A Review on Present Status and Recent Development of Colorimetric Pesticide Assay Test for Safe and Sustainable Agriculture with special reference to Clean Food Production

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Review or study of the pesticide colorimetric assay test towards sustainable agriculture in clean food production.

ABSTRACT

Background: The controversy of use and abuse of pesticides had surfaced within a few years of the green revolution and with time the ill-effects of pesticides on natural resources, food chain toxicity, human health, and agricultural sustainability has become widely apparent. And now under the existential Climate Change, the FAO indicates, 'For the world's poor, adapting to climate change and ensuring food security go hand in hand and thus a paradigm shift towards agriculture and food systems that are more resilient, more productive, and more sustainable is required. Moreover, the World Health Organization (WHO) states that "If it is not safe, it is not food", as it does not serve its purpose to provide proper and safe nutrition. The FAO reiterates that Sustainable Agriculture that seeks to increase yields while limiting the need for application of pesticides or synthetic fertilizers; only can relate Food Security with Food Safety. The 'One Health' approach of FAO promotes effective food safety regulations across sectors.

As the second largest agrarian country of the world, India has also become one of the largest users of pesticides. Surveys have shown that Indian food is laced with one of the highest amount of toxic pesticide residues in the world. Hence, analysis of pesticide residues in food has become the governing criteria for ensuring food safety. The Food Safety and Standards Authority of India (FSSAI) have laid down science based standards towards food safety based on the Codex standards, which are the reference for the international trade in food. However, it has been found that pesticide monitoring in food is most difficult in countries where that monitoring is arguably most needed. This is because the present chromatographic techniques can precisely determine the presence of every chemical at the minute level but the process is hugely expensive, complex, time-consuming and require specific resources and infrastructure which offer major hindrance towards regular analysis for monitoring of food safety. Especially for a country like India, with absolute dominance of marginal farmers in vegetable cultivation, lack of awareness, resource scarcity, inability to take economic risk and flaws in maintaining the standard practices w.r.t. chemical usage enhances the availability of pesticides in food product. Moreover the short time gap between the field harvest of vegetables and consumption, limits the scope for safety analysis even if the infrastructure and economics is not considered.

In this background an effective, simple, and affordable method is needed to enable pesticide residue analysis in situations of limited resources more so for Safe & Sustainable Agriculture to comply the requirement for SDG-2 of the United Nations, more meaningfully SDG- Target 2.4 (*Sustainable food production and resilient agricultural practices*).

Scope & Approach: In this scenario, the Colorimetric Pesticide Assay Test can be a Real Game Changer in the Food Safety Arena and a Crucial 'Sustainability Tool' for Safe & Sustainable Agriculture. This test method although utilized round the globe to identify the pesticides residues both in a quantitative and qualitative manner, lack a standard protocol towards safety evaluation of vegetables in terms of detecting the presence/absence of the

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major pesticide groups. Another crucial point is how to measure in the most affordable and transparent manner. Then it has to be made available for small, marginal and resource poor farmers, who are more than 95% of the total farming community.

Inhana Organic Research Foundation (IORF), Kolkata in collaboration with Nadia Krishi Vigyan Kendra (ICAR) initiated a research work in June, 2020 to develop a Protocol for Colorimetric Pesticide Assay Test of vegetables with the objectives of (i) Most Authentic and Speedy Measurement of the major groups of pesticides *viz*. organochlorine, organophosphate, synthetic pyrethroids, carbamates and neonicotinoids, that are used during vegetable production, (ii) Identifying the collective presence/ absence of the pesticide residues up to the lowest- group specific permissible limits (same type of pesticides in terms of chemical structure) and (iii) Standardization of the Method towards its effective utilization for large scale Pesticide Residue Study in the most economical manner. The Support from IBM, India for Clean Food Production – A Safe and Sustainable Agricultural Initiative; helped in the efforts to standardize the Colorimetric Assay Test Protocol towards safety evaluation of the vegetables. The standardization process involved the analysis of more than 1200 samples of 30 major vegetables produced in India. Vegetable samples were sourced from open markets, certified organic counters and from the farmers' field where the concept of Clean Food Program was 1st initiated by IORF in collaboration with Nadia KVK (ICAR). Also the vegetable samples were sourced during different seasons i.e., winter (Period : November – February), monsoon (Period : July – October) and summer (Period : March – June).

Key Findings & Conclusion: The newly standardized Colorimetric Pesticide Assay Test Protocol can enable detection of the collective presence/ absence of pesticides up to group specific- lowest permissible limit; for more than 90 percent of the pesticides- permitted for use in India, for most of the banned chemicals, as well as chances of residual presence in case of chemicals like DDT and its isomer. In addition; this Assay Test protocol can also be utilized for detecting the presence/ absence of toxic heavy metals such as Hg^{2+}, Cd^{2+}, Cu^{2+}, Pb^{2+} and a wide range of other toxic substance of known/unknown origin related to human health and safety. Moreover the Colorimetric Pesticide Assay Test Protocol opens up the scope for large scale and frequent food safety analysis due to the affordable cost (1/10th to 1/15th of the Conventional Cost of Residue Analysis) and significant reduction in the analysis time (1/10th of the time required for Residue Analysis using HPLC). Thus this Colorimetric Pesticide Assay Test can be a sustainable tool for any sustainable agriculture initiative to ensure safety in real time and in the most authentic and economic manner.

KEYWORDS

Pesticide Residue, Food Safety, Pesticide Monitoring, Speedy and Economical Testing, Sustainability Tool

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1. INTRODUCTION

The use of synthetic pesticides in agriculture has increased rapidly during the last four decades and has overshadowed the traditional plant protection methods used to reduce crop damages due to insects, pests, diseases and weeds. Though pesticide use is claimed to have contributed significantly to food security by way of reducing crop and postharvest losses there is a growing concern about the ill-effects of pesticides on human health, natural resources and sustainability of agricultural production (Chand and Birthal, 1997).

More than half of the pesticides consumed globally are utilized in Asia. India stands 12th in pesticide use globally and 3rd in Asia after China and Turkey (Nayak, and Solanki, 2021). At the same time, India is now the second largest manufacturer of pesticides in Asia after China and ranks twelfth globally (Mathur, 1999). There has been a steady growth in the production of technical grade pesticides in India, from 5,000 metric tons in 1958 to 102,240 metric tons in 1998. The total as well as per hectare consumption of pesticides in India shows a significant increase after 2009-10. The recent increase in pesticide use is said to be because of higher use of herbicides, as cost of manual weed control has risen due to increase in agricultural wages (Kumar, 2020).

Total pesticide consumption is the highest in Maharashtra, followed by Uttar Pradesh, Punjab and Haryana. During 2016-17, the per hectare consumption of pesticides was the highest in Punjab (0.74 kg), followed by Haryana (0.62 kg) and Maharashtra (0.57 kg). Like other developing countries, the most often used pesticides in India are insecticides. Insecticides used are mainly organochlorines, organophosphorus, carbamates and synthetic pyrethroids (Ntow, 2001; Afful et. al., 2010).

But, the irony is despite a clear increase in pesticide use, crop losses have not decreased significantly over the last 40 years. (OERKE, 2006). Some data say that the share of crop yields lost to insects has nearly doubled during the last 40 years despite more than a ten-fold increase in both the amount and toxicity of the synthetic insecticide used (Pimentel et. al., 1991). The increase in crop losses due to insects have been offset by increased crop yields obtained through the use of higher-yielding crop varieties and the greater use of fertilizers and other inputs (Pimentel et. al., 1993).

However, the continuous uses of these pesticides have resulted in contamination of the environment, crops and also caused potential risk to human health (Narenderan *et al*, 2020). Various reports suggest the risk behind the intake of different pesticides with different modes of action. Continuous exposure to pesticides causes depression and neurological deficits, diabetes, respiratory diseases such as rhinitis and in extreme cases, it causes cancer, fetal death, spontaneous abortion and genetic diseases (Ntzani et. al., 2013).

Moreover, repeated surveys have shown that Indian food is now laced with one of the highest amount of toxic pesticide residues in the world. Not only that, study done by Ministry of Agriculture shows more than 18.7 % food items have pesticide residues (FASSI, 2019). According to a report published by Indian Express, pesticide in Indian food is 40 times more than in US, UK (Nilesh, 2018).

So the importance of food quality has become a serious issue due to the widespread use of pesticides. Though, the farmers have a conventional understanding of agriculture; it's the lack in the technical understanding of the pesticides, their usage and safety aspects, which makes them vulnerable (FAO, 2014). For this reason, strict regulations are developed and regulated to monitor these compounds. (Narenderan et al., 2020). WHO, in collaboration with FAO, is responsible for assessing the risks to humans of pesticides – both through direct exposure, and through residues in food – and for recommending adequate protections (WHO, 2018). Governments and international risk managers, such as the Codex Alimentarius Commission (the intergovernmental standards-setting body for food), Environmental Protection Agency (EPA) European Union Commission (EU), Gulf Cooperation Council (GCC) have established the maximum residue limits (MRLs) for pesticides in food. Codex standards are the reference for the international trade in food, so that consumers everywhere can be confident that the food they buy meets the agreed standards for safety and quality, no matter where it was produced. In India the Food Safety and Standards Authority of India (FSSAI) was established in 2008 under the aegis of the Ministry of Health and Family Welfare with the mandate for laying down science based standards towards food safety.

However, routine, residue monitoring does remain a relatively expensive and complex process, typically relying on chromatographic techniques for detection. This limits the ability to effectively monitor pesticides in situations with inadequate resources, such as in developing countries. Unfortunately, these are locales where risks from pesticide exposure are of greatest concern due to increased use (Nweke and Sanders 2009), weak regulation and poor education about safe application practices (Williamson et al. 2008). Studies of pesticide residues in developing countries illustrate situations where pesticide residues are routinely found on market vegetables (e.g., Amoah et. al. 2006; Srivastava et. al. 2011; Hossain et. al. 2015). Thus, pesticide monitoring is most difficult in countries where that monitoring is arguably most needed. As has been noted by other authors, an effective, simple, and inexpensive method is needed to enable pesticide residue analysis in situations of limited resources (Hennion and Barcelo 1998; Mallat et. al. 2001; Qian et al. 2009; Xu et. al. 2012).

Specially in country like India and specially in the state of West Bengal where the small and marginal farmers constitute more than 96% of the total farming community, with a critical land holding size of <0.26 hec. and a high Cropping Intensity, ensuring the Safety Aspect of the produced food through routine chromatographic analysis, is beyond imagination.

In this scenario, the Colorimetric Pesticide Assay Test can be a Real Game Changer in the Food Safety Arena and a Crucial 'Sustainability Tool' for Safe & Sustainable Agriculture, especially in the Indian Perspective. Colorimetric Pesticide Assay Test has been utilized round the globe to identify the pesticides residues in food products both in a quantitative and qualitative manner. However, there is lack of information regarding any comprehensive approach towards utilization of this test method in formulating a protocol towards safety evaluation of vegetables in terms of detecting the presence/absence of the major pesticide groups

In this background, IORF, in collaboration with Krishi Vigyan Kendra (ICAR), Nadia; initiated a research work in June, 2020 to develop a Protocol for Colorimetric Pesticide Assay Test of vegetables. The study was initiated with the objectives of (i) Most Authentic and Speedy Measurement of the major groups of pesticides, that are used during vegetable crop production, (ii) Identifying the collective presence/ absence of the pesticide residues up to the lowest- group specific permissible limits (same type of pesticides in terms of chemical structure) and (iii) Standardization of the Method towards its effective utilization for large scale Pesticide Residue Study in the most economical manner.

To standardize the Protocol of Colorimetric Pesticide Assay Test, the scientists of IORF have, during the past 18 months tested more than 1200 samples of 30 major Vegetables produced in India. The samples were collected from the different local markets during different seasons, organic certified vegetables as well as vegetable produced under the small scale Clean Food Initiative of IORF in collaboration with Nadia KVK (ICAR) in the Nadia District of West Bengal (India). The vegetable samples were analyzed for chemical pesticides, heavy metals and other toxic components i.e., five major groups of pesticides (Organophosphate, Organochlorine, Synthetic Pyrethroids, Carbamates & Neonicotinoids) and other groups of chemicals, covering about 650 different types of pesticides and their combinations. That means a BDL of this study will confirm the absence of these 650 chemicals in the Test Food Products (e.g. vegetables/ fruits, etc.).

2. CLIMATE CHANGE INCREASES THE FOOD SECURITY CHALLENGE

The FAO estimates that, to satisfy the growing demand driven by population growth and dietary changes, food production will have to increase by 60 percent by 2050 (FAO, 2009). However, despite efforts made over the last decades, food insecurity is still a pressing issue especially in the developing countries. Food insecurity is a symptom of the dysfunction of the global food system (Capone et. al., 2018; El Bilali et. al., 2018; El Bilali, 2019), which is under the unprecedented confluence of various pressures (FAO, 2014) climate change (FAO, 2016) being the primary one. Agriculture is the key channel through which climate change affects food security. Climate change is profoundly modifying the conditions under which agricultural activities are conducted. Climate change generates considerable uncertainty about future water availability in many regions, affects precipitation, runoff and snow/ice melt, with effects on hydrological aspects. Moreover, the indirect effects of climate change can play a more critical role considering that they are much more difficult to assess and project, given the high number of interacting parameters and links; many of which are still unknown (El Bilali et. al., 2020). While food security challenge is already spiraling, there is a direct link between food safety, food security, and nutrition security. This means that it is not just enough to produce sufficient food and ensure everyone has access to it, but the food must be safe and nutritious (Beekmann K., 2021). Pesticides widely used in agriculture to mitigate crop losses due to pest/ disease have become the primary cause for food chain toxicity. Although pesticides are developed through very strict regulation processes to function with reasonable certainty and minimal impact on human health and the environment, serious concerns have been raised about health risks resulting from residues in food and drinking water. As per the FAO (2014) "We need to expand and accelerate the transition to sustainable food and agriculture which ensures world food security, provides economic and social opportunities, and protects the ecosystem services on which agriculture depends". Studies conducted by the environmental and public health departments indicate that a reduced usage of pesticides is sufficient to mitigate the negative impacts (Gerage et. al., 2017) set in motion under the conventional farming system. The vicious cycle of problem-solution-negative impacts has to be broken at some point of time. For example, a second green revolution is focused on in various countries (Ameen and Raza, 2017; Armanda et. al., 2019). Instead of this, techniques to promote sustainable agriculture can be considered. Hence, there has to be a wake-up call before the repetition of history.

3. HISTORICAL PERSPECTIVE OF PESTICIDE USAGE

The history of pesticide use can be divided into three periods of time. During the first period i.e., before the 1870s, pests were controlled by using various natural compounds (Tudi et. al., 2021). The first recorded use of insecticides was about 4500 years ago by Sumerians (Unsworth, 2010). They used sulfur compounds to control insects and mites. During the second period, i.e. between 1870 and 1945, people began to use inorganic synthetic materials (Tudi et. al., 2021) as pesticides including the Bordeaux mixture, based on copper sulfate and lime arsenic, and they are still being used to prevent numerous fungal diseases (Bernardes et. al., 2015).The third period started after 1945 (Unsworth, 2010), represented by the use of synthetic pesticides with the discovery of the effects of Dichlorodiphenyltrichloroethane (DDT), β-Hexachlorocyclohexane (BHC), aldrin, dieldrin, endrin, chlordane, parathion, captan, and 2,4-D (Zhang et. al., 2017). Between the 1970s and 1990s, new families of chemicals, such as triazolopyrimidine, triketone and isoxazole herbicides, strobilurin and azolone fungicides, chloronicotinyl, spinosyn, fiprole diacylhydrazine, and organophosphate insecticides, were introduced to the market and most of the new chemicals could be used in grams rather than kilograms per hectare (Zhang et. al., 2011)

During World War II, knowledge of synthetic chemicals increased rapidly as a result of research into chemical weapons. For example, many of the early insecticides were organophosphates, which are closely related to nerve gases. The number of products and their use increased sharply after the war. In particular, new herbicides were developed and the use of these increased during the 1960s (Mesnage, 2021).

4. TYPES, NUMBERS AND COMBINATION OF PESTICIDES

According to the World Health Organization (WHO) pesticides are considered as a special class of chemical compounds used to kill a wide range of pests that include insects, weeds and rodents (WHO, 2008). According to FAO (2021), pesticides are any substance or mixture of substances of chemical or biological ingredients intended for repelling, destroying or controlling any pest, or for regulating plant growth. The term pesticide applies to insecticides, herbicides, fungicides, rodenticides, molluscicides, wood preservatives and various other substances used to control pests.

Pesticides are classified primarily based on (i) use or target pests, (ii) Mode of Action, (iii) Toxicity and (iv) Chemistry/Chemical structure. Pesticide use is not just a modern practice (Hayes, 1991), perhaps the first recorded use of pesticide was around 1550 B.C., when Egyptians used unspecified chemicals to drive fleas from homes (Freedman, 1995). However, pesticides began to be applied more broadly from the 1940s due to the growth of synthetic chemical pesticides and rapid development of bio-pesticides in the past decade. Today, there are more than one thousand pesticides available on the market (including chemical, microbial, semi-chemical and botanical pesticides) (FAO, 2021). In India, as per the record of Directorate of Plant Protection, Quarantine & Storage (2022), 277 different Pesticides along with 589 formulations registered under section 9(3) of the Insecticides Act, 1968 for use in India as on July, 2019. At the same time 203 different combinations of pesticides are also registered under the same act for us in India.

5. HOW PESTICIDES BECAME A NECESSITY IN CHEMICAL AGRICULTURE ?

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Changes in cultivation techniques have resulted in higher pest incidence and susceptibility of plants to damage from pests. Large-scale cropping of genetically uniform plants, multiple cropping, reduced crop rotation and/or reduced tillage cultivation have increased the inoculum of pests in the upper soil layer. Expansion of crops into less suitable regions with higher incidence of other pests, where plants are less adapted and high-yielding varieties have replaced well-adapted local varieties. Lastly, increases in the demand for higher quality food have led to an increase in the crop not suitable for consumption (Yudelman et. al. 1998).

Pesticides are not a modern invention. Ancient Sumerians used elemental sulfur to protect crops from insects, and medieval farmers and scientists experimented with chemicals like arsenic. Nineteenth-century research focused on compounds made from plants, including chrysanthemum. Because of the urgency to improve food production and control insect-borne diseases, the development of pesticides increased during World War II (1939-1945). Additionally, from the 1940s onwards, the increased use of synthetic crop protection chemicals permitted a further increase in food production (Bernardes et. al., 2015). Moreover, worldwide pesticide production increased at a rate of about 11% per year, from 0.2 million tons in the 1950s to more than 5 million tons by 2000 (Carvalho, 2017). Three billion kilograms of pesticides are used worldwide every year (Hayes et. al., 2017), while only 1% of total pesticides are effectively used to control insect pests on target plants (Bernardes et. al., 2015).

6. DOES HIGHER USE OF PESTICIDE REALLY HELP TO MINIMIZE CROP LOSS ?

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Pesticides have become a key tool for plant protection and improvement of crops in the process of agricultural development. (Sharma et. al., 2019). The intensity of protection for crops has increased significantly in order to make agriculture more productive and profitable but despite a 15-20-fold increase in pesticide use around the world, crop losses have not decreased significantly over the last 40 years. (OERKE, 2006). Actual crop protection depends on the importance of pest groups or its perception by farmers and on the availability of crop protection methods. As the availability of control measures greatly varies among regions, actual losses differ to a higher extent than the site-specific loss potentials. As per the previous studies documented by Pimentel et al (1993) it is technologically feasible to reduce pesticide use in the US by 35-50% without reducing crop yields (Office of Technology Assessment (OTA), 1979. According to them in USA, an estimated 37% of all crop production is lost annually to pests (13% to insects, 12% to plant pathogens, and 12% to weeds) despite the use of pesticides and non-chemical controls (Pimentel, 1986). Although pesticide use has increased during the past four decades, crop losses have not shown a concurrent decline.

The share of crop yields lost to insects has nearly doubled during the last 40 years (Table 2) despite a more than ten-fold increase in both the amount and toxicity of synthetic insecticides used (Pimentel et al., 1991). The increases in crop losses due to insects have been offset by increased crop yields obtained through the use of higher-yielding crop varieties and the greater use of fertilizers and other inputs (USDA, 1989). This increase in crop losses despite increased insecticide use is due to several major changes that have taken place in agricultural practices. These include the planting of some crop varieties that are more susceptible to insect pests, the destruction of natural enemies of certain pests (which creates the need for additional pesticide treatments), the increase in numbers of pests resistant to pesticides, the reduction in crop rotations, the increase in crops grown in monoculture and reduced crop diversity . According to Lechenet et. al. (2014), they failed to detect any positive correlation between pesticide use intensity and both productivity and profitability in conventional farms. In comparison to conventional systems, integrated strategies showed a decrease in the use of both pesticides and nitrogen fertilizers and were frequently more energy efficient; therefore appeared as the best compromise in sustainability trade-offs (Lechenet et. al., 2014). These data demonstrate that food production and ecosystem sustainability are not necessarily conflicting goals (Pecenka et. al., 2021). The study indicated that in 77% of farms, high profitability and productivity were achieved with low pesticide use and the authors estimated that pesticides use could be reduced by 42% without negatively affecting productivity or profitability on 59% of the farms surveyed (Lechenet, 2017).

7. CLIMATE CHANGE IMPACT ON PESTICIDE USAGE

Climate factors have been found to influence both pest incidence (pathogens, weeds, fungi, and insects) and the effectiveness of chemical treatments (Van Maanen, and Xu, 2003; Bloomfield et. al., 2006; Matzrafi, 2019). Climate change has been found to alter pest incidence, abundance, and damages (Deutch et. al., 2008). A number of studies have investigated climate influences on pests, pesticide costs and cost variability (Poggi et. al., 2018). All review evidence, mainly on insect abundance, show that climate change enhances populations (Lauren & Bruce, 2020). Chen and McCarl (2003) examined the effects of climate change on pesticide expenditures and found that pesticide expenditures rise with increased temperatures and precipitation for the majority of crops. The climate also influences herbicide, insecticide, and fungicide use through changes in their effectiveness and persistence (Lauren & Bruce, 2020). Walker and Eagle (1983), Ahmad et al. (2003) and Bailey (2004) found that increase in temperature and changes in rainfall pattern (Cabras et. al., 2001) can decrease persistence and, in turn, increase the required number of applications. Climate change impact can also increase the incidence of crop susceptibility to disease [Van Maanen, 2003] with changing temperature, precipitation, and humidity. Additionally, increased atmospheric CO_2 can enhance weed growth (Wolfe et. al., 2008) and increase weed tolerance to herbicides (Ziska et. al., 2016).

8. FATE OF PESTICIDE IN THE ENVIRONMENT

When pesticides are applied to a target plant or disposed, they have the potential to enter the environment (Tudi, 2021). On entering the environment, pesticides can undergo processes such as transfer (or movement) and degradation (Singh, 2012). Pesticide characteristics (water solubility, tendency to adsorb to the soil and pesticide persistence) and soil characteristics (clay, sand and organic matter) are important in determining the fate of the chemicals in the environment (Anonymous 2009). The contamination of water bodies with pesticides can pose a significant threat to aquatic ecosystems and drinking water resources (Tiryaki, 2010). However many factors, such as soil and pesticides properties, and crop management practices, govern the potential for groundwater or surface water contamination by pesticides (Kerle et. al. 2007).

Moreover, pesticides tend to turn into transformation products (TPs) by abiotic or biotic transformation in the environment, which can imply greater hazards to non-target organisms than the parent molecule (Fenner et. al., 2013). Soil microorganisms play an important role in the dissipation processes as they can contribute to the environmental biodegradation of pesticides, which can constitute a nutrient and energy source to them (Copley, 2009). Extensive knowledge about these processes is crucial to predict potential risks for the environment and needs to be comprehensively involved in the environmental risk assessment of pesticides (Storck et. al., 2016).

9. IMPACT OF PESTICIDE RESIDUE ON HUMAN HEALTH

The use of chemicals on a large scale has not been started a long ago however this approach has brought havoc in the biosphere, leading to a decline in the quality of life (Pimentel, 2005). The continuous usage of these pesticides has resulted in contamination of the environment, crops and also caused potential risk to human health. Hence, the importance of food quality has become a serious issue. Though, the farmers have a conventional understanding of agriculture; it's the lack in the technical understanding of the pesticides, their usage and safety aspects which makes them vulnerable (FAO, 2014). For this reason, strict regulations are developed and regulated to monitor these compounds. (Narenderan et. al., 2020). Various reports suggest the risk behind the intake of different pesticides with different modes of action. Continuous exposure to pesticides causes depression and neurological deficits, diabetes, respiratory diseases such as rhinitis and in extreme cases; it causes cancer, fetal death, spontaneous abortion and genetic diseases (Ntzani et. al., 2013).

Pesticide exposures have been linked to many human diseases such as Alzheimer, Parkinson, amyotrophic lateral sclerosis, asthma, bronchitis, infertility, birth defects, attention deficit hyperactivity disorder, autism, diabetes and obesity, respiratory diseases, organ diseases and system failures (Shah, 2020). Especially the people who are exposed to pesticides like agriculture workers are at a greater risk to develop various cancers including non-Hodgkin lymphoma (NHL), leukemia, brain tumors, and cancers of the breast, prostate, lung, stomach, colorectal, liver, and the urinary bladder.

The type of pesticide, the duration and route of exposure, and the individual health status (e.g., nutritional deficiencies and healthy/damaged skin) are determining factors in the possible health outcome. Within a human or animal body, pesticides may be metabolized, excreted, stored, or bioaccumulated in body fat (Alewu and Nosiri, 2011). The general class of organochlorine pesticides has been associated with health effects, such as endocrine disorders (Mnif et. al., 2011), effects on embryonic development (Tiemann, 2008), lipid metabolism (Karami-Mohajeri and Abdollahi, 2011), and hematological and hepatic alterations (Freire et. al., 2015). Their carcinogenic potential is questioned, but concerns about possible carcinogenic action should not be underestimated (Witczak and Abdel-Gawad, 2014).Organophosphates, which were promoted as a more ecological alternative to organochlorines (Jaga and Dharmani, 2003), has been associated with effects on the function of cholinesterase enzymes (Jaga and Dharmani, 2003), decrease in insulin secretion, disruption of normal cellular metabolism of proteins, carbohydrates and fats (Karami-Mohajeri and Abdollahi, 2011), and also with genotoxic effects (Li et. al., 2015) and effects on mitochondrial function, causing cellular oxidative stress and problems to the nervous and endocrine systems (Karami-Mohajeri and Abdollahi, 2011). This leads to serious health effects including cardiovascular diseases (Hung et. al., 2015), negative effects on the male reproductive system (Jamal et. al., 2015) nervous system (Wesseling et. al., 2002), dementia (Lin et. al., 2015), and also a possible increased risk for non-Hodgkin's lymphoma (Waddell et. al., 2001). Synthetic pyrethroids, are considered to be among the safer insecticides currently available for agricultural and public health purposes (Kolaczinski and Curtis, 2004). However, there is evidence for their ability to display endocrine-disrupting activity (Pandey and Mohanty, 2014), and to affect reproductive parameters. A recent study showed relationship with multiple pyrethroid metabolites to DNA damages in human sperm and developmental neurotoxicity (Syed et. al., 2015). Neonicotinoid pesticides are relatively new and also the most extensively used insecticides (Goulson, 2013) that were promoted for their low risk for non-target organisms (Jeschke and Nauen 2008). However, there is plenty of evidence to the contrary (Wright et. al., 2015). Moreover, a recent study demonstrated that neonicotinoids are able to increase the expression of the enzyme aromatase, which is engaged in breast cancer and also plays an important role during developmental periods (Caron-Beaudoin et. al., 2016). Studies have shown that persistent

Comentado [MDLAVH6]: Missing a comma before the year exposure of these pollutants can lead to their accumulation in the tissues and induce harmful effects on growth, development as well as the metabolism of the body (La Merrill et. al., 2013). The pesticides have been linked to several disorders, which are associated with cardiovascular, central nervous (Keifer and Firestone, 2007) and pulmonary system (Ye et. al., 2013). These compounds have also been observed to be carcinogenic, mutagenic and teratogenic in nature (Baird et. al., 2005; Parker et. al., 2017).

10. IMPACT OF PESTICIDE RESIDUE ON THE ENVIRONMENT

Worldwide, about twenty five million agricultural workers experience unintentional pesticide poisonings each year. It is estimated that approximately 1.8 billion people engage in agriculture and most use pesticides to protect food and commercial products that they produce. A large quantity of pesticides is lost via spray drift, off-target deposition, run-off, and photodegradation, for instance, which can have undesirable effects on some species, communities, or ecosystems as a whole, as well as on the humans (Hernández et. al., 2013). In early 1986, Pimental and Levitan (Pimentel and Levitan, 1986) found that only 0.1% of pesticides reach the target whereas, larger parts of them cause contamination of the environment (Pimentel, 1995). Now, Countless chemicals are environmentally stable, prone to bioaccumulation, and toxic (Fenik et. al., 2011), because some pesticides can persist in the environment and can remain there for years.

Rachel Carson provided clear evidence of the far-reaching environmental impact of pesticides in her pioneering work 50 years ago. In 'Silent spring' she showed that organochlorines, a large group of insecticides, accumulated in wildlife and the food chain. This had a devastating effect on many species. Only a decade after the 'green revolution' began it became obvious that large-scale spraying of pesticides was causing serious damage.

Environmental modeling indicates that over 60% of global agricultural land (~24.5 million km^2) is "at risk of pesticide pollution by more than one active ingredient", and that over 30% is at "high risk" of which a third are in high-biodiversity regions. (Ratcliffe, 1967; Levengood & Beasley, 2007)

11. IMPACT OF PESTICIDE ON PLANT HEALTH

Pesticides are applied all over the world to protect plants from pests. However, their application also causes toxicity to plants, which negatively affects their growth and development (Sharma et. al., 2019). Pesticide induced toxicity to plants, can be seen in the form of necrosis, chlorosis, stunting, burns and twisting of leaves (Sharma et. al., 2018a). The excessive use of pesticides is one of the major causes of reduction of the diversity of structural vegetation (Donald, 2004).

Pesticide stress also generates reactive oxygen species which causes oxidative stress to plants (Sharma et. al., 2019). This oxidative stress results in degradation of chlorophyll pigments and proteins and it ultimately causes a reduction in the photosynthetic efficiency of plants (Xia et. al., 2006). Pesticides impact the crop physiology through various disruptions, such as perturbation in the development of the reproductive organs, growth reduction, and alteration of the carbon and/or nitrogen metabolism, leading to a lower nutrient availability for plant growth (Miguel et. al., 2020). These disruptions will partly depend on the type of pesticide used [Petit et al, 2012]. Some important effects may only become apparent after repeated treatments and not necessarily translate into visible necrosis [Ferree, 1979].

At the same time, nitrogen fixation, which is required for the growth of higher plants, is hindered by pesticides in soil (Rockets and Rusty 2007). Many insecticides have been shown to interfere with legume-rhizobium chemical signaling. Reduction of these symbiotic chemical signaling results in reduced nitrogen fixation and thus reduced crop yields. Root nodule formation in these plants saves the world economy $10 billion in synthetic nitrogen fertilizer every year (Fox et. al., 2007). On the other side, pesticides have some direct harmful effect on plant including poor root hair development, shoot yellowing and reduced plant growth (Walley et. al., 2006).

12. TREND OF GLOBAL PESTICIDE USAGE

Global pesticides use increased during the period 2000–2019 by 36 percent (Fig.1), going up to about 4.2 million tonnes in 2019 (FAO, 2021). Nearly all the increase took place between 2000 and 2012, with a plateau afterwards. The highest contributions came from Asia, followed by the America, Europe, Africa and Oceania. The regional contributions to the world total changed slightly over time, but Asia, the largest contributor, remained stable at 52–53 percent. The share of the Americas increased from 29 percent to 33 percent of global pesticides consumption while that of Europe decreased slightly from 14 percent to 11 percent. Africa and Oceania applied small amounts of pesticides over time, but Oceania nonetheless had the highest growth in pesticides applications (+85 percent). China was the largest pesticide user in 2019 with 1.8 million tonnes, or 42 percent of the world total, far ahead of the United States of America and Brazil (0.4 million tonnes each) (FAO, 2021).

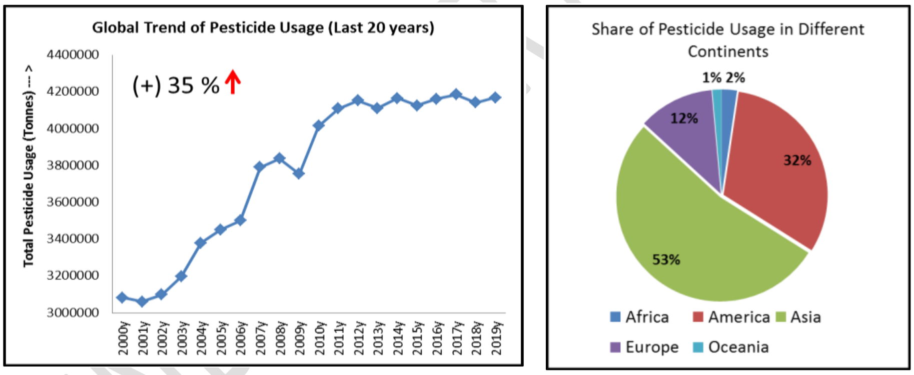

Fig. 1 : Scenario of global pesticide usage

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As per Pesticide usage per ha crop land in the first 50 countries having more than 90 of total agricultural land in the world, the highest usage was in China (13.1 kg/ ha) followed by Malaysia (8.1 kg/ha), Italy (6.1 kg/ha), Brazil (6.0 kg/ha), Argentina (4.9 kg/ha), Germany 94.0 kg/ha), France (3.6 kg/ha), Spain (3.6 kg/ha), UK (3.2 kg/ha), US (2.6 kg/ha) and Canada (2.4 kg/ha). India having the largest cultivated land in the world stood in 28th position with 0.30 kg/ha pesticide usage. The Crop Protection Chemicals Market was valued at USD 61,298.1 million in 2020, and it is projected to reach USD 73,530.7 million in 2026, registering a CAGR of 3.7% during the forecast period (2021-2026) (Mordor Intelligence, 2021).

13. TREND OF PESTICIDE USAGE IN INDIA

Globally more than half of the pesticides are utilized in Asia (Fig. 2). India stands 12th in pesticide use globally and 3rd in Asia after China and Turkey (Nayak & Solanki 2021). There are 277 pesticides registered in India, and it is reported that 104 pesticides are still being produced/used in the country despite being prohibited in two or more nations around the world (GOI, 2021). Out of total insecticides used for pest management in India, 50% are diverted to cotton pest management (Mooventhan, Murali, Kumar, & Kaushal, 2020).

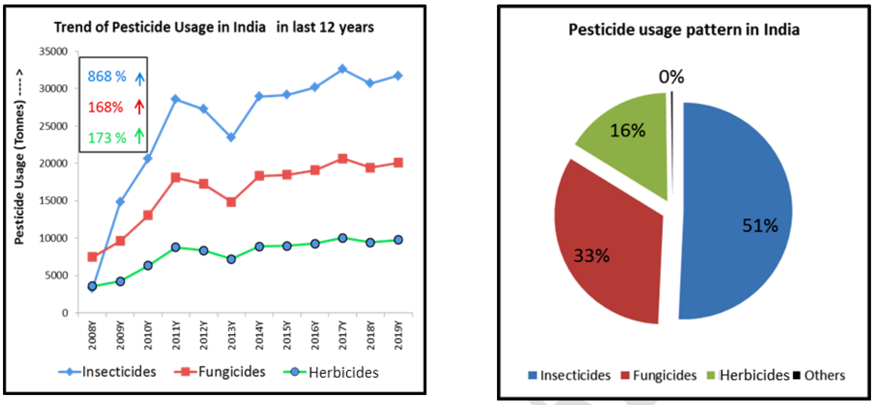

Fig. 2: Pesticide usage trend in India in last 12 years

Pesticide usage patterns in India differ from those in the world as a whole. In India, insecticides account 51 % of total pesticide usage followed by 33% account for fungicides+ bactericides and 16 % comprise herbicides. In the case of the global pesticide usage, the highest usage is herbicides (53 %) followed by fungicides (22 %), insecticides (17 %) and others (8 %) (FAO, 2021). Chlorpyriphos is the most widely used insecticide and its consumption has risen from 471 MT in 2014-15 to 1431 MT in 2019-20 (Nayak & Solanki 2021). Sulphur is the most often used fungicide, with a consumption of 1548 MT in 2014-15, which has climbed to 3878 Mt in 2019-20. In India, a high concentration of 2, 4-D amine salts is used as a weedicide (herbicide). Its usage was 1MT in 2014-15, but it increased to 1067 MT in 2019-20 (Nayak & Solanki 2021). Zinc phosphide has been the most often used rodenticide, with consumption ranging from 65 to 200 MT from 2014 to 2020 (GOI, 2021).

The most often used insecticides are organophosphates, followed by neonicotinoids and pyrethroids. According to one study, cotton is the most pesticide-consuming agri-product (93.27 percent), followed by vegetables (87.2 percent), wheat (66.4 percent), millet (52.6 percent), and mustard (12.6 percent) (Maurya and Malik, 2016; Yadav and Dutta, 2019; Nayak et. al., 2020)

14. COMPARATIVE PESTICIDE USAGE IN THE DIFFERENT STATES OF INDIA

Comparative study showed that state wise total pesticide consumption (Fig. 3) is the highest in Maharashtra (Avg 13367 MT), followed by Uttar Pradesh (Avg 11252 MT), Punjab (Avg 5482 MT), Telangana (Avg 4619 MT) and Haryana (Avg 4068 MT). On the other hand, per hectare consumption of pesticides (based on gross cropped area) was the highest in Jammu & Kashmir (2.18 kg/ha), followed by Telangana (0.94 Kg/ha), Punjab (0.70 kg/ha), Tripura (0.69 kg/ha), Haryana (0.62 kg/ha) and Maharashtra (0.57 kg/ha). West Bengal stood in 11th place with an average pesticide consumption of 0.32 kg per ha (Directorate of Plant Protection, Quarantine & Storage, 2022).

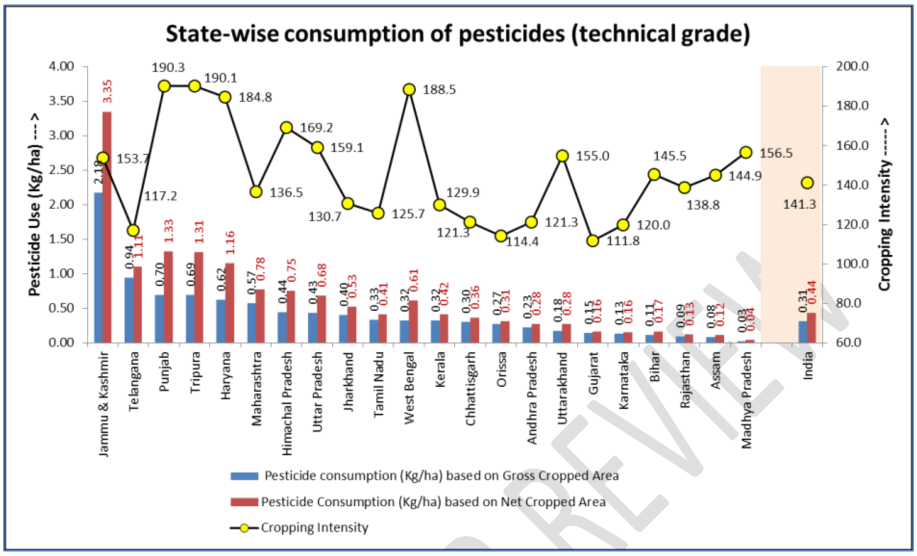

Fig. 3 : State wise pesticide consumption based on gross and net cropped area along with cropping intensity.

During the last decade, the total consumption increased in Maharashtra and Uttar Pradesh, while it declined slightly in Punjab and Haryana. However states like West Bengal, Gujarat and Karnataka have seen a steep decline in the total consumption. On the other hand, Chhattisgarh and Kerala showed a steep increase in total pesticide consumption (Subash et al, 2018).

15. MONITORING OF PESTICIDES IN FOODS – A GLOBAL PERSPECTIVE

Monitoring pesticide residues is the only way to effectively control the concentrations of pesticides in foods. Recently more programs have been established to monitor pesticides, with surveillance focusing on proper use of pesticides with regards to application rates and compliance with MRLs (Chen et. al., 2011). In USA, the U.S. Food and Drug Administration uses three approaches to monitor pesticide residues in foods: regulatory monitoring, incidence/level monitoring, and the Total Diet Study (Yess et. al., 1993). U. S. Environmental Protection Agency (EPA) approves the use of pesticides and may establish tolerances for pesticide chemical residues that could remain in or on food. A tolerance is the EPA established maximum residue level of a specific pesticide chemical that is permitted in or on a human or animal food in the United States. Within the European Union, a 2-tiered approach is used for the approval and authorization of pesticides. Firstly, before an actual pesticide can be developed and put on the European market, the active substance of the pesticide needs to be approved for the European Union. Only after approval of an active substance, a procedure of approval of the Plant Protection Product (PPP) can begin in the individual Member States. In case of approval, there is a monitoring program to make sure the pesticide residues in food are below the limits set by the European Food Safety Authority (EFSA).

For the past 30 years, FAO has assisted countries in the Asia and Pacific region in establishing pesticide legislation and regulations, and in managing these products in accordance with the Code of Conduct on the Distribution and Use of Pesticides and other international conventions and treaties. With the advance of globalization and the free movement of goods and services, it has become more and more important to harmonize pesticide regulatory management in order to stay competitive in the international marketplace (FAO, 2013) Most of the country in the Asia and Pacific region follow the basic standard set as per FAO/WHO specifications and maximum residue limits (MRL) defined by the Codex Alimentarius.

16. PESTICIDE RESIDUE AND FOOD SAFETY

Pesticide residue refers to the pesticides or metabolic products of the pesticides that may remain in food grains, vegetables and fruits after they are applied to crops (Grewal, 2017). Many of these chemical residues, especially derivatives of chlorinated pesticides, exhibit bioaccumulation which could build up to harmful levels in the body as well as in the environment (Sachs et. al., 2010). Persistent chemicals can be magnified through the food chain and have been detected in products ranging from meat, poultry, and fish, to vegetable oils, nuts, and various fruits and vegetables (Crinnion, 2009). Monitoring of Pesticide Residues at National Level, conducted by the Ministry of Agriculture & Farmers Welfare has revealed presence of pesticide residues in food commodities beyond the specified limits in some of the states. Out of a total of 23,660 samples analyzed, pesticide residues were detected in 4,510 samples which is about 19.10% out of which the residues in 523 (2.2%) samples were found exceeding maximum residue limits (MRL) (Mittal, 2019).

According to the FSSAI report 2019 (Mittal, 2019), among the vegetable samples studied brinjal showed the maximum number of pesticide residues; followed by the samples of tomato, okra, cabbage, cauliflower and cucumber. In spices a maximum number of MRL exceedance was found in cardamom samples followed by the cumin. The report also showed that the most commonly detected residues were acephate, chlorpyriphos, imidacloprid, carbendazim, acetamiprid, profenophos, methamidophos and thiamethoxam, while non-approved pesticides detected were mainly ethion, carbendazim, acetamiprid, triazophos, bifenthrin, imidacloprid, cypermethrin, chlorpyriphos, profenofos, hexaconazole and profenofos.

17. FOOD SAFETY – THE GUIDING PRINCIPLES IN INDIA

In India, the food safety is based on the guiding principle of risk analysis of the Codex Alimentarius Commission (CAC). The Codex Alimentarius Commission (CAC) was created in 1961/62 by Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), to develop food standards, guidelines and related texts and it is a collection of internationally adopted food standards and related texts presented in a uniform manner. These food standards and related texts aim at protecting consumers' health and ensuring fair practices in the food trade. The Food Safety and Standards Authority of India (FSSAI) under the administration of Ministry of Health and Family Welfare have been designated as the nodal point for liaison with the Codex, known as "National Codex Contact Point of India" (NCCP). According to Food Safety and Standards (Contaminants, Toxins And Residues) Regulations, 2011 developed by Food Safety and Standards Authority of India, lowest limits of pesticide residue in vegetables is 0.1 ppm except very few cases. This was in accordance with Codex Alimentarius maximum residual limit (0.1 ppm) in case of vegetables.

During 2008 to 2018, a total of 1,81,656 samples of the various food commodities were collected from various parts of the country and analyzed for the presence of pesticide residues, out of which 3,844 (2.1%) samples were found above MRL as prescribed under Food Safety Standard Authority of India (FSSAI), Ministry of Health and Family welfare. However Maximum Residual Limits (MRLs) of Insecticides in Organic Foods as per the Food Safety and Standards (Organic Foods) Regulations, 2017 are based on the standards of National Programme for Organic Production (NPOP) and Participatory Guarantee System (PGS-India) and lowest limit is mostly 0.01ppm in case of vegetables.

18. LIMITATIONS OF ROUTINE CHROMATOGRAPHIC TECHNIQUES FOR PESTICIDE RESIDUE ANALYSIS TOWARDS REGULAR MONITORING OF PESTICIDE RESIDUE IN VEGETABLES

Gas chromatography is the most widely adopted technique in pesticide residue analysis. But in any case, although chromatographic methods coupled to MS detectors provide the aforementioned merits, they are also time-consuming, laborious, and expensive methods. At the same time, these methods require highly skilled personnel, indicating the need to seek for alternatives providing simple, low-cost, rapid, and on-site results. Thus it is necessary to seek for alternatives that can combine sufficient detectability with cost-efficiency, simplicity, and applicability at the point of need (Tsagkaris et. al., 2021). Similar views was expressed by other workers in this field and according to them, Chromatographic-based methods have complicated steps, require skilled technical help, are equipment intensive, and require a significant amount of time. Therefore, a low number of samples can be analyzed per day, and thus a high number of samples cannot be processed in a short time (Hongsibsong et. al., 2020). Additionally, it is also very expensive per sample and is thus not economically viable for any frequent study protocol.

In this way, screening methods have been introduced in food contaminant analysis featuring a great potential (Tsagkaris et. al., 2019). According to the Decision 2002/657/EC (European Commission, 2002), "screening methods are used to detect the presence of a substance or class of substances at the level of interest" (Tsagkaris et. al., 2021). There are several methods fitting within this concept aiming to achieve rapid, selective, cost-efficient, and sensitive screening in the food safety field (Nelis et. al., 2019). Such methods are usually based on bio-affinity interactions between selective biomolecules, e.g., antibodies (Fang et. al., 2020) or enzymes (Cao et. al., 2020), and pesticide residues, while bio recognition events are typically monitored by either optical or electrochemical transducers (Capoferri et. al., 2018).

19. DEVELOPMENT OF SAMPLING PROTOCOL AND EXTRACTION PROCESS

The sample extraction process is considered as the essential step in pesticide residue analysis, as it provides the base for the detection of the pesticides at trace level (Narenderan et. al., 2019). Over the past few years, the use of QuECHERs method increased highly due to its micro scale extraction process (Lehotay et. al., 2010). Extraction of organic compounds from different matrices (e.g. food, biological, and environmental) is a time - consuming process, but the QuECHERs method reduces the analysis time, minimizes the number of analysis steps with the use of fewer reagents in smaller amounts and in turn provides high recovery.

Originally, QuEChERS was introduced for pesticides residues analysis in fruits and vegetables with high water content. However, more recently it is gaining significant popularity in the analysis of pesticides and other compounds in a huge variety of food products and others with different types of matrices. QuEChERS method enables yielding high recovery rates for wide range of analytes and is characterized by very accurate (true and precise) results thanks to the use of an internal standard (IS) for elimination of problematic commodity differences (Lehotay et. al., 2004). Internal standard addition is also important for minimization of error generation in the multiple steps of the QuEChERS (Majors, 2013). Another important advantage of the QuEChERS technique is its rapid character and high sample output. Using this method, a batch of 10−20 samples could be extracted in 30−40 minutes by a single analyst (Lehotay et. al., 2004). QuEChERS approach is also in accordance with so-called green chemistry due to low solvent consumption and absence of chlorinated solvents and a very small waste generation (Schenck and Hobbs, 2004).

20. BACKGROUND BEHIND STANDARDIZATION OF PROTOCOL FOR COLORIMETRIC ASSAY TEST OF PESTICIDE RESIDUE IN VEGETABLES.

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HPLC study helps in the quantitative estimation of individual pesticides. But major limiting factor is the cost of analysis and the time required considering the present infrastructure of the Indian Pesticide Analysis Facilities. Thus none of the Certification Systems round the globe indicate regular batch wise pesticide analysis in its 'Must Do Criteria', primarily considering the related economics. Another limiting point w.r.t. the study of individual pesticide residue is that, their individual presence might be below the detectable limit (0.01 ppm) or MRL, but the value might go up in respect of their collective presence as a group; whichever is considered for 'SAFETY' evaluation. On the contrary, Enzyme-based detection methods, such as ELISA or Acetylcholinesterase (AChE) inhibition tests, present an alternative method for monitoring pesticides, and have been used for monitoring pesticides in vegetables (Watanabe et. al., 2006; Graber Neufeld et. al., 2010) and water samples (Mallat et. al., 2001). Enzyme-based tests are typically faster and less expensive, and often have high specificity and sensitivity (Qian et. al., 2009; Wang et. al., 2011). As an example Acetylcholinesterase (AChE) inhibition was used as a simple colorimetric test for organophosphates/carbamates (OP/C).

In this regard, pre development of Colorimetric Assay Test Protocol, the literature search indicated that in India, as per the last five years pesticide use trend, more than 25000 MT pesticides (technical grade) was consumed. As per Insecticides / Pesticides Registered under section 9(3) of the Insecticides Act, 1968 for use in the Country (As on 30.11.2020)- a total of 750 formulations were registered for use in India. However, excluding the bio-pesticides, sulphur and neem based formulations, five major groups of chemicals *viz*. Organochlorine, Organophosphate, Carbamate, Synthetic pyrethroids and Neonicotinoids cover more than 90% of the synthetic pesticides consumed in India. So, Colorimetric Assay Test of five major groups *viz*. Organochlorine, Organophosphate, Carbamate, Synthetic Pyrethroids and Neonicotinoids will serve to authenticate the non-presence of every single variant out of more than 650 pesticides formulations covering major insecticides, fungicides and Herbicides; whose presence in food products have been indicated round the globe. Not only the pesticide, but also the presence/ absence of harmful heavy metals *viz*. Hg^{2+}, Cd^{2+}, Cu^{2+} and Pb^{2+} can also be done using the colorimetric qualitative test. Apart from that; Triazines, Paraquat and many other known and unknown toxic substances which inhibit our central and peripheral nervous system; if present in food product; can also be brought under the scanner under the Colorimetric Assay Test.

For the 1st time in the history of any Agricultural Certification Process, Colorimetric Assay Test of Pesticide Groups will be taken up for authenticating the purity of Clean Food on a regular batch wise testing protocol. During Standardization of the Colorimetric Assay Test Protocol, we studied more than 1200 vegetable samples among which 42% samples were sourced from open markets and 47% samples were sourced directly from farmers' field in the villages where the concept of Clean Food program was 1st initiated by Inhana Organic Research Foundation in collaboration with KVK (ICAR), Nadia. Also 11% samples were organic; which were sourced both from certified organic counters and small indigenous organic farms (Fig. 4).

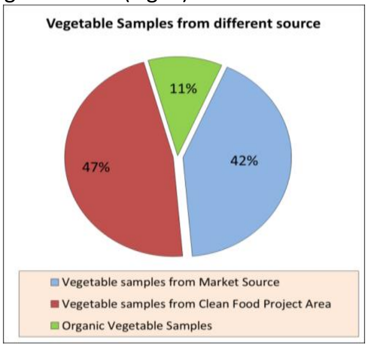

Fig. 4: Vegetable samples from different sources for Study.

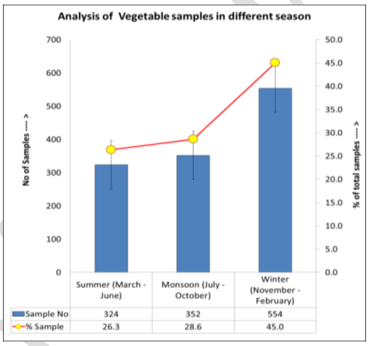

Fig. 5: Vegetable samples sourced in different seasons.

Vegetable samples were sourced during different seasons with majority samples (45 %) during winter (Period : November – February), followed by 28.6 % samples during monsoon (Period : July – October) and 26.3 % samples during summer (Period : March – June) (Fig. 5.). Also for standardization of the protocol we took 30 different test vegetables belonging to 13 different vegetable families. Details of the sampling of vegetables are given in Table 1A & 1B. The details indicate that we tried to induct the highest possible diversity in terms of vegetable type, season and sampling sources during standardization of the Protocol.

For authentication of Clean Food Safety, IORF follows the Food Safety and Standards Authority of India (FSSAI)- Organic Standard, of 0.01 ppm as Tolerance limit. But there is a clear difference in that under FSSAI Organic Standard, the MRL of 0.1 ppm is the ceiling for individual pesticide, whereas under Clean Food Safety Standard 0.01 ppm is the MRL for the total presence of residues (irrespective of the number of pesticides groups present). Hence, the Standard maintained for Clean Food Safety is perhaps the most stringent in the Indian food safety arena. A Comparative study of MRL for the different vegetable families collected from different Sources was done in respect of the Clean Food Standard (<0.01 ppm) *vis-a-vis* Standards of CODEX ALIMENTARIUS FAO-WHO & FSSAI (<0.10 ppm); utilizing the Protocol of Colorimetric Pesticide Assay Test (Fig. 6 & 7).

Table 1A : Colorimetric Pesticide Assay Test: Analysis of more than 1200 samples of 30 different vegetables in different seasons to standardize the Protocol.

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Total No of Sample Analyzed	Summer (Period : March - June)				Monsoon (Period : July - October)				Winter (Period : November - February)				Grand Total
	Vegetable from Market Source	Vegetable from Clean Food Project	Organic Vegetables	Total	Vegetable from Market Source	Vegetable from Clean Food Project	Organic Vegetables	Total	Vegetable from Market Source	Vegetable from Clean Food Project	Organic Vegetables	Total	
THE SOLANACEAE FAMILY													
Brinjal (Solanum melongena L.)	12	8	2	22	10	12	2	24	12	18	2	32	78
Tomato (Solanum lycopersicum)	6	4	2	12	6	4	2	12	8	12	2	22	46
Potato (Solanum tuberosum)	6	4	2	12	4	0	2	6	8	12	2	22	40
Green Chilli (Capsicum annuum)	8	8	2	18	10	8	2	20	10	10	4	24	62
THE CUCURBITACEAE FAMILY													
Bitter gourd (Momordica charantia)	6	4	2	12	8	8	2	18	8	12	2	22	52
Cucumber (Cucumis sativus)	10	12	2	24	8	8	2	18	0	0	0	0	42
Pumkin (Cucurbita pepo L.)	4	6	2	12	6	6	2	14	8	10	2	20	46
Bottle gourd (Lagenaria siceraria)	4	4	2	10	6	6	2	14	6	6	2	14	38
Pointed gourd (Trichosanthes dioica Roxb.)	12	10	2	24	10	10	2	22	10	16	2	28	74
Ridge Gourd (Luffa acutangula (L.) Roxb.)	12	16	2	30	8	10	2	20	0	0	0	0	50
Spine Gourd (Momordica dioica)	8	8	2	18	10	12	2	24	0	0	0	0	42
Snake Gourd (Trichosanthes cucumerina)	8	8	2	18	10	10	2	22	0	0	0	0	40
THE FABACEAE FAMILY													
Broad beans (Vicia faba)	8	6	2	16	0	0	0	0	12	16	2	30	46
Peas (Pisum sativum)	0	0	0	0	0	0	0	0	10	14	2	26	26
French beans (Phaseolus vulgaris)	0	0	0	0	0	0	0	0	8	12	2	22	22
Yardlong bean (Vigna unguiculata ssp. Sesquipedalis)	0	0	0	0	8	8	2	18	8	10	2	20	38
THE BRASSICACEAE FAMILY													
Cabbage (Brassica oleracea var. capitata)	2	0	0	2	2	0	0	2	10	12	2	24	28
Cauliflower (Brassica oleracea var. botrytis)	2	0	0	2	2	0	0	2	12	16	2	30	34
knol-khol (Brassica oleracea L.)	0	0	0	0	0	0	0	0	10	12	2	24	24

Table 1B : Colorimetric Pesticide Assay Test: Analysis of more than 1200 samples of 30 different vegetables in different seasons to standardize the Protocol

Total No of Sample Analyzed	Summer (Period : March - June)				Monsoon (Period : July - October)				Winter (Period : November - February)				Grand Total
	Vegetable from Market Source	Vegetable from Clean Food Project	Organic Vegetables	Total	Vegetable from Market Source	Vegetable from Clean Food Project	Organic Vegetables	Total	Vegetable from Market Source	Vegetable from Clean Food Project	Organic Vegetables	Total	
THE MALVACEAE FAMILY													
Okra (Abelmoschus esculentus)	8	10	2	20	10	12	2	24	12	12	2	26	70
THE LILIACEAE FAMILY													
Onion flower stalk (Allium sativum)	0	0	0	0	0	0	0	0	8	14	2	24	24
Onion (Allium cepa)	6	6	2	14	0	0	0	0	8	12	2	22	36
THE ARACEAE FAMILY													
Colocasia (Colocasia esculenta)	0	0	0	0	6	8	2	16	8	10	2	20	36
THE UMBELLIFERAE FAMILY													
Carrot (Daucus carota subsp. Sativus)	0	0	0	0	0	0	0	0	8	14	2	24	24
THE CHENOPODIACEAE FAMILY													
Spinach (Spinacia oleracea)	6	6	2	14	4	4	2	10	8	10	2	20	44
THE DIOSCOREACEAE FAMILY													
Yam (Dioscorea)	0	0	0	0	6	8	2	16	8	8	2	18	34
THE CARICACEAE FAMILY													
Raw Papaya (Carica papaya. L.)	6	6	2	14	8	10	2	20	8	8	2	18	52
THE MUSACEAE FAMILY													
Raw Banana (Musa balbisiana)	4	4	2	10	4	4	2	10	2	2	2	6	26
Plantain Flower (Musa balbisiana)	4	4	2	10	4	4	2	10	2	2	2	6	26
THE AMARANTHACEAE FAMILY													
Red Amaranthas (Amaranthus cruentus)	4	4	2	10	4	4	2	10	4	4	2	10	30
TOTAL	**146**	**138**	**40**	**324**	**154**	**156**	**42**	**352**	**216**	**284**	**54**	**554**	**1230**

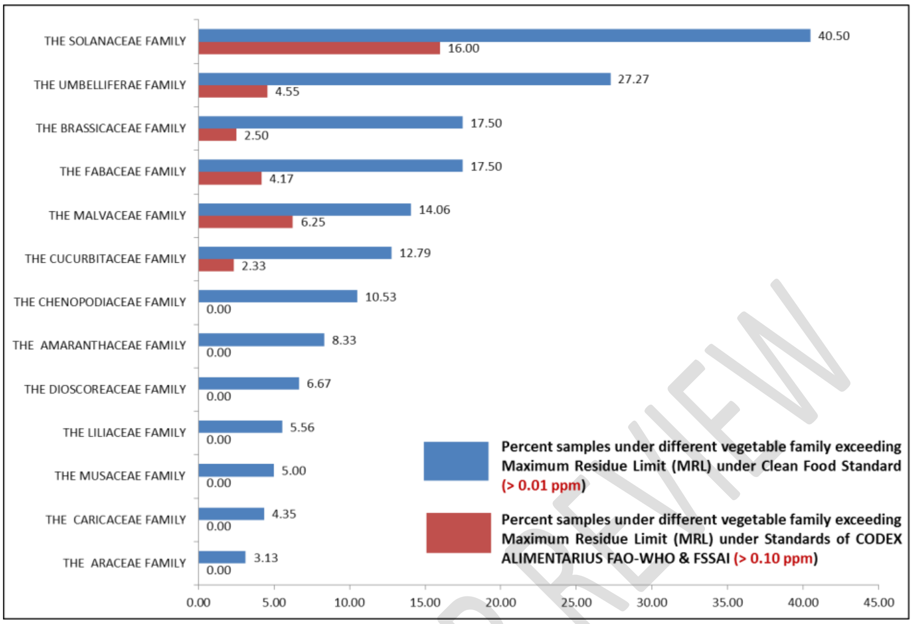

Fig. 6: Comparative Study of different Vegetable Families in terms of Percent Samples exceeding the Maximum Residue Limit (MRL) as per Standards of CODEX ALIMENTARIUS FAO-WHO & FSSAI and Clean Food Standard .

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The colorimetric pesticide assay test can be a potent tool for any sustainable initiative as the protocol can ensure detection of the pesticides groups, at a very low cost (only about 6 - 10% of the conventional cost of residue analysis) and provide speedy results. This particular factor open up the scope for batch wise testing of Clean Food– ensures consumers compliance by authenticating 100% Product Safety. This Test Method can also enable the detection of a wide range of toxic substances of known/unknown origin related to human health and safety. The standard protocol was first implemented under the Clean Food Program IORF) in collaboration of KVK (ICAR), Nadia. The comparative analysis of different vegetables from different sources w.r.t. most toxic load as per Standards of CODEX ALIMENTARIUS FAO-WHO & FSSAI (> 0.10 ppm) was done as per the Colorimetric Pesticide Assay Test Protocol and the progress was assessed w.r.t. the project objectives.

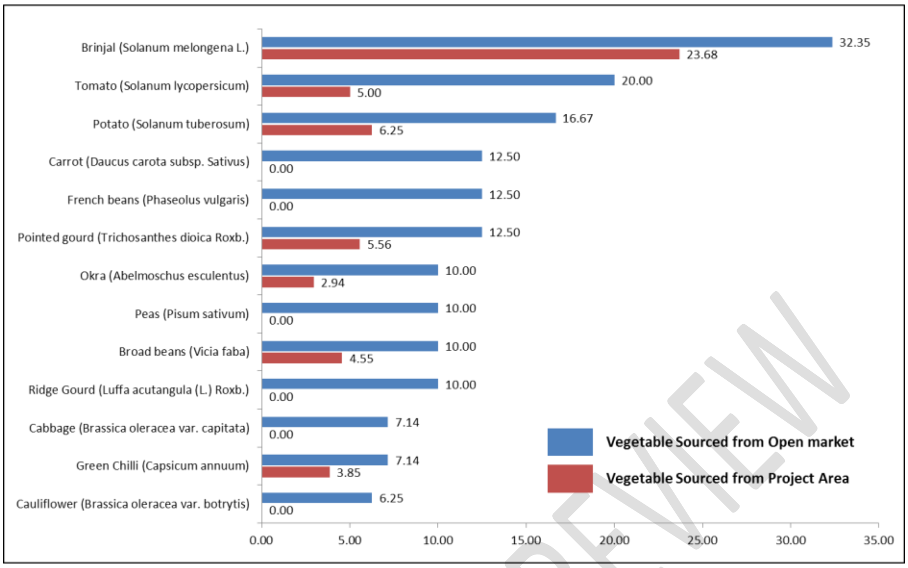

Fig. 7: Vegetables with most toxic load as per Standards of CODEX ALIMENTARIUS FAO-WHO & FSSAI (> 0.10 ppm)

20.1 Colorimetric Enzymatic – Assay Test using Acetylcholinesterase (AChE) for analysis of Organophosphate and Carbamates

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Acetylcholinesterase (AChE) is a key enzyme in the nervous system of animals, terminating impulse transmission by rapid hydrolysis of the neurotransmitter acetylcholine. Organophosphate (OP) and carbamate esters can inhibit acetylcholinesterase (AChE) by binding covalently to a serine residue in the enzyme active site, and their inhibitory potency depends largely on affinity for the enzyme and the reactivity of the ester. AChE inhibition can be quantified by an assay method first described by Ellman et. al. (1961). AChE hydrolyses, ATCh (acetylenethiocholine) to produce thiocholine base and acetate (fig.8). Then the free sulphydryl group of thiocholine reduces the Ellman's reagent called 5, 5'-dithiobis 2 - nitrobenzoic acid (DTNB) to a yellow chromophore of 5 - thio - 2 - nitrobenzoate (TNB) which has the maximal absorbance at 412 nm (Badawy, 2014). The colour development is at maximum level at the beginning, and the enzyme inhibitor addition reduces the final colour developed (Fig. 8).

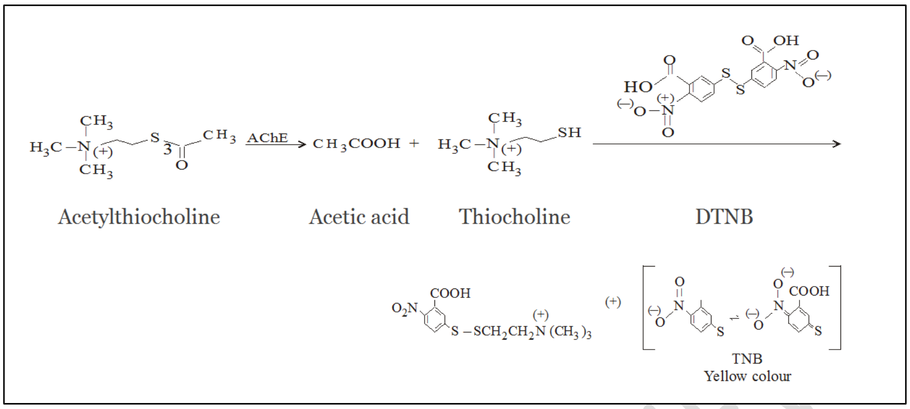

Fig. 8: AChE hydrolyses the ATCh and forms thiocholine base, which then reacts with DTNB to generate TNB, an anion, which is yellow in colour.

Comentado [MDLAVH13]: remove the point

Based on this knowledge system, many test kit has been developed round the world to monitor pesticide residue especially organophosphate (OP) and carbamate (Gabaldón 1999). The process has clear advantage in terms of practical usability as immunoassays are specific, sensitive, easy to perform, and relatively inexpensive. Compared with chromatographic techniques, immunoassays are, in general, advantageous if large series of samples have to be analyzed. Also, no complex or sophisticated instrumentation is required and the use of organic solvents is minimal (Gabaldón, 1999).

20.2 Colorimetric Enzymatic – Assay Test using AChE for analysis of other toxic chemicals and heavy metals.

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Measurement of AChE inhibition as a tool to identify toxic presence of other toxic chemical substance and heavy metals has also been explored by different researchers. It has been discovered that AChE is useful for detecting heavy metals, especially its sensitivity towards copper, silver and chromium (Tham, 2009). The potential of some metallic ions, such as Hg^{2+}, Cd^{2+}, Cu^{2+}, and Pb^{2+}, to depress the activity of AChE *in vitro* and/or *in vivo* conditions has been demonstrated in several studies on humans and animals (Ademuyiwa et. al., 2007; Reddy and Philip, 1994). Ademuyiwa et. al. (2007) studied the potential effect of lead on erythrocyte and AChE activity during occupational exposure to this metal and suggested that erythrocyte AChE activity could be used as a biomarker of lead-induced neurotoxicity in occupational exposed subjects. Other workers also reported that inhibition of AChE activity is due to heavy metal exposure, including Pb, As, Hg, and Cd (Carageorgiou et al, 2004; Richetti et. al., 2011; Phyu and Tangpong 2014), which can be utilized to make AChE based biomarker.

AChE activity may also be affected by other pesticides from different chemical families, such as pyrethroids (Reddy and Philip, 1994), triazines (Davies and Cook, 1993) and Paraquat (Szabo et. al., 1992). Hernández et. al. [2005] suggested the usefulness of AChE as a biomarker for presence of pesticides other than organophosphate and carbamate (Lionetto et. al., 2013).

20.3 Colorimetric Assay Test for Type-II Pyrethroid

The conventional chromatography based methods for pyrethroid analysis (Dubey et. al., 2018) offer high accuracy and precision, good sensitivity, and a very low detection limit, but they are expensive, complicated, and laborious. Thus, these methods may not be suitable for rapid screening and the detection of pyrethroids in developing countries which have a low-resource setting but high risk of pesticide exposure (Pengpumkiat et. al., 2020). Type II pyrethroids are a class of pyrethroids which contain an alpha cyano ester group, obtained by esterification of a cyanohydrine, often m-phenoxy benzaldehyde cyanohydrine, with a modified pyrethroic acid derivative. The most commonly used type-II pyrethroids are deltamethrin, cypermethrin, cyhalothrin, acrinathrin, fenpropathrin, β-cyfluthrin, fenvalerate, esfenvalerate, and fluvalinate (Pengpumkiat et. al., 2020). Under basic conditions these type II pyrethroids are easily hydrolyzed resulting in the formation of pyrethroic acid derivative, m-phenoxybenzaldehyde and cyanide. A test to detect the type II pyrethroids has been developed on basis of the formation and detection of cyanide upon hydrolysis of the type II pyrethroids (Kaur and Eggelte, 2009). The cyanide ions were then detected by reacting with ninhydrin (2,2-dihydroxy-1,3-indanedione) to form a colored complex. The color intensity was quantitatively measured corresponding to the pyrethroid concentration (Pengpumkiat et. al., 2020).

Interestingly, the reaction mechanism of ninhydrin considerably varies among organic chemistry and biochemistry contexts (Bottom et. al., 1978). Recently, ninhydrin has been applied to quantitatively determine free cyanide in a medium of sodium carbonate (Drochioiu, 2002; Mihaescu et. al., 2009). The pyrethroid hydrolysis product, cyanide, works as a selective reducing agent for ninhydrin to form 2-hydroxy-1,3-indanedione (II). It later couples with another molecule of ninhydrin and a free ammonium ion resulting in diketohydrindylidene-diketohydrindamine or Ruhemann's purple (III). The color intensity of Ruhemann's purple stoichiometrically corresponds to the pyrethroid concentration (Pengpumkiat et. al., 2020).

20.4 Colorimetric Assay Test for Organochlorine

Organochlorine pesticides (OCPs; aldrin, chlordane, DDT, dieldrin, endrin, heptachlor, mirex, toxaphene, hexachlorobenzene (HCB)) constitute ten of the twelve chemical substances/groups currently defined under the Stockholm Convention on Persistent Organic Pollutants (POPs) (Muir and Sverko, 2006). Among the wide range of chemical compounds, the organochlorine pesticides (OCPs) are able to alter the proper functioning of the endocrine system in animals and humans (Toppari et. al., 1996; Safe, 2005; Mrema et. al., 2013). This group of pesticide is very stable in the environment because they are not readily metabolized, and their lipophilic character makes them highly accumulable in fat tissues, animal milk, meat and eggs (Olivero-Verbel et. al., 2011). Hence, the Analytical methods for the analysis of organochlorine pesticides (OCPs) are widely available and are the result of a vast amount of environmental analytical method development and research on persistent organic pollutants (POPs) over the past 30–40 years. However, application of this methodology at currently acceptable international standards is a relatively expensive undertaking. Furthermore, the current trend to use isotope-labeled analytical standards and high-resolution mass spectrometry for routine POPs analysis is particularly expensive. These costs limit participation of scientists in developing countries. With the signing of the Stockholm convention on POPs and the development of global monitoring programs, there is an increased need for laboratories in developing countries to determine OCPs. And in this context major focus need to be diverted towards low-cost methods that can be easily implemented in developing countries (Muir and Sverko, 2006).

The analytical work on organochlorine determination depends on chlorine estimation. Either the labile chlorine split out on dehydrochlorintion by alcoholic alkali can be determined or else the total chloride can be determined. Both labile and total organochlorine must be determined in order to detect the presence of organochlorine or its decomposition. In the Colorimetric method for estimation of organochlorine, the insecticide was first decomposed in an organic solvent medium by NaOH or metallic sodium. In the second step, the chloride formed was reacted with mercuric thiocyanate, addition of ferric ion to the liberated thiocyanate to form a complex, leading to colour development. The optical density was determined in a photoelectric colorimeter (Lumetron 401-A) using filter 420.

20.5 Colorimetric Assay Test for Neonicotinoid- Development of a New Method

The word neonicotinoid means "new nicotine-like insecticide". Historically, neonicotinoid insecticides were viewed as ideal replacements for some insecticides (e.g., organophosphates and carbamates) due in part to both their perceived low risk to the environment and to non-target organisms (Jeschke et. al., 2008). However, given their systemic activity (Delso et al, 2015), and presence in the environment (Bonmatin et. al., 2007), and the fact that breakdown products for neonicotinoids are potentially more toxic than the parent compounds (Bonmatin et. al., 2015); neonicotinoids represent a unique human exposure and health risk (ArturoAnadón et. al., 2020). Fruits and vegetables are critical to promoting good health, but translocation of neonicotinoids into plant tissues may potentially be subject to human consumption and subsequently dietary intake. Hence, measurement of neonicotinoids especially in such food items is extremely crucial. Neonicotinoids are generally estimated following the QuEChERS procedure, using the Liquid Chromatography Mass Spectropmetry (LC-MS/MS) method- a critically expensive method, that limits frequent and large scale determination of this insecticide. Thus, these methods may not be suitable for rapid screening and the detection of neonicotinoid in developing countries which have a low-resource setting but high risk of pesticide exposure (Pengpumkiat et. al., 2020). The Colorimetric Method offers a low cost scientific solution in this respect, however; the protocol for assay of neonicotinoid is unavailable. In the Indian context a significant chunk of the food, especially vegetables, are produced by the marginal farms, hence; ensuring safety of the produced food through routine chromatographic analysis, is beyond imagination.

So we took up the initiative to develop a protocol for assessment of neonicotinoid using the Colorimetric Assay Test. Taking cue from the fact that neonicotinoids are proposed to promote stress tolerance of plants (e.g., to drought) by increasing NAD(P) to compensate for a stress-induced decrease in NAD(P) levels, presumably with a neonicotinoid metabolite functioning as a nicotinamide analog that feeds into the NAD salvage pathway (Ford AND Casida, 2006), we used the standard colorimetric test method for nicotinamide (Nwanisobi and Egbuna, 2015) for qualitative assessment of neonicotinoid. The reagent 2,3-dochloro-5,6- dicyano-1,4- benzoquinone (DDQ) was used and the method is based on charge transfer reaction between nicotinamide and DDQ.

21. SCOPE OF COLORIMETRIC PESTICIDE ASSAY TEST FOR SAFE AND SUSTAINABLE AGRICULTURE

Food safety and security are two complementing elements for our sustainable future. The major guideline of any sustainable initiative is, whether the produce is safe**.** Hence, we need novel solutions for our future food security and sustainability without compromising food safety to achieve the United Nations Sustainable Development Goals (SDG) including eradication of hunger and poverty, clean water, sustainable land use, responsible production and consumption, mitigating climate change, and sustainable life on land and water (Vågsholm et. al., 2020). According to WHO (2022), regular monitoring of residues in food and the environment is also required to avoid the associated risk and health hazards. The present HPLC based pesticide analysis system being costly, time consuming, complex and resource intensive are unsuitable and economically nonviable for regular batch wise testing; especially relevant for perishable items like vegetables and fruits. Safety Assessment is becoming a necessary component for any sustainable initiative and Colorimetric Assay Test can be an apt tool in this respect due to the option of both qualitative and quantitative (in totality) expression, process simplicity, lesser time consumption and low analytical cost.

22. CONCLUSION

Safe and Sustainable Agriculture has become a necessity towards the food security goal especially under the existential climate change impacts. Food safety is a critical component of Safe & Sustainable Agriculture considering that food is sustainable only when it is safe. However, the chromatographic techniques for detection of pesticide residues offer very limited scope for regular monitoring of food safety considering the high analytical cost, complex time-taking processes and requirement of specific resources; which are especially difficult to comply in a country like India. Thus, pesticide monitoring is most difficult where that monitoring is arguably most needed considering the marginal to small land holdings, the related acute resource scarcity, extreme reliance on unsustainable inputs *vis-à-vis* lack of awareness regarding the food safety aspect. Now in the context of vegetable crops with a short time gap between harvest and consumption, only regular batch wise testing can ensure 100% safety compliance for the consumers, which is understandably quite beyond imagination. The situation therefore calls for a scientific yet speedy, transparent and conclusive, and an economical method for pesticide residue assessment. The Colorimetric Pesticide Assay Test was standardized to fulfill all the above criteria, measure the five major groups of pesticides *viz*. Organophosphate, Organochlorine, Synthetic Pyrethroids, Carbamates & Neonicotinoids, which comprise more than 90% of the synthetic pesticides used in India, detect the presence of toxic heavy metals as well as a wide range of other toxic substance of known/unknown origin related to human health and safety. The importance of this test can be judged from the fact that apart from the pesticide groups this method can additionally reveal the presence of heavy metal toxicity or any other toxic agents, which is so far the most authentic and transparent confirmation of food safety. Finally this test also opens up the scope for large scale and frequent food safety analysis due to the affordable cost ($1/10^{th}$ to $1/15^{th}$ of the Conventional Cost of Residue Analysis) and significant reduction in the analysis time ($1/10^{th}$ of the time required for Residue Analysis using HPLC). Thus the Colorimetric Pesticide Assay Test can be a sustainable tool for any sustainable agriculture initiative to ensure food safety in real time and in the most authentic and economic manner.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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