen in Bhakkar district

The map in (Figure 35) is the representation of mutagens on their respective locations denoted by numbers. Safe and risk zones are assigned based on their HRI values Color indicates the risk factor while the size of the pollutant shows pollutant count. Blue color shows that mutagens

are in safer limits. The size of the circle is showing us the pollutant count like the number of specific pollutants at corresponding location. Map in (figure 35) shows that among all sites only chak no 209 was observed as polluted in terms of mutagens. No mutagens were observed at at chak no 45**(18)**, and darya khan 2 **(19)** chak no 183 **(28),** chak no 185 **(29),** dagar rehtas**(13),** Anar Shah **(1)**, Notak **(3)**, Mithu Bindu **(5)**, Janu Sahrif **(12)**, Kirari Kot **(16)**, Mankera **(23)** ,Khansar **(6)**, Chak No 4 **(7)**, Dajal **(9)**, Dagar Rehtas **(13)**, Shahani **(22)**, Kotla Jam **(21)**, Nawan Gusu **(24)**, Bhakkar city **(27)**, Chak No 183 **(28)**, Chak No 185 **(29)**, Panja Giraen **(20)**, Jafar Wala **(30)**, Naurang Wala **(25)**, Chak No 45 **(18)**, Kirari Kot **(16)**, Mehraab Wala **(15)** and Chak No 01 **(10),** Ali khel, **(8),** bhattiyan wala, **(11**) bhaktawar wala**(26)**

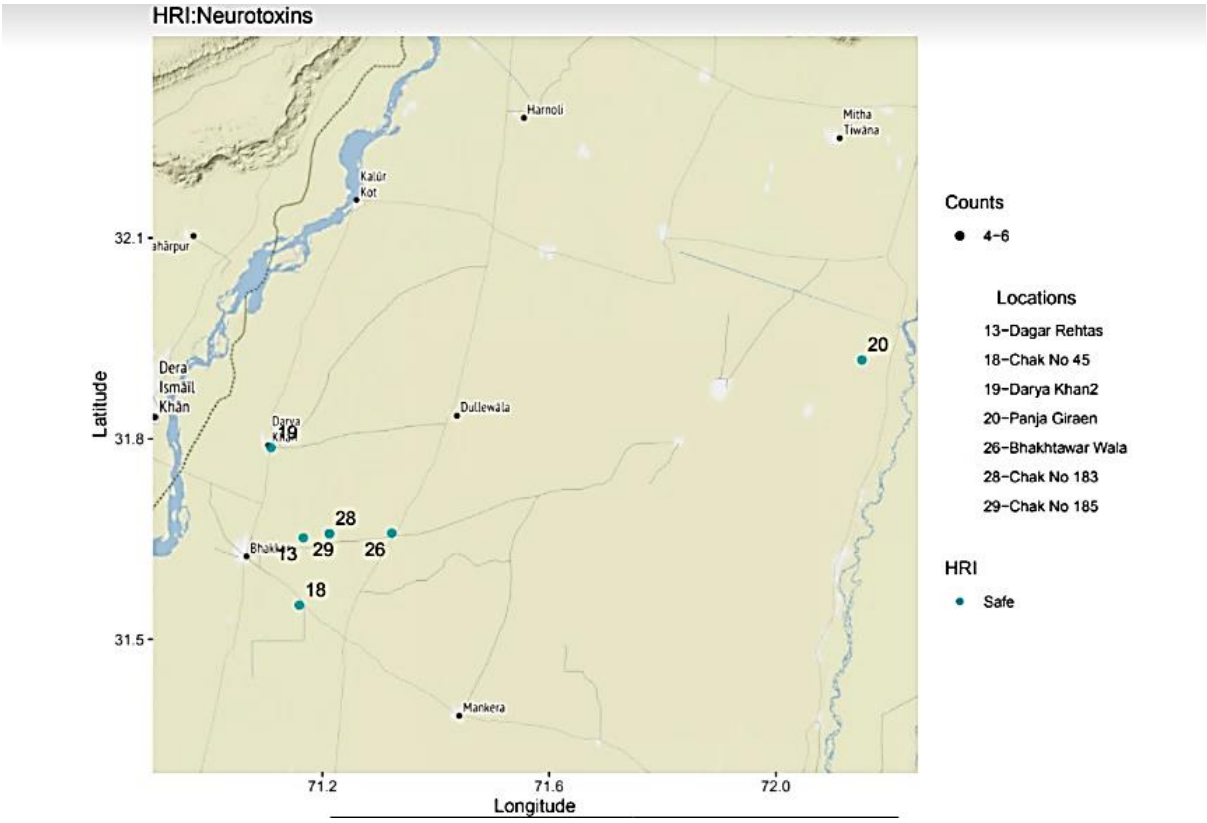

Figure *36:* Location base representation of neurotoxins in Bhakkar district

The map in (Figure 36) is the representation of neurotoxins on their corresponding locations denoted by numbers. Safe and risk zones are assigned based on their HRI values Color indicates the risk factor while the size of the pollutant shows pollutant count. Blue color shows that neurotoxins are in safer limits. The size of the circle is showing us the pollutant count like the number of specific pollutants at corresponding location. Map shows that among all locations dagar rehtas, chak no 45**(18)**, darya khan 2 **(19)** Panj Giraen **(20)**, bhaktawar wala, Chak No 183 **(28)**, and)**,** chak no 185 **(29), were** observed as more polluted in terms of neurotoxins. All

were in safe zone. No neurotoxin were observed at and chak no 183 **(28)** dagar rehtas**(13),** Anar Shah **(1)**, Notak **(3)**, Mithu Bindu **(5)**, Janu Sahrif **(12)**, Kirari Kot **(16)**, Mankera **(23)** ,Khansar **(6)**, Chak No 4 **(7)**, Dajal **(9)**, Dagar Rehtas **(13)**, Shahani **(22)**, Kotla Jam **(21)**, Nawan Gusu **(24)**, Bhakkar city **(27**Chak No 185 **(29)**, Jafar Wala **(30)**, Naurang Wala **(25)**, Chak No 45 **(18)**, Kirari Kot **(16)**, Mehraab Wala **(15)** and Chak No 01 **(10),** Ali khel, **(8),** bhattiyan wala, **(11),** bhaktawar wala**(26)**

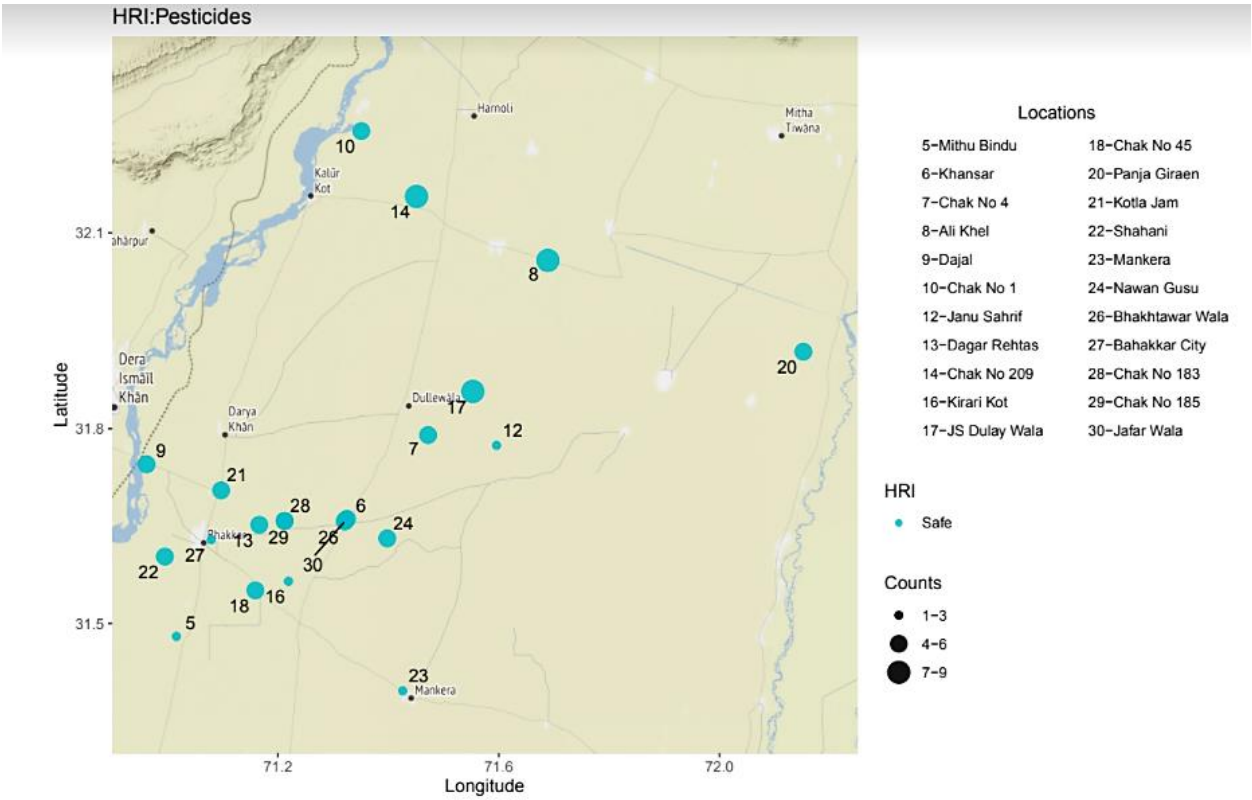

Figure 35 :Location based representation of Pesticides in Bhakkar District

The map in (Figure 37) is the representation of pesticides on their respective locations denoted by numbers. Color indicates the risk factor while the size of the pollutant shows pollutant count. Blue color shows that pesticides are in safer limits. Almost all the locations which contain

pesticides are in safer zone despite of the usage of pesticide in that targeted agricultural area. The size of the circle is showing us the pollutant count like the number of specific pollutants at corresponding location. The smallest sized circle represents pollutants which are 1 to 3 in number. While medium sized circle represents 4 to 6 and larger sized circles represents those pollutants which are 7 to 9 in number. Map shows that chak no 1 **(10),** chak no 209**(14),** ali khel **(8**), dagar rehtas **(13),** dulay wala (**17**), chak no 45**(18)** were observed as more polluted in terms of pesticides. Moderate pesticides count was observed at jafar wala **(30),** shahaani (**22),** chak 183**(28),** chak 185**(29)** and khansar**(6).** Few pesticeds were observed in the samples collected from janu sharif **(22**), kirrai kot (**16),** mithu pindu **((5),** Bhakkar city **(27)** and chak no 4**(7).** No pesticide was observed at Mehraab Wala **(15)**, Darya khan **(4),** anaar sha, **(2),** rakh dala, (**3**) notak **(4),** bhattiyan wala **(11)** Mehraab Wala **(15).**

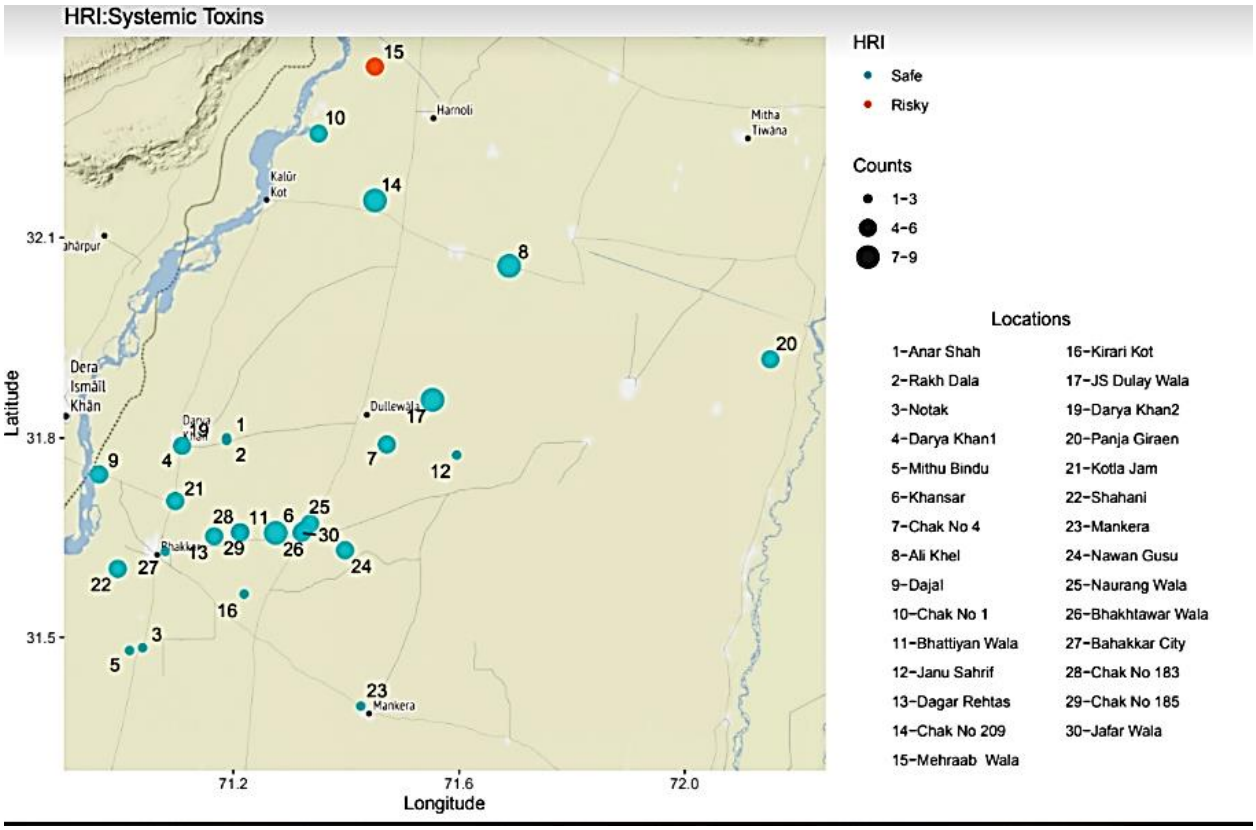

Figure 36: Location based representation of Systemic Toxins in Bhakkar district

The map in (Figure 38) is the representation of systemic toxins on their respective locations denoted by numbers. Color indicates the risk factor while the size of the pollutant shows pollutant count. Blue color circles show that systemic toxins are in safer limits while orange color circle indicates a location which is at danger zone based on HRI value. The size of the circle is showing us the pollutant count like the number of specific pollutants at corresponding location. The smallest sized circle represents pollutants which are 1 to 3 in number. While medium sized circle represents 4 to 6 and larger sized circles represents those pollutants which are 7 to 9 in number. Map shows that chak no 4 **(7),** JS dulay wala (**17**), ali khel **(8),** bhaktawar wala (**26),** bhattiyan wala **(11),** were observed as more polluted sites in terms of systemic toxins. Chak no 1**(10),** chak no 4**(7),** kotla jam **(21),** nawan gassu **(24),** darya khan 1**(4),** darya khan 2**(19),** jafar wala **(30),** shahani (**22**), dagar rehtas (**13),** chak no 183(***28),*** chak no 185**(29**), panj giraen (20) were observed as moderately polluted in terms of systemic toxins. Few systemic toxins were observed at janju sharif (**12**), mithu pindu **(5),** notak, **(3**) kirari kot (**16),** rakh dala (**2**), anaar shah (**1),** Bhakkar city **(27)** and rakh dala **(2).** no systemic toxin was observed at chak no 45(1**8**).

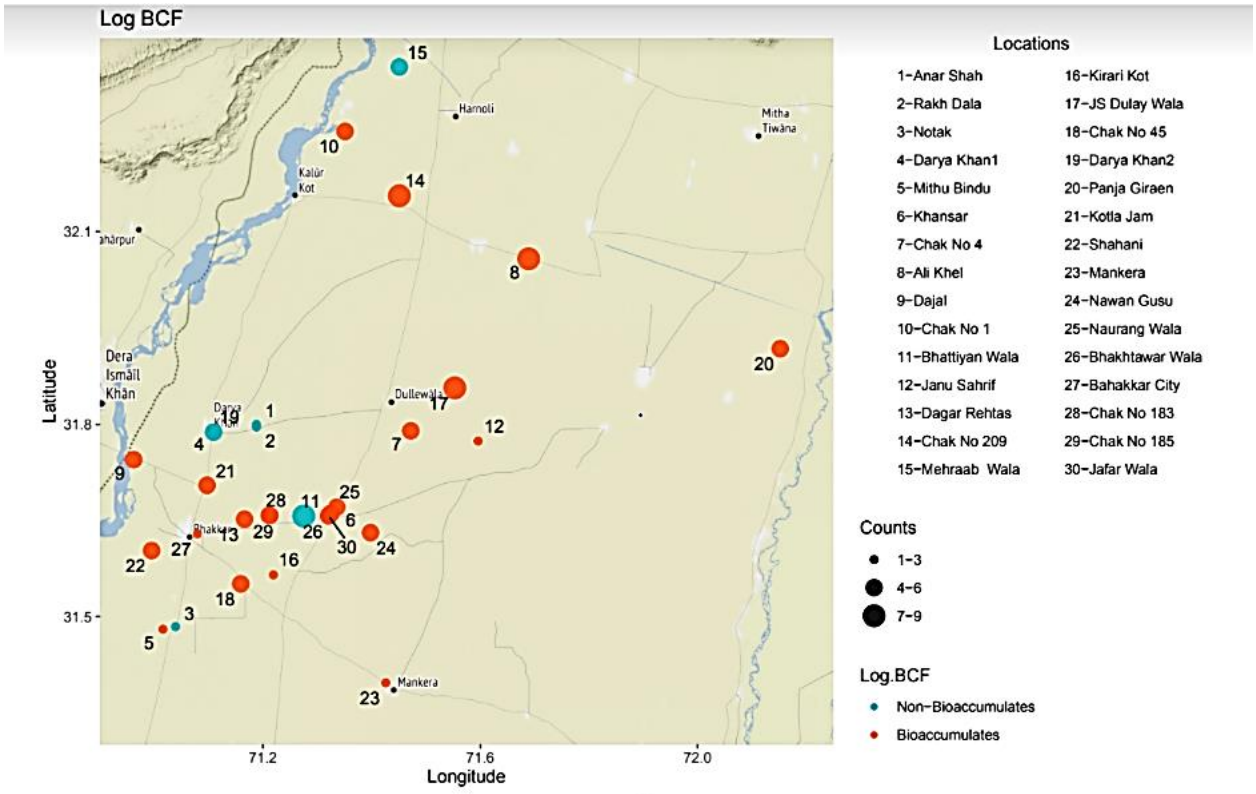

Figure 37 :Differentiation of bioaccumulates and non-bioaccumulates according to BCF values

Pollutants were categorized into bioaccumulates and non-bioaccumulates according to their log BCF values determined through the EPI-SUITE modeling technique.

Log BCF> 3 = Highly Bio accumulative

A map in (Figure 39) represents these two categories. Locations are denoted by numbers. Color indictes the nature of the pollutant i-e bioaccumulate or non-bioaccumulate while the size of the circle is showing the pollutant count. Blue color circles indicate those locations which contain non-bio accumulative pollutants in their samples while orange color circles indicate locations which contain bio-accumulative pollutants in their samples. The smallest sized circle represents pollutants which are 1 to 3 in number. While medium sized circle represents 4 to 6 and larger sized circles represents those pollutants which are 7 to 9 in number.

Map shows that few bioaccumultes were observed at mithu bindu(**5)**, Kirari Kot **(16** Janu Sahrif **(12)**, Bhakkar city **(27)**Chak and Mankera **(23)**Maximum number of bioaccumulates were observed in the samples of Ali khel**(8),** chak no 209**(14),** Moderate number of bioaccumulates were observed in the samples of dagar rehtas, chak no 45**(18)**, darya khan 2 **(19)** Panj Giraen **(20)**, bhaktawar wala, , Chak No 183 **(28)**, and **),** chak no 185 **(29),** chak no 183 **(28** dagar rehtas**(13),** Notak **(3)**, ,Khansar **(6)**, Chak No 4 **(7)**, Dajal **(9)**, Dagar Rehtas **(13)**, Shahani **(22)**, Kotla Jam **(21)**, Nawan Gusu **(24)**, Chak No 185 **(29)**, Jafar Wala **(30)**, Naurang Wala **(25)**, Chak No 45 **(18)**, Kirari Kot **(16)**, Mehraab Wala **(15)** and Chak No 01 **(10).** Minimumum number of non- bioaccumulates were observed at annar shah **(1),** rakh dala **(2).** Moderate count of non bioaccumulates were observed at darya khan 2**(19).** Maximim amount of non-bioaccumulates were observed in the samples of bhattiyan wala, **(11),** and bhaktawar wala **(26).**

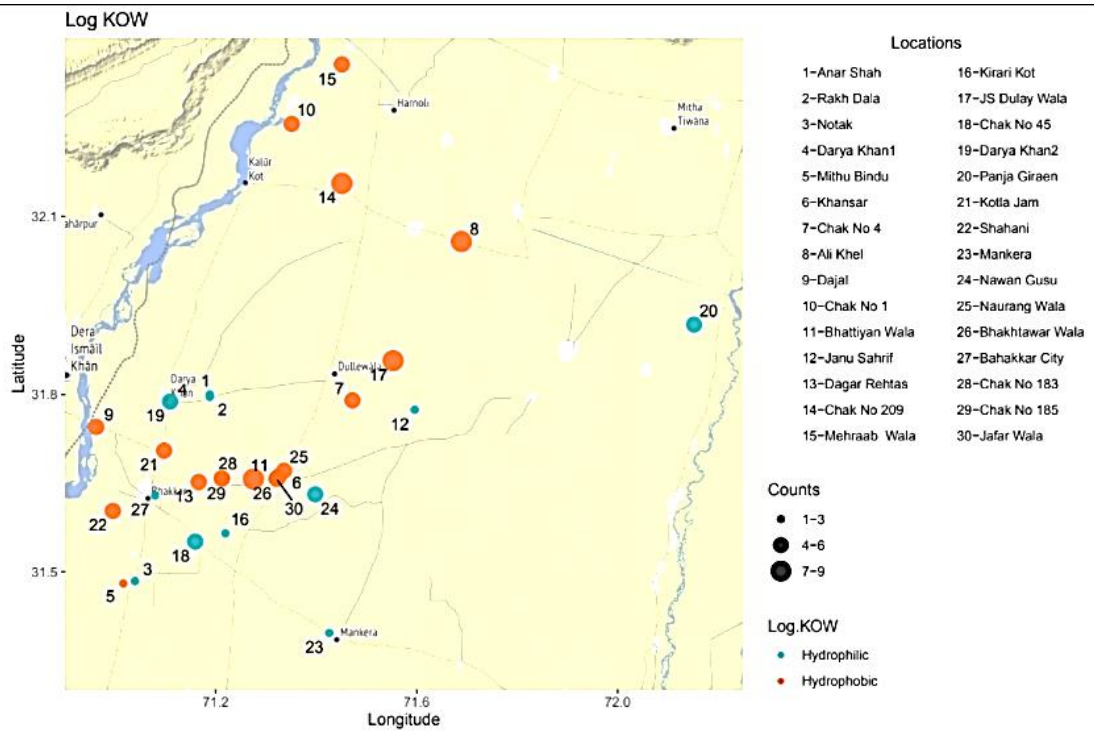

Figure 38: Classification of pollutants according to their log K_{ow} values

Pollutants were categorized according to their log K_{ow} values determined through the EPI-SUITE modeling technique. A map in (Figure 40) represents these two categories. Locations are denoted by numbers. Color is indicating the nature of the pollutant i-e into hydrophobic or hydrophilic while the size of the circle is showing us the pollutant count. If log K_{ow} value is greater than 3 then the pollutant or chemical is said to be hydrophobic in nature while it is said to be hydrophilic if the log K_{ow} value is less than 1. Blue color circles are indicating those locations which contain hydrophilic nature pollutant in their samples while orange color circles indicate those locations where samples contain hydrophobic nature pollutants. The smallest sized circle represents pollutants which are 1 to 3 in number. While medium sized circle represents 4 to 6 and larger sized circles represents those pollutants which are 7 to 9 in number.

Log K_{ow} > 3= Hydrophobic

Log K_{ow}< 1 = Hydrophilic

Map shows that maximum number of hydrophobic pollutants were observed in the samples of chak no209 **(14),** Ali khel **(8),** js dulay wala**, (17)** bhaktawar wala **(26)** bhattiyan wala **(11).** Moderate number of hydrophobic pollutants were observed at chak no 45**(18)**, darya khan 2 **(19)** Panja Giraen **(20**, , Chak No 183 **(28)**, and **),** chak no 185 **(29 ,**dagar rehtas**(13),** Kirari Kot **(16)**,Khansar **(6)**, Chak No 4 **(7)**, Dajal **(9)**, Dagar Rehtas **(13)**, Shahani **(22)**, Kotla Jam **(21)**, Chak No 185 **(29)**, Jafar Wala **(30)**, Naurang Wala **(25)**, Kirari Kot **(16)**, Mehraab Wala **(15)** and Chak No 01 **(10)** **.**Few hydrophobic pollutants were observed in the sample of mithu bindu**(5**).Moderate number of hydrophilic pollutants were observed in the pollutants of panj giraen, , Chak No 45 **(18)**, , Nawan Gusu **(24)**, darya khan 1, darya khan 2. Few hydrphillic pollutants were observed in the samples of Notak **(3)** Janu Sahrif **(12)**, Kirari Kot **(16)**, annar shah **(1),** Rakh dala **(2)** and Bhakkar city **(27)**

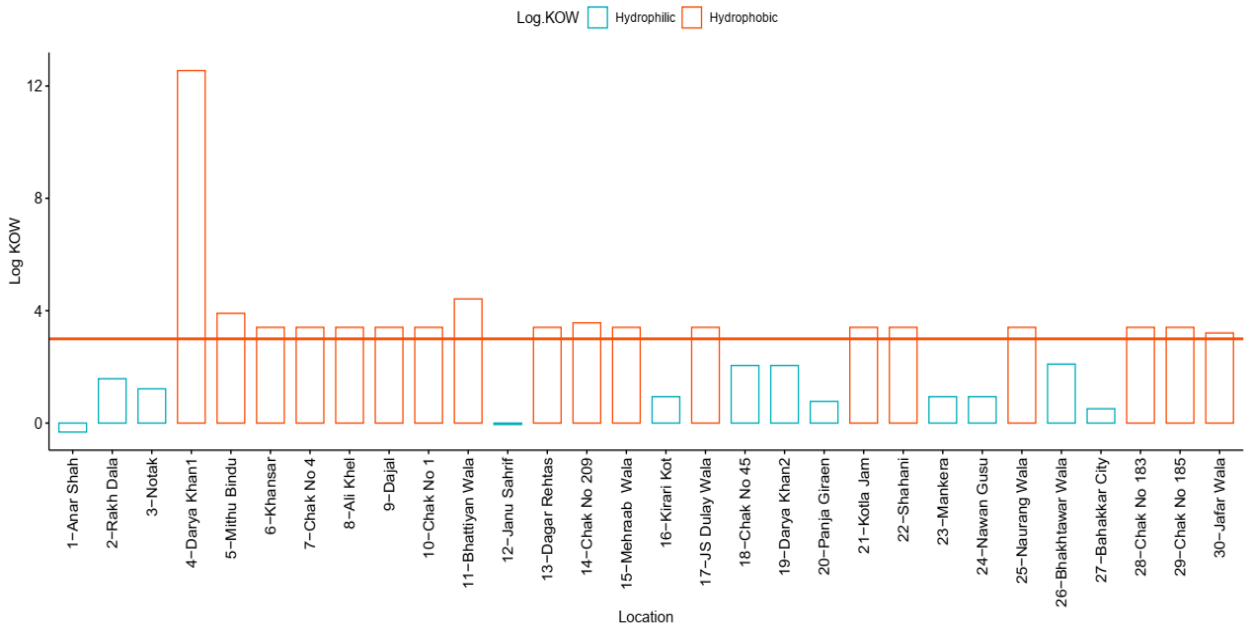

Figure 39 :Log K_{ow} vs Location

(Figure 41) has a graphical representation of log K_{ow} vs Location. Bar color is representing the nature of pollutants i-e hydrophobic or hydrophilic while bar length represents the log k_{ow} value. On x-axis of the graph all the locations are present while on y-axis log K_{ow} values are

present. The locations which exceed the threshold limit (3) are shown by orange bars which contain hydrophobic pollutants in their samples. Hydrophobic substances are considered as more lethal as compared to hydrophilic chemicals. That's why these locations are marked as risky. Blue color bars are indicating those locations which are in safe zone because pollutants do not exceed the threshold limit (3) of Log K_{ow} value.

Chak No 45 **(18)**, Nawan Gusu **(24)**, darya khan (**19**) ,Kirari Kot **(16)**, Notak **(3)** Janu Sahrif **(12)**, Kirari Kot **(16)**, annar shah **(1),** Rakh dala **(2)** and Bhakkar city **(27), (11),** bhaktawar wala **(26),** mankera**(23)** are denoted by sky blue bars which indicate the presence of hydrophilic pollutants in their samples**.** Sites chak no209 **(14),** Ali khel **(8),** js dulay wala**, (17))** darya khan 1 **(4)**, Chak No 183 **(28)**, and **),** chak no 185 **(29 ,**dagar rehtas**(13),** Khansar **(6)**, Chak No 4 **(7)**, Dajal **(9)**, Dagar Rehtas **(13)**, Shahani **(22)**, Kotla Jam **(21)**, Chak No 185 **(29)**, Jafar Wala **(30)**, Naurang Wala **(25)**, Mehraab Wala **(15)** and Chak No 01 **(10),** bhattiyan wala **(11)** are shown by orange bars which surpass the threshold limit and depict the presence of hydrophobic pollutants inside their samples**.**

4.4.1 List of persistent pollutants identified through EPI-SUITE modelling software technique

Sr#	Pollutant name	Location
1	1,7 Dimethylxanthine	Anar shah (1)
2	CynamideI-2-propenyl-	Anar shah (1), Chak # 185 (9), CHAK # 209(14), Nawan gassu (24), Naurnag wala (25), Bhakkar city (27), Bhakhtawar wala (26), Chak # 183 (28),
3	1,3-DIMETHYL-2,4,5-TRIOXOIMIDAZOLIDINE	Rakh dala (2)
4	3H-N-TRIAZOLO(4,5-D)PYRIMIDINE-5,7(4H,6H)-DIONE, 3,6-DIMETHYL-	Notak (3)

5	P-DIOXANE, 2,5-DIVINYL-	Notak (3), Ali khel (8), Dajjal (9), Bhattiyan wala (11), Bakhtawar wala (26) Darya khan (4), Chak 45(18), Naurang wala (25)
6	1,5-HEXADIENE, 3,4-DIMETHYL-	Notak (3), Ali khel (8), Dajjal (9), Bhattiyan wala (11), Bakhtwar wala (26) Darya khan (4), Chak 45(18), Naurang wala (25)
7	2-METHYLTETRACOSANE	Darya khan (4)
8	5 –Amino –3 Azido-1,2,4 –Triazine -6-CARBONITRILE	Mithu pindu (5), Chak # 185 (9) Bhakkar city (27), Bakhtawar wala (26), Naurnag wala (25), Mankera (23), Kotla Jam (21), Shahani (22), Chak 145(18) Dulay wala (17), Dagar rehtas (13), Chak 209 (14) Dajjal (9) Khansar (6)
9	3,3SULPHONYLDIPROPIONITRL	Chak # 4(7), Dajjal (9), Chak 1(10), Bhatiiyan wala (11), Chak no 209, (14) Mehrab wala (15), Dajjal (9), Dulay wala, (17) Kotla jam (21), Mankera (23) Naurang wala (25), Bakhtawar wala (26), Jafar wala (30)
10	1,2-DIOXOLAN-3-ONE, 5,5-DIETHYL-4-METHYLENE	Bhattiyan wala (11)
11	1H-1,2,4-TRIAZOLE,1-(2-PROPENYL)-	Bhattiyan wala (11)
12	PIPERAZINE,2,5-DIMETHYL	Dagar rehtas (13)

13	BUTANENITRILE,3 METHYL-2-METHYLENE-	Dagar rehtas (13)
14	CYCLOBUTANEACETONITRILE, -1-METHYL-2-(1-METHYLETHENYL)-	Dagar rehtaas
15	2-propenyl-3-vinyloxirane	Chak no 209(14)

4.4.2 Principal component analysis

PCA or principal component analysis is a dimension reduction technique in which data is reduced but not destroyed or lost. In principle component analysis original variables are transformed into latent variables. Latent are the linear combinations of originals ones. PCA analysis was performed to identify influential variables and influential samples/locations. In factor PCA there are two components or dimensions for data expression crossing each other on origin named principal factors. The first component is explaining 35 percent variation and the second component is explaining 25 percent variation in data. On an individual basis contribution of each variable is shown by the color scheme. The red color variables show more contribution in data which are log K_{ow} and pollutant count while blue color variable log BCF shows the least contribution in data. Yellow colored variables stand in the middle or in between based on their contribution.

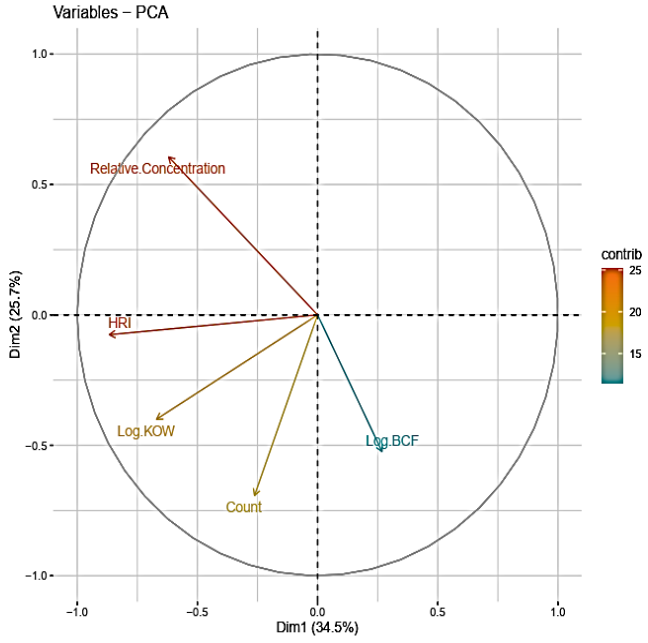

Figure 40: Variables-Principal component analysis

Principal component analysis was also performed for locations from which samples were collected. The red-colored locations contribute the most (0.75)towards respective variable discussed in factors PCA while the blue color locations contribute least (0.25)towards variables in the data which are nearest to the origion. Yellow color locations cause variation in between both red and blue colored locations(0.50) mentioned in (Figure 43). Hence, through PCA we can get the idea of most influential and least influetial variables in data.

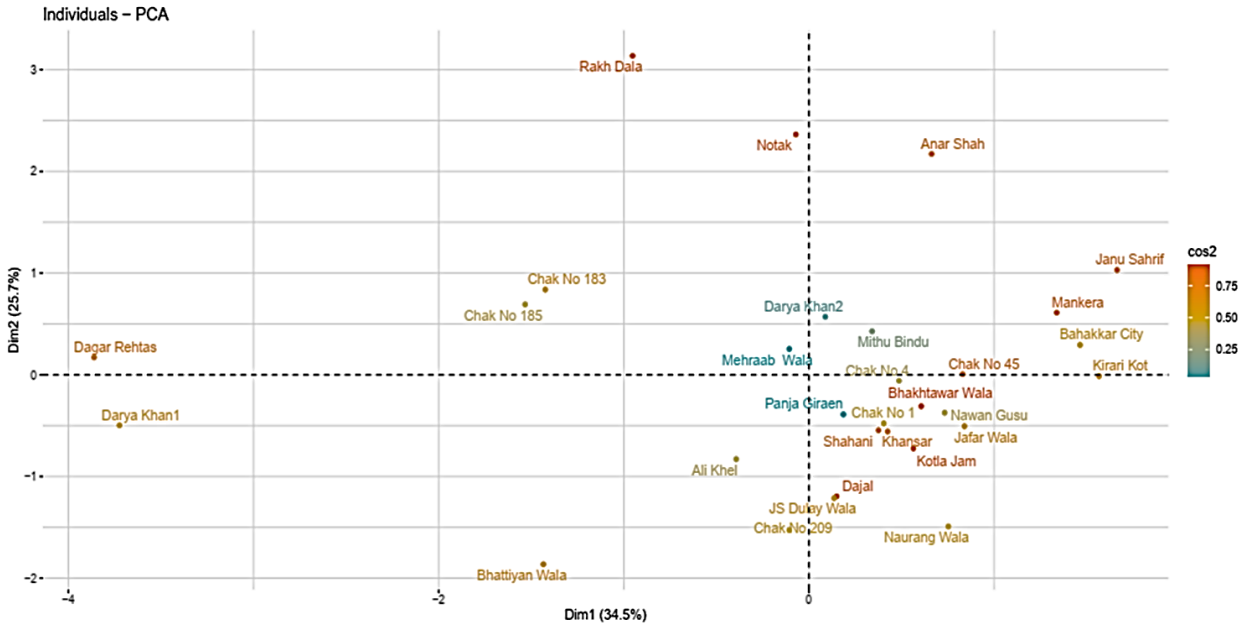

Figure 41: Individual-Principal component analysis for influential locations

CHAPTER 05

5-Conclusion

The organic pollutants study via honey sample was performed for the first time in Pakistan in Bhakkar district of Punjab. The presence or absence of organic airborne pollutants was determined with the help of honey as a biomonitor. Organic content was extracted from the samples with the help of QuEChERS method of extraction. Results of Gas chromatography Mass spectrometry have confirmed the presence of organic pollutants in honey samples which were further supported through FTIR and UV-Visible spectroscopy respectively. The results obtained have shown the presence of different types of pollutants in 30 samples of honey which were proved to be carcinogenic, mutagenic, cytotoxins, systemic toxins, ecotoxins based on literature. Health risk assesment was assumed by calculating HRI values using relative concentrations of the pollutants due to the lack of standards. Morover, Honey proved to be a good indicator of environment due to honey bee's mobility, intense foraging activity and morphological features.

5.1 Limitations of study

Accurate quantification of samples was not performed due to absence of standards and repetitive aanalysis. HRI values were assumed with the help of relative concentrations of pollutants identified through GC/MS.

5.2 Future recommendations

With the help of standards, accurate quantification of samples can be performed which can be used to calculate HRI values which are helpful in health risk assesement of the pollutants present in environment.

REFERENCES

1. Briggs, D., Environmental pollution and the global burden of disease. *Br Med Bull* **2003,** *68*, 1-24.
2. Deknock, A.; De Troyer, N.; Houbraken, M.; Dominguez-Granda, L.; Nolivos, I.; Van Echelpoel, W.; Forio, M. A. E.; Spanoghe, P.; Goethals, P., Distribution of agricultural pesticides in the freshwater environment of the Guayas river basin (Ecuador). *Sci Total Environ* **2019,** *646*, 996-1008.
3. Özkara, A.; Akyil, D.; Konuk, M., Pesticides, Environmental Pollution, and Health. In *Environmental Health Risk - Hazardous Factors to Living Species*, 2016.
4. Ruiz-Toledo, J.; Vandame, R.; Castro-Chan, R. A.; Penilla-Navarro, R. P.; Gomez, J.; Sanchez, D., Organochlorine Pesticides in Honey and Pollen Samples from Managed Colonies of the Honey Bee Apis mellifera Linnaeus and the Stingless Bee Scaptotrigona mexicana Guerin from Southern, Mexico. *Insects* **2018,** *9* (2).
5. Muralikrishna, I. V.; Manickam, V., Introduction. In *Environmental Management*, 2017; pp 1-4.
6. Al Alam, J.; Fajloun, Z.; Chabni, A.; Millet, M., The use of honey as environmental biomonitor of pesticides contamination in northern Lebanon. *Euro-Mediterranean Journal for Environmental Integration* **2017,** *2* (1).
7. Machine"., a. t. W., Microsoft Encarta Online Encyclopedia 2009.

. *Pollution Archived* **2009-10-2**.
8. 1. G. Tyler Miller, J. a. D. H., " "Outdoor Air Pollution in Living in the Environment.
9. Ghorani-Azam, A.; Riahi-Zanjani, B.; Balali-Mood, M., Effects of air pollution on human health and practical measures for prevention in Iran. *J Res Med Sci* **2016,** *21*, 65.
10. Forouzanfar, M. H.; Afshin, A.; Alexander, L. T.; Anderson, H. R.; Bhutta, Z. A.; Biryukov, S.; Brauer, M.; Burnett, R.; Cercy, K.; Charlson, F. J. J. T. l., Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks, 1990–2015: a systematic analysis for the Global Burden of Disease Study 2015. **2016,** *388* (10053), 1659-1724.
11. Ghorani-Azam, A.; Riahi-Zanjani, B.; Balali-Mood, M. J. J. o. r. i. m. s. t. o. j. o. I. U. o. M. S., Effects of air pollution on human health and practical measures for prevention in Iran. **2016,** *21*.
12. Theron, J.; Cloete, T. J. C. R. i. M., Emerging waterborne infections: contributing factors, agents, and detection tools. **2002,** *28* (1), 1-26.
13. Maipa, V.; Alamanos, Y.; Bezirtzoglou, E. J. M. e. i. h.; disease, Seasonal fluctuation of bacterial indicators in coastal waters. **2001,** *13* (3), 143-146.
14. Atlas, R. M., *Microbial ecology: fundamentals and applications*. Pearson Education India: 1998.
15. Manisalidis, I.; Stavropoulou, E.; Stavropoulos, A.; Bezirtzoglou, E. J. F. i. p. h., Environmental and health impacts of air pollution: a review. **2020,** *8*, 14.
16. Batzias, F. A.; Siontorou, C. G., A knowledge-based approach to environmental biomonitoring. *Environmental Monitoring and Assessment* **2006,** *123* (1), 167-197.
17. Bayoumi, A. E. J. A. C. H.; Safety, Counterfeit Pesticides. **2021**.
18. Nadaf, H. A.; Yadav, G. S.; Kumari, B. J. J. o. A. R., Validation and monitoring of pesticide residues in honey using QuEChERS and gas chromatographic analysis. **2015,** *54* (3), 260-266.

19. Al-Alam, J.; Fajloun, Z.; Chbani, A.; Millet, M. J. J. o. E. S.; Health, P. A., Determination of 16 PAHs and 22 PCBs in honey samples originated from different region of Lebanon and used as environmental biomonitors sentinel. **2019,** *54* (1), 9-15.
20. .
21. Biziuk, M.; Stocka, J., Multiresidue Methods for Determination of Currently Used Pesticides in Fruits and Vegetables Using QuEChERS Technique. *International Journal of Environmental Science and Development* **2015,** *6* (1), 18-22.
22. Norli, H. R.; Christiansen, A.; Deribe, E., Application of QuEChERS method for extraction of selected persistent organic pollutants in fish tissue and analysis by gas chromatography mass spectrometry. *J Chromatogr A* **2011,** *1218* (41), 7234-41.
23. Sheldon, M.; Pinion Jr, C.; Klyza, J.; Zimeri, A. M. J. J. o. E. H., Pesticide Contamination in Central Kentucky Urban Honey: A Pilot Study. **2019,** *82* (1).
24. Raja, P. M. V.; Barron, A. R., Principles of Gas Chromatography. 2021.
25. Griffiths, P. d. H., J. A. , https://books.google.com/books?id=C_c0GVe8MX0C. **18 May 2007**.
26. Mathias, J.
27. CHOUDHARY, A., The principle of Ultra Violet (UV) Spectrophotometer.
28. Card, M. L.; Gomez-Alvarez, V.; Lee, W. H.; Lynch, D. G.; Orentas, N. S.; Lee, M. T.; Wong, E. M.; Boethling, R. S., History of EPI Suite and future perspectives on chemical property estimation in US Toxic Substances Control Act new chemical risk assessments. *Environmental science. Processes & impacts* **2017,** *19* (3), 203-212.
29. Briggs, D. J. B. m. b., Environmental pollution and the global burden of disease. **2003,** *68* (1), 1-24.
30. Rissato, S. R.; Galhiane, M. S.; de Almeida, M. V.; Gerenutti, M.; Apon, B. M. J. F. C., Multiresidue determination of pesticides in honey samples by gas chromatography–mass spectrometry and application in environmental contamination. **2007,** *101* (4), 1719-1726.
31. Eissa, F.; El-Sawi, S.; Zidan, N. E.-H. J. P. J. o. E. S., Determining Pesticide Residues in Honey and their Potential Risk to Consumers. **2014,** *23* (5).
32. Malhat, F. M.; Haggag, M. N.; Loutfy, N. M.; Osman, M. A.; Ahmed, M. T. J. C., Residues of organochlorine and synthetic pyrethroid pesticides in honey, an indicator of ambient environment, a pilot study. **2015,** *120*, 457-461.
33. Al Alam, J.; Fajloun, Z.; Chabni, A.; Millet, M. J. E.-M. J. f. E. I., The use of honey as environmental biomonitor of pesticides contamination in northern Lebanon. **2017,** *2* (1), 1-7.
34. JURAK, G.; BOSNIR, J.; RACZ, A.; BRKIC, D.; PRSKALO, I.; KISc, D.; OZIMEC, S.; KALAMBURA, S. J. J. o. E. P.; Ecology, BIOINDICATOR DETECTION OF PESTICIDE RESIDUES IN THE ENVIRONMENT USING HONEY BEES. **2021,** *22* (2), 458-466.
35. Kumar, A.; Gill, J. P. S.; Bedi, J. S.; Kumar, A. J. E. S.; Research, P., Pesticide residues in Indian raw honeys, an indicator of environmental pollution. **2018,** *25* (34), 34005-34016.
36. Panseri, S.; Catalano, A.; Giorgi, A.; Arioli, F.; Procopio, A.; Britti, D.; Chiesa, L. J. F. C., Occurrence of pesticide residues in Italian honey from different areas in relation to its potential contamination sources. **2014,** *38*, 150-156.
37. Ruiz-Toledo, J.; Vandame, R.; Castro-Chan, R. A.; Penilla-Navarro, R. P.; Gómez, J.; Sánchez, D. J. I., Organochlorine pesticides in honey and pollen samples from managed colonies of the honey bee Apis mellifera Linnaeus and the stingless bee Scaptotrigona mexicana Guérin from Southern, Mexico. **2018,** *9* (2), 54.

38. Kazazic, M.; Djapo-Lavic, M.; Mehic, E.; Jesenkovic-Habul, L. J. C.; Ecology, Monitoring of honey contamination with polycyclic aromatic hydrocarbons in Herzegovina region. **2020,** *36* (8), 726-732.
39. Hungerford, N. L.; Tinggi, U.; Tan, B. L.; Farrell, M.; Fletcher, M. T. J. I. j. o. e. r.; health, p., Mineral and Trace element analysis of Australian/Queensland Apis mellifera honey. **2020,** *17* (17), 6304.
40. Lasheras, R. J.; Lázaro, R.; Burillo, J. C.; Bayarri, S. J. F., Occurrence of Pesticide Residues in Spanish Honey Measured by QuEChERS Method Followed by Liquid and Gas Chromatography–Tandem Mass Spectrometry. **2021,** *10* (10), 2262.
41. Chiesa, L. M.; Labella, G. F.; Panseri, S.; Britti, D.; Galbiati, F.; Villa, R.; Arioli, F. J. F. A.; A, C. P., Accelerated solvent extraction by using an 'in-line'clean-up approach for multiresidue analysis of pesticides in organic honey. **2017,** *34* (5), 809-818.
42. Schanzer, S.; Kröner, E.; Wibbelt, G.; Koch, M.; Kiefer, A.; Bracher, F.; Müller, C. J. C., Miniaturized multiresidue method for the analysis of pesticides and persistent organic pollutants in non-target wildlife animal liver tissues using GC-MS/MS. **2021,** *279*, 130434.
43. Calatayud-Vernich, P.; Calatayud, F.; Simó, E.; Picó, Y. J. M., Efficiency of QuEChERS approach for determining 52 pesticide residues in honey and honey bees. **2016,** *3*, 452-458.
44. Calatayud-Vernich, P.; Andrés-Costa, M. J.; Calatayud, F.; Simó, E.; Picó, Y., Pesticide occurrence in different apicultural matrices (honey bees, wax and pollen). **2018**.
45. Jovetić, M. S.; Redžepović, A. S.; Nedić, N. M.; Vojt, D.; Đurđić, S. Z.; Brčeski, I. D.; Milojković-Opsenica, D. M. J. A. z. h. r. i. t., Urban honey-the aspects of its safety. **2018,** *69* (3), 264-274.
46. Grenier, É.; Giovenazzo, P.; Julien, C.; Goupil-Sormany, I. J. E. M.; Assessment, Honeybees as a biomonitoring species to assess environmental airborne pollution in different socioeconomic city districts. **2021,** *193* (11), 1-14.
47.available at https://www.spectroscopyonline.com/authors/brian-c-smith-0, The Carbonyl Group, Part I: Introduction. **Spectroscopy-09-01-2017,,** *Volume 32* (Issue 9).
48. U. O. Aroke, A. A. R. O. O., Fourier-transform Infrared Characterization of Kaolin, Granite, Bentonite and

Barite.
49. Hubicki, Z.; Wołowicz, A. J. H., Adsorption of palladium (II) from chloride solutions on Amberlyst A 29 and Amberlyst A 21 resins. **2009,** *96* (1-2), 159-165.
50. IR Spectroscopy Tutorial: Carboxylic Acids.
51. Janaki, K.; Velraj, G. J. R. R. i. S.; Technology, Spectroscopic studies of some fired clay artifacts recently excavated at Tittagudi in Tamilnadu. **2011,** *3* (3).
52. İşçi, S.; Ünlü, C.; Atici, O.; Güngör, N. J. B. o. M. S., Rheology and structure of aqueous bentonite-polyvinyl alcohol dispersions. **2006,** *29* (5), 449-456.