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Assessment of 27 pesticide residues in fruit juices & vegetables paste by gas chromatography with mass spectrometry (GC-MS)

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Abstract
This review presents an overview of analytical methods for the analysis of pesticide residues in fruit juices and vegetable paste. The most widely used detection technique for the determination of pesticides is mass spectrometry combined with gas chromatography. QuEChERS method was used for determining twenty seven pesticide residues as per standard guidelines of Association of Official Analytical Chemists (AOAC). The method involves extraction with acetonitrile, liquid-liquid partition with addition of $MgSO_4$ and NaCl followed by dispersive SPE cleanup with PSA sorbent and the analysis was carried out with a GC-MS triple quad equipment. Three major groups of pesticides (organochlorine, organophosphorus and synthetic pyrethoids) were taken for study. The method was applied to 9 fruit juices (Apple, Pomegranate, Tomato, Guava, Mixed fruit, Mango, Litchi, Cranberry and Orange) & two vegetable pastes (Ginger and Ginger-garlic). Most of the pesticides were found below the tolerance limit i.e. 0.01 to 1.0 mg/kg, as per Food Safety and Standard Authority of India (FSSAI). Some pesticides like Chlorpyrifos in orange juice (1.08 mg/kg) & Deltamethrin (1.28 mg/kg) in ginger garlic were detected above the permissible limit of FSSAI

Keywords: Vegetable Paste, Fruit juices, QuEChERS, Pesticide, GC-MS, d-SPE, OCs, Ops, SPs, ppm

1. Introduction
The rapid population growth has resulted in increasing demand for food almost all over the world. In order to fulfill the increasing demand of food, the agricultural productivity needs to be increased. It has been found that many countries of the world have been extensively using chemical pesticides to increase the agricultural productivity so as to fulfill the growing demand for food. About 900 chemical pesticides are used worldwide, legally and illegally, in various food products and for the treatment of crops and soil (Thurman et. al., 2008) [59].
Pesticide is a chemical agent that kills pests and are used to control organisms that are considered to be harmful. They are a class of biocide. They are characterized by pronounced persistence against chemical/biological degradation, high environment mobility, strong tendency for bioaccumulation in human and animal tissues, and significant impacts on human health and the environment, even at extremely low concentrations (H. Liu *et al*., 2009) [28]. The most common use of pesticides is as plant protection products which in general protect plants from damaging influences such as weeds, fungi or insects. According to FAO (Food and Agricultural Organization), a pesticide is any substance or mixture of substances that are intended for preventing, destroying & controlling pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm or otherwise interfering with the production, processing, storage, transport or marketing of food, or substances which may be administered for the control of insects, arachnids or other pests. The term includes substances intended for use as a plant growth regulator, defoliant, desiccant or agent for ripening of fruit, and substances applied to crops either before or after harvest to protect the commodity from deterioration during storage and transport. Pesticides are chemical substances defined as poisons and used in certain circumstances to kill specifically targeted pests. According to the Stockholm Convention on Persistent Organic Pollutants, nine Organochlorines pesticides in twelve POPs are the most dangerous and persistent organic pollutant (POPs).
Herbicides are commonly applied in ponds and lakes to control algae and plants such as water grasses. Natural Herbicides, also commonly known as weed killers, are chemical substances

used to control unwanted plants. Synthetic herbicides like 2, 4-D, Aminopyralid, Atrazine etc are also used. Herbicides are widely used in India by farmers for crop protection from unwanted weeds & shrubs. The application of herbicides is a routine for controlling harmful grass in sugarcane crops, and also other types of pesticides are applied for pest and disease control. Systematic pesticides applied on crops are absorbed either by the plant roots or foliar parts and are incorporated into the tissue, and in case of sugarcane it can result in the presence of their residues in the juice.(Furlani R.P.Z. *et al*, 2011) [23]. The most common fruit juices analyzed are: Orange, Grape, Apple and Tomato and in general, the pesticides levels detected and reported in the studies are considered low.(Alberto, Sanchez-Brunete, & Tadeo, 2003, 2005; Gilbert-Lopez, Garcia-Reyes, Mezcua, Molina-Diaz, & Fernandez-Alba, 2007; Pico & Kozmutza, 2007; Rawn, Roscoe, Krakalovich, & Hanson, 2004; Tadeo. Sanchez–Brunete, Alberto, & Gonzalez, 2004) [2, 3, 25, 48, 50, 58].

Organochlorines pesticides (OCs) are an organic compound containing at least one covalently bonded atom of chlorine as the functional group. Some well known OCs are Lindane, DDT, DDD, HCH, Endosulfan, DDE, Aldrin etc. DDT is the most popular example of organochlorine pollutants, characterized for long persistence in the environment after application. (M. Suwalskya *et al*, 2005; S. Chen *et al*, 2007) [48]. Pesticides are often highly stable compounds that can last for years and decades before breaking down. These substances are highly mobile and capable of bioaccumulation. They circulate globally and pesticides that are released in one part of the world can be easily transported to the other part of the world by a repeated process of evaporation and deposition through the atmosphere to regions far away from the original source (Williams, 2000) [65].

Although organochlorine insecticides like DDT (Dichlorodiphenyltrichloroethane) and its metabolites, Lindane & Aldrin have been banned years ago in many countries because of their mutagenic, carcinogenic and endocrine disrupting properties, they still can be found in environmental samples like river water, ground water, drinking water & river sediment, due to their persistence and lipophilic properties. In India, the use of pesticides has become inevitable to sustain and improve current label of crop from pests. Being a subtropical country, India observes varying temperature and humidity, profile throughout the year, which brings a vast array of pest to be tagged. Annually, approximately 500 different pesticides are applied or found on fields throughout the world. India is now the large manufacturer and consumer of pesticides in South Asia. Despite of proliferation of different types of pesticides, organochlorine pesticides such as, HCH and DDT still account for 2/3rd of total consumption of country because of their low cost and versatility in action against various pest. Few earlier studies have indicated the contamination of Indian food and feeds by HCH and DDT. (Dikshith *et al*., 1989, Battu *et al*., 1989., Kaphalia *et al*., 1990) [18, 9, 32].

Organophosphates pesticides (OPs) are known as esters of phosphoric acid. Some examples of OPs are Chlorfenvinphos, Chlorpyrifos etc. OPs protect crops from pests by inhibiting acetyl-cholinesterase enzyme activity in insects. They are sprayed over crops or soils, causing residues to be found in surface and groundwater, fruits, vegetables and in drinking water (Yao *et al*., 2001) [67]. General population is mainly exposed to organophosphorus pesticide residues through the ingestion of contaminated foods (such as cereals, vegetables and fruits), which are directly treated with OPPs pesticides or are grown in contaminated fields. Compared with organochlorine pesticides, OPPs demonstrate relatively low environmental persistence but a higher toxicity acute. Therefore, the OPPs residue in food has been strictly regulated by government in all countries in order to determine whether the concentrations of the pesticides used exceed their maximum residue limits (MRLs). (European Commission directive (1993) [20] 93/58/EEC Official, Journal of the European Communities L.211/6-39; FAO, "Agriculture towards 2010," in Proceedings of the 27th Session of the FAO Conference, Rome. Italy, 1993 [20], C 93/24).

A survey on pesticide residues carried out by the Department of Agriculture Sarawak reported that 95% of total residue violation is caused by organophosphate pesticides (Lian and Seng, 2003) [39]. The pesticides are traditionally extracted using liquid-liquid extraction (LLE) (Fenoll *et al*., 2007; Wang *et al*., 2008; Hassan *et al*., 2010; Pirad *et al*., 2007) [22]. The LLE procedures consume large amounts of solvents, involve several steps and difficult to automated. Alternatively, solid –phase extraction (SPE) involving the use of different types of sorbents like amine, PSA and C18 have been used. (Lopez-Blanco *et al*., 2006; Albero et. al., 2005; Wang et. al., 2009) [28]. QuEChERS with dispersive solid-phase extraction(d-SPE) technique using different type of sorbents such as PSA, C18, silica gel, graphitized carbon black,florisil and amine modified graphene were also reported. (Anastassiades and Lehotay, 2003; Walorczyk *et al*., 2011; Guan *et al*., 2013; Chai and Elie, 2013; Cieslik *et al*., 2011) [5, 63, 27, 14, 15]. Since the majority of the OPs are volatile and thermally stable, they are amenable to gas chromatography analysis. OPs in fruits, vegetables and water using GC with either electron capture (ECD) or mass spectrometry (MS) detectors have been used. (Nguyen *et al*., 2008; Melo *et al*., 2012) [46, 43]. In one of the study, OPPs were found to be most frequently employed worldwide, and are normally sprayed over banana trees which constitute a hazard to the environment and also to human health. (Borges, Cabrera, Delgado, Suarez & Sauco, 2009; Tock, Lai, Lee, Tan & Bhatia, 2010; Tsoukali &Tsoungas, 1996) [11, 60, 62]. The presence of several of pesticides used in banana production were fungicides thiabendazole, propiconazole and imazalil; the nematicide terbufos and cadusafos and the insecticide Chlorpyrifos. (Castillo *et al*., 2006) [13]. To minimize such problems various organizations have set stringent regulatory controls on pesticide use in order to minimize exposure of the population to pesticide residues in food (Kmellar, Pareja, Ferrer, Fodor, & Alba, 2011) [33]. However, mass spectrometry (MS) is preferred as it provides confirmatory evidence of the identity of the compound (Fang *et al*., 2012) [21].

In carbamates group of pesticides, carbamate ester (e.g., ethyl carbamate), and carbamic acids are functional groups that are inter-related structurally and often interconverted chemically, i.e. Sodium dimethyldithiocarbamate. Pyrethoids are new type of insecticide, it prevent and treat insects in modern agriculture due to their broad-spectrum insecticidal capacity and high effectiveness. (Ye, Xie, Wu & Lin, 2006) [66]. Synthetic Pyrethroids are synthetically produced molecules that are chemically similar to pyrethrins. Some well-known pyrethroids are Cypermethrin, Cyfluthrin, and Cyhalothrin etc. However, pyrethoids residues are considered to be one of the most important sources of pollution in agricultural production, and may be a potential threat to public health (Kolaczinsiki & Curtis, 2004).Therefore it is necessary to develop sensitive and selective methods for the analysis of

pyrethoid residues usually present in trace amounts. Potential analytical methods include gas chromatography-mass spectrometry (GC-MS) (Cunha, Fernandes, & Oliveira 2009; Kok, Hiemstra, & Bodegraven, 2005) [34], and gas chromatography-tandem mass spectrometry (GC-MS/MS) (Paya *et al*., 2007) [47]. Quick and effective sample preparation coupled with a reliable analytical method is imperative. Liquid –liquid extraction (LLE) (Rezaee *et al*., 2006) [52] and solid-phase extraction (SPE) (Sharif, Man, Hamid, & Keat, 2006) [57] are the most common sample preparation methods widely used for residue analysis.

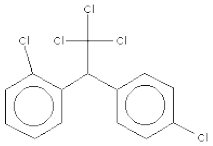
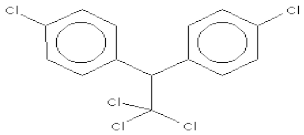
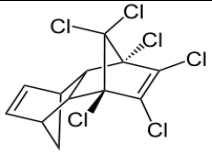
At present, "quick, easy, cheap, effective, rugged and safe" (QuEChERS) sample preparation, is the most common technique for multi-residue pesticides analysis in food, especially fruit and vegetable (Anastassiades, Lehotay, Stajnbaher & Schenck, 2003) [5]. This technology is widely accepted & approved by AOAC. Originally, QuEChERS was introduced for pesticide residues analysis in fruits and vegetables with high water content. Now-a-days it is gaining popularity in analysis of pesticides and other compounds in huge variety of food products and others with different types of matrices. This method has important advantages over most traditional extraction methods as it yields high recovery rates for wide range of analytes. Using this method, a batch of 10-20 samples could be extracted in 30-40 minutes by a single analyst. (Lehotay et. al., 2004) [38]. QuEChERS involves an acetonitrile salting-out extraction of a solid sample in an aqueous environment followed by dispersive solid phase extraction (d-SPE) to remove a majority of the remaining matrix interferences (Lehotay et. al., 2010) [36]. Acetonitrile as a solvent for the first step of QuEChERS was made on the basis of its selectivity as it cover broad scope of pesticides (Anastassiades *et al*., 2006; Anastassiades *et al*., 2003) [4, 5]. Other advantage of acetonitrile is its compatibility with the chromatographic applications and also it gives large solvent expansion volume during GC vaporization. (Anastassiades *et al*., 2006; Majors *et al*., 2007) [4, 41] Liquid-liquid extraction (LLE) has long been an effective method of separating compounds with different solubilities in two immiscible liquids. (Majors *et al*., 2013) [42].

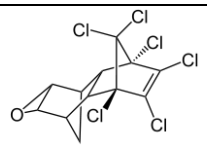
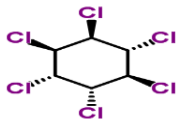
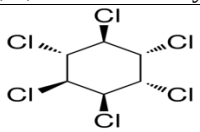
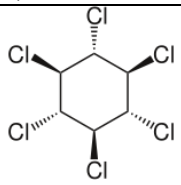
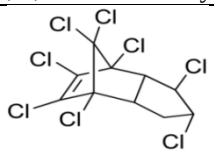
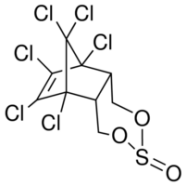
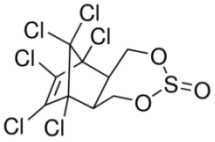
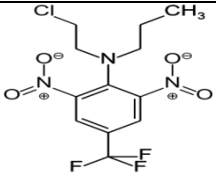
Lehotay in 2005 conducted validation experiments of the QuEChERS method for the determination of residues from 229 pesticides in fruit juices and vegetables using gas chromatography and mass spectrometric detection. (Lehotay *et al*., 2005) [37].

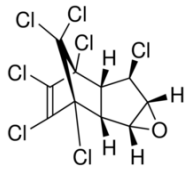
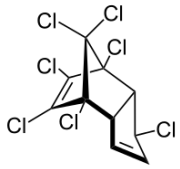
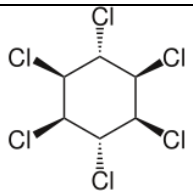
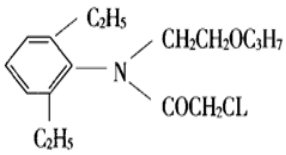
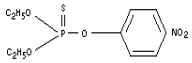
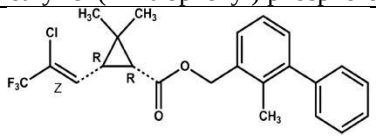
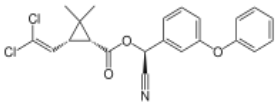
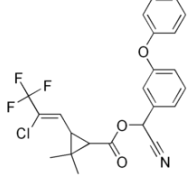
In another method, 14 pesticides (13 pesticides and 1 insecticide) were investigated from peel to pulp in grapes. Pesticides such as cymoxanil and oxadixyl were found in the pulp, while only the contact pesticide folpet was detected in the peel and not in the whole grapes. (Teixeira et. al., 2004) [44].

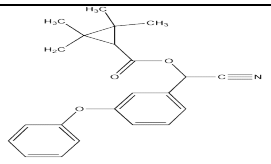
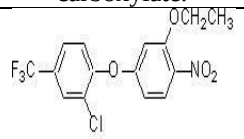
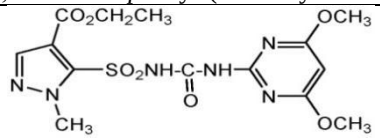
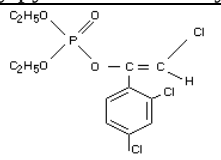
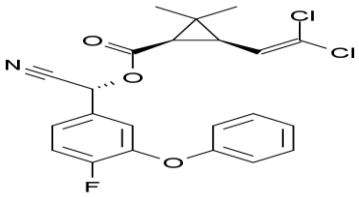
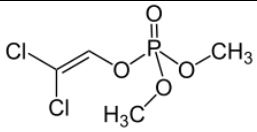
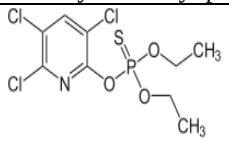
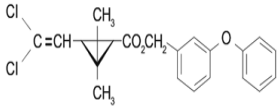
Two surveys for table grapes carried out in three different regions in Turkey showed that Chlorpyrifos-methyl and Chlorpyrifos-ethyl, besides Deltamethrin and ƛ- cyhalothrin, were the most frequently found pesticide. (Edison *et al*., 2011) [56]. In fact, a market survey of commercial sources of products labeled as containing pomegranate found that only six out of twenty-three met the proposed standards for authenticity (Zhang *et al*., 2009) [68]. Pomegranate juice looks similar to grape juice, which often contains pesticides. Since grape juice costs much less than pomegranate juice, it would be a cost effective choice for adulteration. One way to look for adulteration and fraud is to look for pesticides, so GC-MS is needed. It clearly indicates the use of the banned pesticides by the farmers. Even in ppb level pesticides are neuro & hepato toxic because it accumulates in the human cells. In this project we assess the level of pesticides in Fruit juices available in the market for human consumption. The data will be useful for society to understand the contamination level of pesticides & its comparison with the permissible limits as per FSSAI in Fruit juices available in the market. The proposed project work is related with the requirement of pesticides (described in Table 1) in the food products, especially in juices as per FSSAI, 2015 (Food Safety Standard Authority of India).

Table 1: General information about the pesticides taken for the study

S. No	Name Of Pesticides	Molecular Formula	Structure
1.	2,4'-DDT (OC)	$C_{14}H_9Cl_5$ (354 g/mol)	 **IUPAC name:** 1, 3-Dichlorodiphenyltri-chloro ethane
2.	4,4′-DDT (OC)	$C_{14}H_9Cl_5$ (354 g/mol)	 **IUPAC name:** 1,1'-(2, 2, 2-trichloroethane-1,1-diyl) bis (4-chlorobenzene
3.	Aldrin (OC)	$C_{12}H_8Cl_6$ (364 g/mol)	 **IUPAC name:** 1, 2, 3, 4, 10, 10-Hexachloro-1, 4, 4a, 5, 8a-hexahydro1, 4endo, exo-5, 8-dimethane-naphthalene.

4.	Dieldrin (OC)	$C_{12}H_8Cl_6O$ (378 g/mol)	 **IUPAC name:** (1Ar, 2R, 2aS, 3S, 6R, 6R, 7S, 7As)-3, 4, 5, 6, 9, 9-hexachloro-1a, 2, 2a, 3, 6, 6a, 7, 7a-octahydro 2, 7, 3, 6-dimethanenapth (2, 3-b)oxene.
5.	Alpha-BHC Solution (OC)	$C_6H_6Cl_6$ (290 g/mol)	 **IUPAC name:** α-1, 2, 3, 4, 5, 6-hexachlorocyclohexane
6.	δ- BHC solution (OC)	$C_6H_6Cl_6$ (290 g/mol)	 **IUPAC name:** δ-1, 2, 3, 4, 5, 6-hexachlorocyclohexane
7.	β-Hch Solution (OC)	$C_6H_6Cl_6$ (288 g/mol)	 **IUPAC name**: β-1, 2, 3, 4, 5, 6-hexachlorocyclohexane
8.	Chlordane- (CIS + Trans) (OC)	$C_{10}H_6Cl_8$ (409 g/mol)	 **IUPAC name**: 1, 2, 4, 5, 6, 7, 8, 8-Octachloro-3a, 4, 7, 7a-tetrahydro-4, 7-methanoindane
9.	α- Endosulfan (OC)	$C_9H_6Cl_6O_3S$ (404 g/mol)	 **IUPAC name:** (5a*R*, 6*S*, 9*R*, 9a*S*)-6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6, 9-methano-2, 4, 3-benzodioxathiepine 3-oxide
10.	β- Endosulfan (OC)	$C_9H_6Cl_6O_3S$ (404 g/mol)	 **IUPAC name :** (5a*R*, 6*S*, 9*R*, 9a*S*)-6, 7, 8, 9, 10, 10-hexachloro-1, 5, 5a, 6, 9, 9a-hexahydro-6, 9-methano-2, 4, 3-benzodioxathiepine 3-oxide
11.	Fluchloralin (OC)	$C_{12}H_{13}ClF_3N_3O_4$ (355 $gmol^{-1}$)	 **IUPAC name :** N-(2-chloroethyl)-2, 6-dinitro-N-propyl-4-(trifluoromethyl) aniline.

12.	Heptachlor epoxide isomer b solution (OC)	$C_{10}H_5Cl_7O$ (386 g/mol)	 **IUPAC name:** 1, 4, 5, 6, 7, 8, 8-Heptachloro-3a, 4, 7, 7a-tetrahydro-4, 7-methano-lH-indene
13.	Heptachlor Solution (OC)	$C_{10}H_5Cl_7$ (373 g/mol)	 **IUPAC name:** 1, 4, 5, 6, 7, 8, 8-Heptachloro-3a, 4, 7, 7a-tetrahydro-4, 7-methano-1*H*-indene
14.	Lindane (OC)	$C_6H_6Cl_6$ (288 g/mol)	 **IUPAC name:** 2*R*, 3*S*, 4*r*, 5*R*, 6*S*)-1, 2, 3, 4, 5, 6-hexachlorocyclohexane
15.	Pretilachlor (OC)	$C_{17}H_{26}ClNO_2$ (311 g/mol)	 **IUPAC name:** 2-chloro-N-(2, 6-diethylphenyl)-N-(2-propoxyethyl) acetamide
16.	Parathion Solution (OP)	$C_{10}H_{14}NO_5PS$ (291 g/mol)	 **IUPAC name:** *O*, *O*-Diethyl *O*-(4-nitrophenyl) phosphorothioate
17.	Bifenthrin Solution (SP)	$C_{23}H_{22}ClF_3O_2$ (422 g/mol)	 **IUPAC name :** (2-methyl-3-phenylphenyl)methyl (1S, 3S)-3-[(Z)-2-chloro-3, 3, 3-trifluoroprop-1-enyl]-2, 2-dimethylcyclopropane-1-carboxylate
18.	Cypermethrin Solution (SP)	$C_{22}H_{19}Cl_2NO_3$ (416 g/mol)	 **IUPAC name:** [Cyano-(3-phenoxyphenyl) methyl]3-(2, 2-dichloroethenyl)-2, 2-dimethylcyclopropane-1-carboxylate
19.	Lambda-Cyhalothrin (SP)	$C_{23}H_{19}ClF_3NO_3$ (449 g/mol)	 **IUPAC name :** 3-(2-chloro-3, 3, 3-trifluoro-1-propenyl)-2, 2-dimethyl-cyano(3-phenoxyphenyl) methyl cyclopropanecarboxylate

20.	FENPROPATHRIN (SP)	$C_{22}H_{23}NO_3$ (349 g/mol)	 **IUPAC name:** (Cyno-(3-phenoxyphenyl) methyl] 2, 2, 3, 3-tetramethylcyclopropiene-1-carboxylate.
21.	Oxyfluorfen (OT)	$C_{15}H_{11}ClF_3NO_4$ (361 g/mol)	 **IUPAC name :** 2-chloor-α, α, α- trifluor-*p*-tolyl-(3-ethoxy-4- nitrofenyl)ether
22.	Pyrazosulfron-Ethyl (OT)	$C_{14}H_{18}N_6O_7S$ (414 g/mol)	 **IUPAC name :** ethyl 5-(4,6-dimethoxypyrimidin-2-ylcarbamoylsulfamoyl)-1-methylpyrazole-4-carboxylate
23.	Chlorfenvinphos solution of cis and trans isomer (OP)	$C_{12}H_{14}Cl_3O_4P$ (359 g/mol)	 **IUPAC name:** 2-chloro-1-(2,4 dichlorophenyl) vinyl diethyl phosphate
24.	Beta-Cyfluthrin (SP)	$C_{22}H_{18}Cl_2FNO_3$ (434 g/mol)	 **IUPAC name :** 3-(2,2-dichloro-vinyl)-2,2-dimethyl-cyclopropane-carboxylic acid cyano-(4- fluoro-3-phenoxy-phenyl)-methyl ester
25.	DICHLORVOS (OP)	$C_4H_7Cl_2O_4P$ (220 g/mol)	 **IUPAC Name:** 2, 2-dichlorovinyl dimethyl phosphate.
26.	CHLORPYRIFOS SOLUTION (OP)	$C_9H_{11}Cl_3NO_3PS$ (350 g/mol)	 **IUPAC name:** *O*,*O*-Diethyl *O*-3,5,6-trichloropyridin-2-yl phosphorothioate
27.	PERMETHRIN (SP)	$C_{21}H_{20}Cl_2O_3$ (391 g·mol^{-1})	 **IUPAC name:** 3-Phenoxybenzyl 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate

2. Material and Methods

2.1 Pesticides

Three different classes of pesticides were investigated and informations are given in table below as follows: organochlorine pesticides (2, 4-DDT, 4, 4-DDT, Aldrin, Chlordane-Cis, Heptachlor Epoxide B, Chlordane-trans, Endosulfan-I, Endosulfan-II, Iprodione, Fluchloralin, Pretilachlor, Dieldrin & Dicofol.), organophosphorus

pesticides (Dichlorovos, Chlorpyrifos, Chlorpyrifos-methyl, and Parathion, Chlorfenvinphos cis + trans & Parathion-methyl.),synthetic pyrethoids pesticides (β-Cyfluthrin, λ-Cyhalothrin, Deltamethrin, Cypermethrin, Fenpropathrin, Fenvalerate & Permethrin.) and other pesticides such as; Oxyfluorfen, Pyrazosulfuron-ethyl, Trifluralin & Etofenprox were obtained from the authentic suppliers.

2.2 Standards and Reagents

Pesticides standards (Dichlorvos, Alpha-HCH, Beta-HCH, Delta-HCH, Lindane, Iprodione, Fluchloralin, Heptachlor, Aldrin, Parathion, Chlorpyrifos, Parathion-methyl, Heptachlor epoxide B, Trans-chlordane, Cis- chlordane, Endosulfan-I, Endosulfan-II, Pretilachlor, Dieldrin, Oxyfluorfen, Pyrazosulfuron-ethyl, 2,4-DDT, 4,4-DDT, Cypermethrin, Bifenthrin, Fenpropathrin, Permethrin, Beta-Cyfluthrin, Lamda-Cyhalothrin & Deltamethrin), with a minimum of < 99.5% purity were taken. Certified reference standards of pesticides were procured from the authentic suppliers like MERCK, SUPELCO & SIGMA-ALDRICH. For pesticides analysis by GC-MS, we prepared the standards of 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, and 80.0 and 100 ppb using successive dilution from 10 to 100 ppm (27 mix) Stock solutions of individual standards 10 (mg/l) were prepared in Methyl alcohol, Hexane Ethyl Actate, Toluene and stored in the refrigerator at -2 to 8 °C.The calibration standards stock solutions contained 0.250 ppm pesticides in concentrations ranging from 0.001 to 0.1 mg/l and were prepared in Ethyl Acetate.

All reagents (Acetonitrile & Acetic Acid, 1% acetic acid in acetonitrile, Toluene, MS-grade Methanol, Hexane & Ethyl acetate) used in the study were of Mass-grade (Merck). Ultrapure water was generated by a Millipore Milli-Q system.

2.3 Samples

Tetra packed nine fruit juices of Apple, Pomegranate, Tomato, Guava, Mango, Litchi, Cranberry, Orange and Mixed fruit with two vegetable paste samples of Ginger paste and Ginger-garlic paste were taken due to their commercial importance and potential consumption. Samples freshly prepared and analyzed. For validation experiments Ethyl Acetate used as blank.

2.4 Sample preparation by QuEChERS method

For sample preparation we used the Quick-Easy-Cheap-Effective-Rugged-Safe extraction method that has been developed for the determination of pesticide residues in agricultural commodities (fruits and vegetables). QuEChERS is valued for its simplicity, low cost, low susceptibility to error, and ability to extract pesticides from various matrices. It is a sample preparation approach entailing solvent extraction of high-moisture samples with acetonitrile, ethyl acetate, or acetone and partitioning with magnesium sulphate alone or in combination with other salts followed by clean up using d-SPE.

For extraction, 15ml of sample was transferred to 50ml Teflon tube, 15ml 1% acetic acid in Acetonitrile with 1.5g anhydrous sodium acetate and 6g anhydrous magnesium sulphate (QuEChERS Agilent Pouch -Part No.-5982-0755**)** were added and then shake vigorously for 1 minute. The tube was centrifuged for 5 min at 1500 rcf. (Anastassiades, Lehotay, Stajnbaher, & Schenck, 2003) [5].

The SPE columns were used for the clean-up of multiple types of pesticides in fresh fruits and vegetables, this study included reverse phase columns such as primary secondary amine (PSA). (Schenck *et al*., 2002). Cleanup was performed by transferring 1ml aliquot of upper layer to a polypropylene centrifuge tube containing 150 mg anhydrous $MgSO_4$ and 50 mg PSA (Primary Secondary Amine) (Agilent -Part No.-5982-5022). Extracts was shake for 30 seconds using a vortex. Centrifugation was done at 1500 rcf for 5 minutes. 1 ml extract was transferred to GC vial for analysis and diluted with ethyl Acetate. Transferred 0.5-1.0 ml extract to GC vial for analysis. Analyzed by GC/MS.

2.4 Instrumentation

2.4.1 Gas Cartographic conditions

The pesticides were identified and quantified by a gas chromatograph (Agilent, 7890A) equipped with a 7693 auto sampler and a column oven 230 °C. 27 pesticides were prepared and 27 were detected including their isomers by GC-MS in multiple reactions monitoring (MRM) mode. These compounds were separated on column having DB-5 MS, 30m * 0.250µm, film thickness 0.25 µm. Operating conditions were as follows: initial column temperature of 70 °C with a Injection Volume of 2 µl. The mobile phase consisting of He. The carrier gas He was at flow rate 1.2 ml/minutes. He gas of MS-grade was used and supplied by Sigma. Oven Temperature gradient, injector temperature gradient, transfer line temperature, total run time are shown in table 2.

2.4.2 Mass spectrometric conditions

Mass spectrometric analysis was carried out using a 7000 GC/MS Triple Quad, Agilent. The instrument was operated using Electron Ionization (EI) source in both positive and negative modes using split/split less injection. Electron Ionization is most commonly used for analysis of pesticide residues in grapes than chemical ionization. (A. Angioni et. al, 2011; J. Dong *et al*., 2011) [1, 31] Instrument settings, data acquisitions and processing were controlled by the software Mass Hunter Workstation.

Source ionization was optimized as follows: ion spray voltage, 70 kV for EI (+) and 70 Kv for EI (-); collision gas flow, 1.5 ml/min; Quenching gas flow, 2.5 ml/min respectively; ion source temperature, 230°C. The temperature of both the first and third quadrupoles was 150°C. When MS analyzer is used, the acquisition mode mainly selected is multiple reactions monitoring (MRM) and full scan m/z 100 to 1000.The conformations of pesticides peaks has been done by comparing the fragment ions with NIST library.

2.4.3 Linearity

A test mixture with standard of organochlorines, organophosphates, synthetic pyrethoids and other pesticides were prepared and analyzed under optimized conditions to determine linearity.

Linearity was determined by constructing calibration curves with standard solutions, in Ethyl acetate containing all pesticides in the range of 10 mg/l. Single injections were made at each of the 0.001 mg/l to 0.1 ppm concentration levels.

Table 2: Parameters of GC/MS (Gas Chromatograph/Mass Spectrometer)

GC Parameter	
Analytical column	Agilent DB-5 MS 30m * 0.250µm,0.25 µm
Injection Volume	2 µl
Injector Temperature gradient	250°C
Carrier gas	Helium, 1.2 ml/minutes
Oven Temperature gradient	70°C for 2 minutes 25°C/min to 150°C 3°C/min to 200°C 280°C hold for 10 minute.
Transfer line Temperature	280°C
Total Run Time	42 minutes
MS Parameter	
Source	Electron Ionization (EI)
Collision gas flow	Nitrogen 1.5 ml/min
Quenching gas flow	2.5 ml/min
Ion Source Temperature	230°C
Environmental condition	
Room Temperature	20 -27°C
Relative Humidity	20-60%

3. Results and Discussions

3.1 Method Validation

Method validation (for juices) was carried out using parameter such as: linearity with Good Correlation coefficients were obtained for all of the compounds ranging from 0.990 to 0.999

(Table-3) The obtained values were satisfactory and allow the determination of these pesticides at the limits required. Better linearity was given by Bifenthrin and Dichlorovos compared to other pesticides the effective usage of Linearity as a standard for the determination of pesticides in fruit juices.

Existence of interferences in chromatographic determination of pesticides in fruit and vegetable samples was monitored by running control of blank samples in each calibration. The absence of any chromatographic components at the same retention times in target pesticides suggested that no chemical interferences occurred. GC-MS QQQ is the most frequently used detector for pesticide residues analysis.

Table 3: MS Transition Parameters: precursor ion and product ion, CE (Collision Energy)

S.No.	Name Of Pesticides	Precursor Ion	Product Ion	Collision Energy
1.	DICHLOROVOUS	109	79	5
		109	47	15
2.	α-HCH	180.9	145	16
3.	β-HCH	180.9	145	16
4.	γ-HCH	180.9	145	16
5.	Lindane	180.9	109	30
		314	245	10
		314	56	20
6.	Fluchloralin	352.9	63	34
		306	264	34
		306	206	34
7.	Heptachlor	271.8	236.8	20
		271.8	234.7	20
		271.9	236.8	25
		271.9	116.9	40
		274	239	30
8.	Heptachlor Epoxide B	352.8	281.7	10
		352.8	262.7	14
9.	Aldrin	263	193	30
		263	191	30
10.	Parathion	263	127	6
		263	109	15
		263	79	30
		173.1	125.1	20
11.	Parathion-Methyl	263	109	10
		263	79	35
12.	Trans-Chlordane	372.9	265.9	20
		372.9	265.3	25
	Cis-Chlordane	372.9	265.9	20
		372.9	263.9	25
13.	Endosulfan-I	241	206	15
		241	136	40
14.	Endosulfan-Ii	195	159	10
		195	125	25

15.	Pretilachlor	162	147	10
		262.8	193	30
16.	Dieldrin	262.8	191	30
17.	OXYFLUORFEN	252.1	196	20
		252	170	32
		252	146	32
18.	2,4-DDT	237	165	15
		235	199.1	15
		235	165	15
19.	4,4-DDT	237	165	15
		235	199	15
20.	Cypermethrin-III	181.1	152.1	25
		181.1	127.1	35
21.	Cypermethrin-IV	181.1	152.1	25
		181.1	127.1	35
22.	Bifenthrin	181	166	25
		181	165	25
23.	Fenpropathrin	181	152	26
24.	γ-CYHALOTHRIN	197	171	15
		197	161	10
25.	Pyrozosulfron-Ethyl	252	146	10
26.	Chlorfenvinfos	267	159	20
27	Chlorfenvinfos-Methyl	267	81	20

3.2 Sample Analysis

The developed GC-MS was applied for the determination of three classes of pesticides residues in nine fruit juices and two vegetable pastes. Different variants of fruit juices like Apple, Pomegranate, Tomato, Guava, Mixed fruit, Mango, Litchi, Cranberry and Orange.Two variants of ginger-garlic paste. The samples were immediately processed according to the sample preparation procedure using QuEChERS kit. The analysis was performed and between each run of the sample, a blank was carried out to avoid contamination from extraction process. The results obtained from the positive samples are summarized in below table. The use of chromatographic techniques coupled with Mass Spectrometer produce very reliable method for the determination of pesticides at trace level.

The Organochlorine pesticides (OCPs) were not detected in most of the Juices (<0.01 mg/kg). Total eighteen OCPs were taken for this study (DDT, HCH & its isomer, Heptachlor and Endosulfan are the most available pesticides in the environment). In Tomato juice the Aldrin content was found i.e.; 0.07 mg/kg. (Table no.4). The 2, 4-DDT, 4, 4-DDT and Fluchloralin were detected in cranberry and orange juice (0.01 mg/kg). All the values of organo chlorine pesticides are under the tolerance limit of FSSR (Food Safety And Standards Regulations, 2011) [24]. As per the FSSR regulation the aldrin should not more than the 0.1 mg/kg.

Table 4: Concentration of Organochlorine pesticides in fruit juices & Vegetable Paste

Type of Sample	**Fruit Juices**									**Vegetable Paste**	
Sample	**Apple**	**Pomegranate**	**Tomato**	**Guava**	**Mixed Fruit**	**Mango**	**Litchi**	**Cranberry**	**Orange**	**Ginger Garlic**	**Ginger**
	mg/kg	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**	**mg/kg**
Organochlorine Pesticide											
2,4-DDT	ND	ND	ND	ND	ND	ND	ND	0.01	ND	ND	ND
4,4-DDT	ND	ND	ND	ND	ND	ND	ND	0.01	ND	ND	ND
Aldrin	ND	ND	0.07	ND	ND	ND	ND	ND	ND	ND	ND
Chlordane-Cis	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Heptachlor Epoxide B	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Chlordane-trans	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Endosulfan-I	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Endosulfan-II	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Iprodione	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Fluchloralin	ND	ND	ND	ND	ND	ND	ND	ND	0.01	ND	ND
Pretilachlor	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dieldrin	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Dicofol	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

The organophosphorus pesticides like Dichlorovos, Chlorpyrifos, Chlorpyrifos-methyl, Parathion, Chlorfenvinphos (cis & trans) and Parathion-methyl were taken in study. The parathion and parathion-methyl were not detected in all samples of fruit juices and vegetable paste. Rodrigues D., *et al*. (2010) found parathion-methyl 36.0 µg/kg in mango juices. The Dichlorovos was detected in Guava and Mango juice (0.01mg/kg) and the tolerance limit of Dichlorvos is 0.1 ppm as per FSSR. Dichlorovos content was found in ginger-garlic paste (Table no.5).The concentration of 0.94 mg/kg was noticed whereas the permissible limit in FSSAI is 0.15ppm. Arpad Ambrus, (2010) detected 0.05 ppm of Dichlorovos in ginger paste. The content of Chlorpyrifos was detected in all samples except tomato-juice. The concentration of Chlorpyrifos varies from 0.032-1.08 mg/kg in fruit juices and in vegetable paste it

varies from 0.76 to 0.79mg/kg. As per FSSR guideline the permissible limit is 0.2 mg/kg for vegetable paste and 0.5 ppm for fruit juices. The concentrations of Chlorpyrifos were noticed higher side in case of vegetable paste available in the market. The concentration of Chlorpyrifos methyl was detected in range of 0.02-0.08 mg/kg. (Table no.5). The values are under permissible limit as per FSSR compliance. Rodrigues D., *et al*. (2010) & Mladenova R. *et al*. (2009) were found Chlorpyrifos concentration <0.75 µg/kg in orange juice and 0.77 mg/kg in apple juice respectively and Arpad Ambrus, (2010) detected 0.05 ppm of Chlorpyrifos residues in ginger paste. The concentration of Chlorfenvinphos (cis & trans) was detected in guava (0.07mg/kg), cranberry (0.30 mg/kg) and orange (0.13 mg/kg) more than the permissible limit i.e. 0.05 mg/kg. The data of organophosphorus pesticides clearly indicates its presence in fruit juices and vegetable paste. (Table no.5).

Table 5: Concentration of Organophosphorus pesticides in fruit juices & Vegetable Paste

Type of Sample	Fruit Juices									Vegetable Paste	
Sample	Apple	Pomegranate	Tomato	Guava	Mixed Fruit	Mango	Litchi	Cranberry	Orange	Ginger Garlic	Ginger
	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Organophosphorus Pesticide											
Dichlorovos	ND	ND	ND	0.01	ND	0.01	ND	ND	ND	0.94	ND
Chlorpyrifos	0.41	0.32	ND	0.56	0.42	0.49	0.433	0.85	1.08	0.79	0.76
Chlorpyrifos-methyl	0.03	0.08	ND	0.06	0.02	0.04	0.05	ND	ND	ND	ND
Parathion	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Chlorfenvinphos cis+trans	ND	ND	ND	0.07	0.03	ND	0.04	0.304	0.13	ND	ND
Parathion-methyl	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

The concentration of synthetic pyrethoids pesticides (SPPs) were analyzed for β- Cyfluthrin, Deltamethrin, Cypermethrin, λ- Cyhalothrin, Fenpropathrin, Fenvalerate and Permethrin. β-Cyfluthrin was found in mango juice i.e. 0.03 mg/kg and not detected in rest of the sample. (Table no.6). Arpad Ambrus, (2010) detected 0.05 ppm of β- Cyfluthrin in ginger paste. λ-Cyhalothrin was found in tomato juice (0.11 mg/kg) and ginger-garlic paste (0.01 mg/kg). (Table no.6) fruit juices. Y.Zhang *et al*. found 2ppb of λ- Cyhalothrin in apple juice. Arpad Ambrus, (2010) detected 0.05 ppm of λ- Cyhalothrin in ginger paste. Deltamethrin was not detected but in vegetable paste it was detected in ginger garlic paste (1.28 mg/kg) (Table no.7). Arpad Ambrus, (2010) detected 0.05 ppm of Deltamethrin in ginger paste. In guava (0.01 mg/kg), mango (0.04 mg/kg), litchi (0.10 mg/kg), ginger garlic (0.42 mg/kg), Cypermethrin was detected. (Table no.6). Arpad Ambrus, (2010) detected 0.05 ppm of Cypermethrin in ginger paste. The concentration of Fenpropathrin, Fenvalerate and Permethrin were found in range of 0.01-0.08mg/kg. (Table no.6). Arpad Ambrus, (2010) detected 0.05 ppm of Fenvalerate in ginger paste.

The above seven synthetic pyrethoids pesticides which we taken for the study are having the tolerance limits for other group of product like food grains, cotton seed oils and pulses as per food standard and safety regulations, so we cannot correlate with the observed concentration of pesticides w.r.t. fruit juices and vegetable paste.

Table 6: Concentration of Synthetic pyrethoids pesticides in fruit juices & Vegetable Paste

Type of Sample	Fruit Juices									Vegetable Paste	
Sample	Apple	Pomegranate	Tomato	Guava	Mixed Fruit	Mango	Litchi	Cranberry	Orange	Ginger Garlic	Ginger
	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Synthetic Pyrethoids Pesticides											
β-Cyfluthrin	ND	ND	ND	ND	ND	0.03	ND	ND	ND	ND	ND
λ-Cyhalothrin	ND	ND	0.11	ND	ND	ND	ND	ND	ND	0.01	ND
Deltamethrin	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.28	ND
Cypermethrin	ND	ND	ND	0.01	ND	0.04	0.10	ND	ND	0.42	ND
Fenpropathrin	0.01	0.02	ND	ND	ND	ND	0.01	ND	ND	ND	ND
Fenvalerate	ND	0.01	ND	ND	ND	ND	0.01	0.04	0.01	ND	ND
Permethrin	ND	ND	0.08	ND	ND	0.03	ND	ND	ND	0.04	0.02

In other pesticides (OTPs) the Oxyfluorfen and Pyrazosulfuron-ethyl were not detected in any of the fruit juices and vegetable paste. Trifluralin content was found in mango juice (0.49 mg/kg). (Table no.7).The concentrations of Ethofenprox were found in the range of 0.01-0.2 mg/kg in fruit juice but it was not detected in vegetable paste. (Table no.7). The tolerance limit of the Oxyfluorfen, Pyrazosulfuron-ethyl, Trifluralin & Ethofenprox are given for other food products like rice and wheat so, we are not able to compare these concentrations with the existing limits.

Table 7: Concentration of Other pesticides in fruit juices & Vegetable Paste

Type of Sample	Fruit Juices									Vegetable Paste	
Sample	Apple	Pomegranate	Tomato	Guava	Mixed Fruit	Mango	Litchi	Cranberry	Orange	Ginger Garlic	Ginger
	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Other Pesticides											
Oxyfluorfen	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Pyrazosulfuron-ethyl	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

Trifluralin	ND	ND	ND	ND	ND	0.49	ND	ND	ND	ND	ND
Etofenprox	0.11	ND	0.02	ND	ND	0.05	0.20	0.01	0.12	ND	ND
Inorganic Bromide	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Copper Oxychloride	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

The current finding showed that the concentration of Chlorpyrifos, Chlorpyrifos-methyl and Chlorfenvinphos (cis & trans) were noticed in most of the sample including fruit juices and vegetable paste. It clearly indicates the contamination or presence of pesticides in these samples, which we taken for the study. The level of pesticides residues are controlled by Maximum Residue Level (MRLs), which are established by each country. In Brazil the MRLs are established by ANVISA through the Program for Analysis of Pesticide Residue in Food (PARA), started in 2001, which monitors the levels of pesticides in fruits, vegetables, and grains consumed by Brazilians.Since not all the ANVISA's data for these insecticide residues were available during the fruit studies, it was compared with the MRLs established by the European Union (EU) and US. Different MRLs specific values are given for each fruit/vegetable, and the reason for it is perhaps the quantity of daily intake in average.

3.3 Chromatograms of pesticides present in extracted fruit juices and vegetable pastes with Linearity and Fragmentation pattern

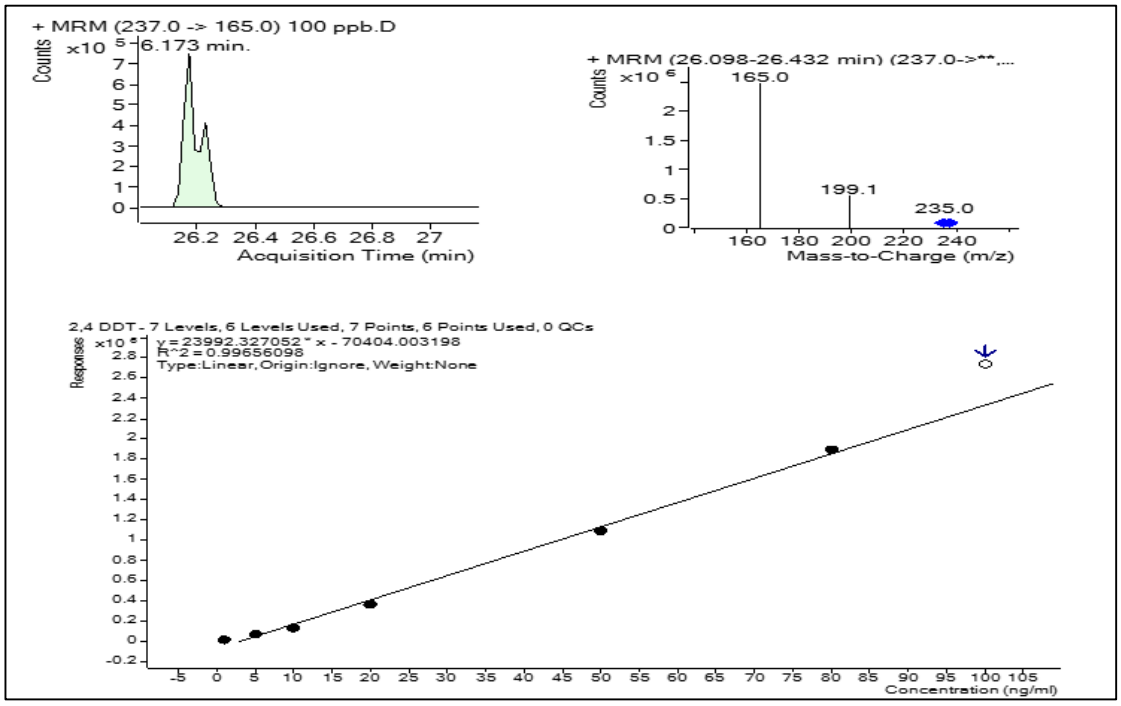


Fig 1: showing the Linearity & Fragmentation pattern for 2, 4 DDT by GC-MS/MS QQQ

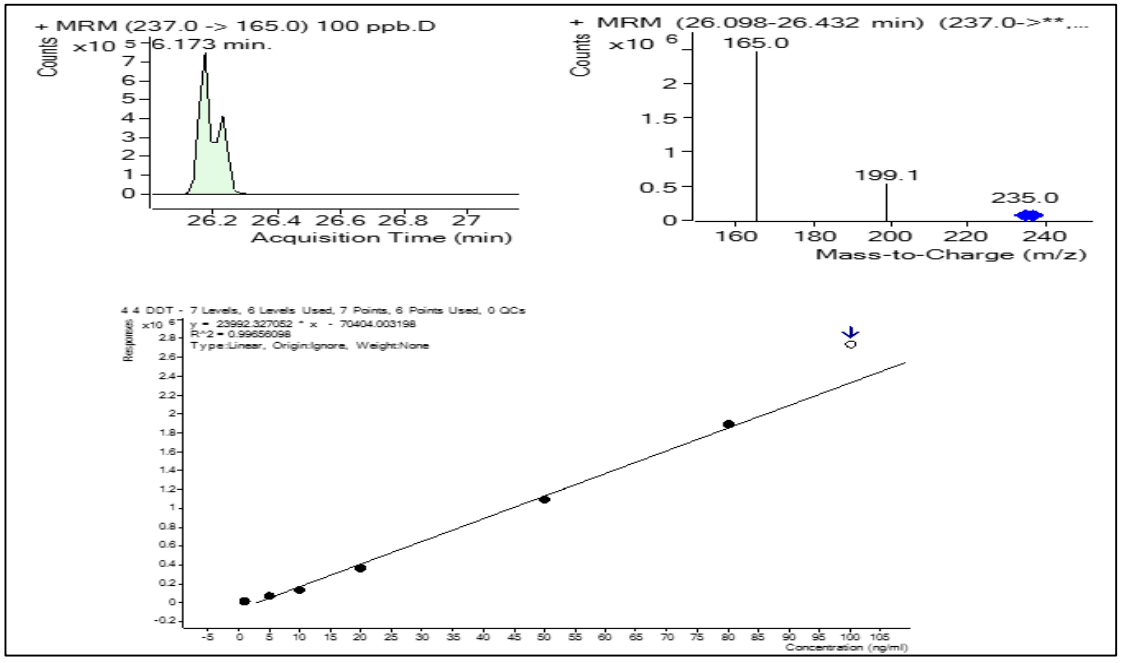


Fig 2: showing the Linearity & Fragmentation pattern for 4, 4 DDT by GC-MS/MS QQQ

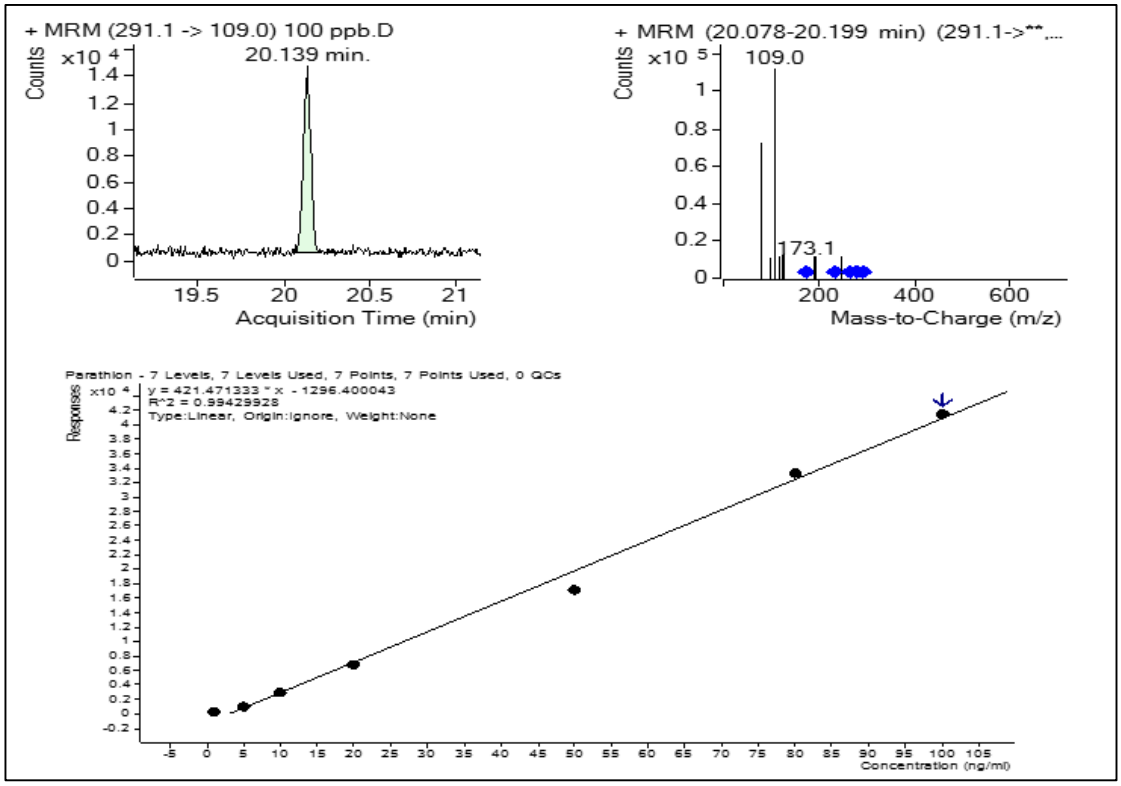


Fig 3: showing the Linearity & Fragmentation pattern for Aldrin by GC-MS/MS QQQ

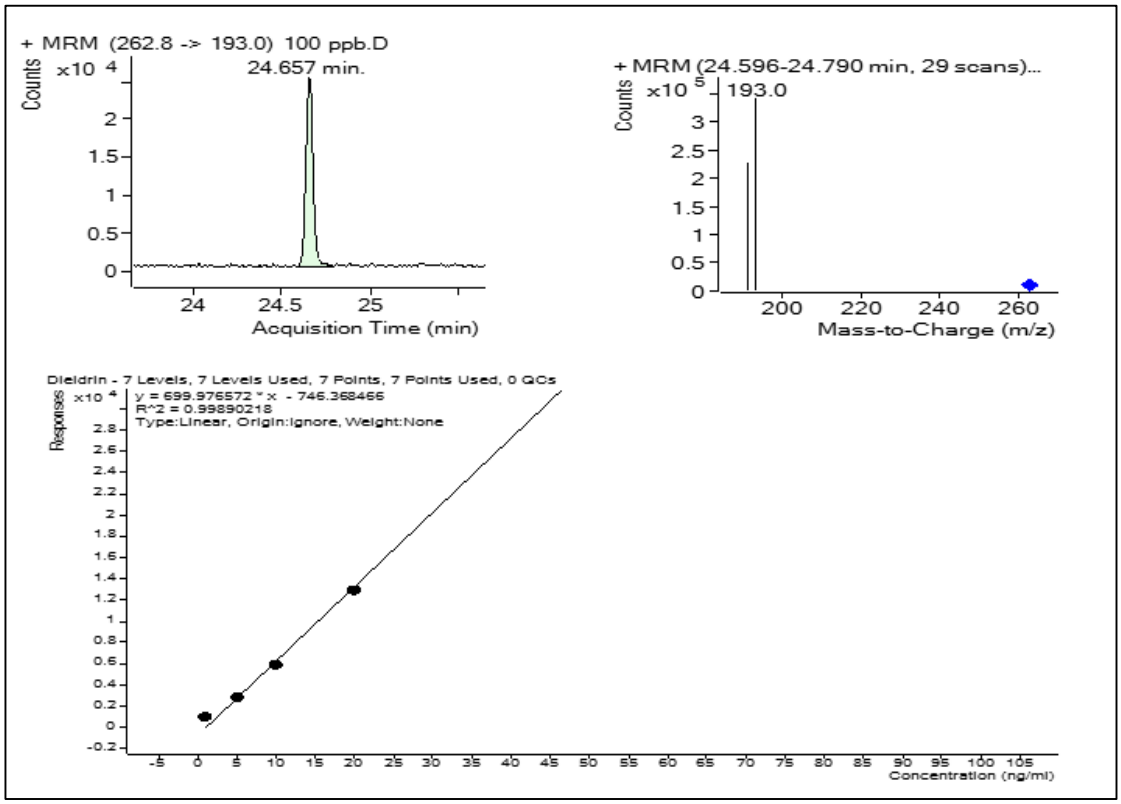


Fig 4: showing the Linearity & Fragmentation pattern for Dieldrin by GC-MS/MS QQQ

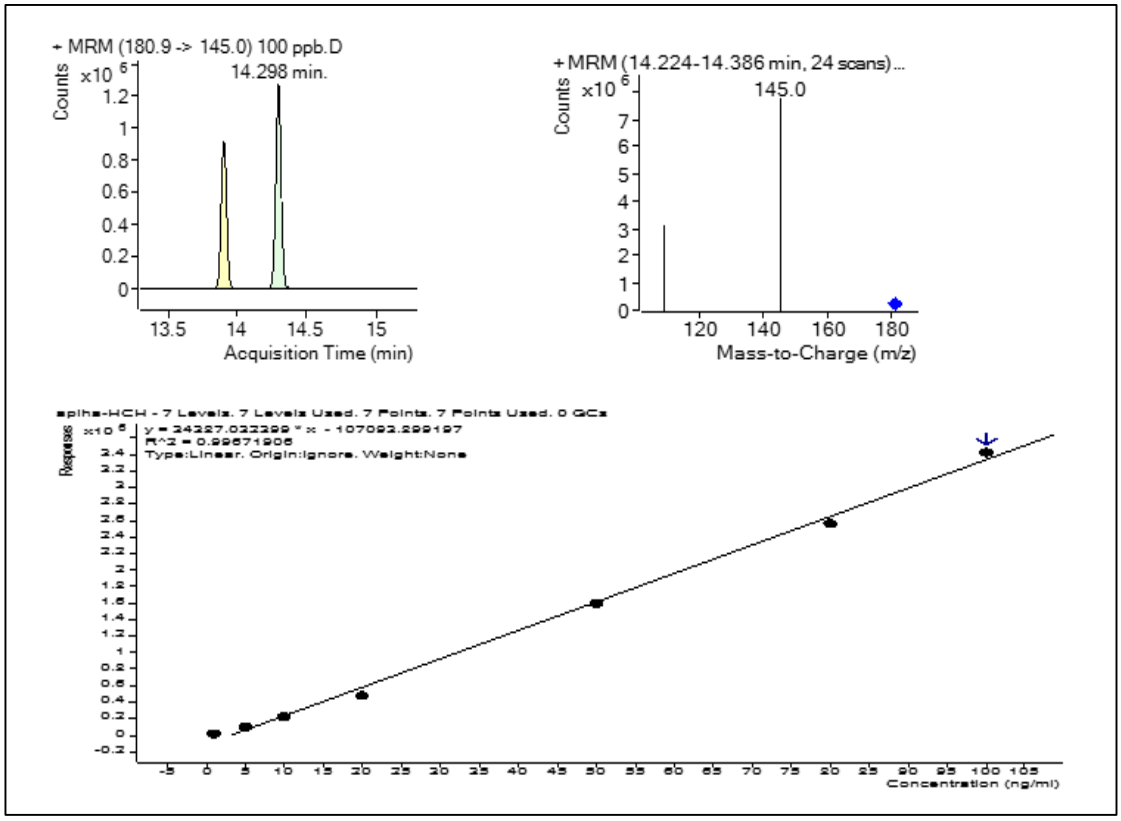


Fig 5: showing the Linearity & Fragmentation pattern for α- HCH by GC-MS/MS QQQ

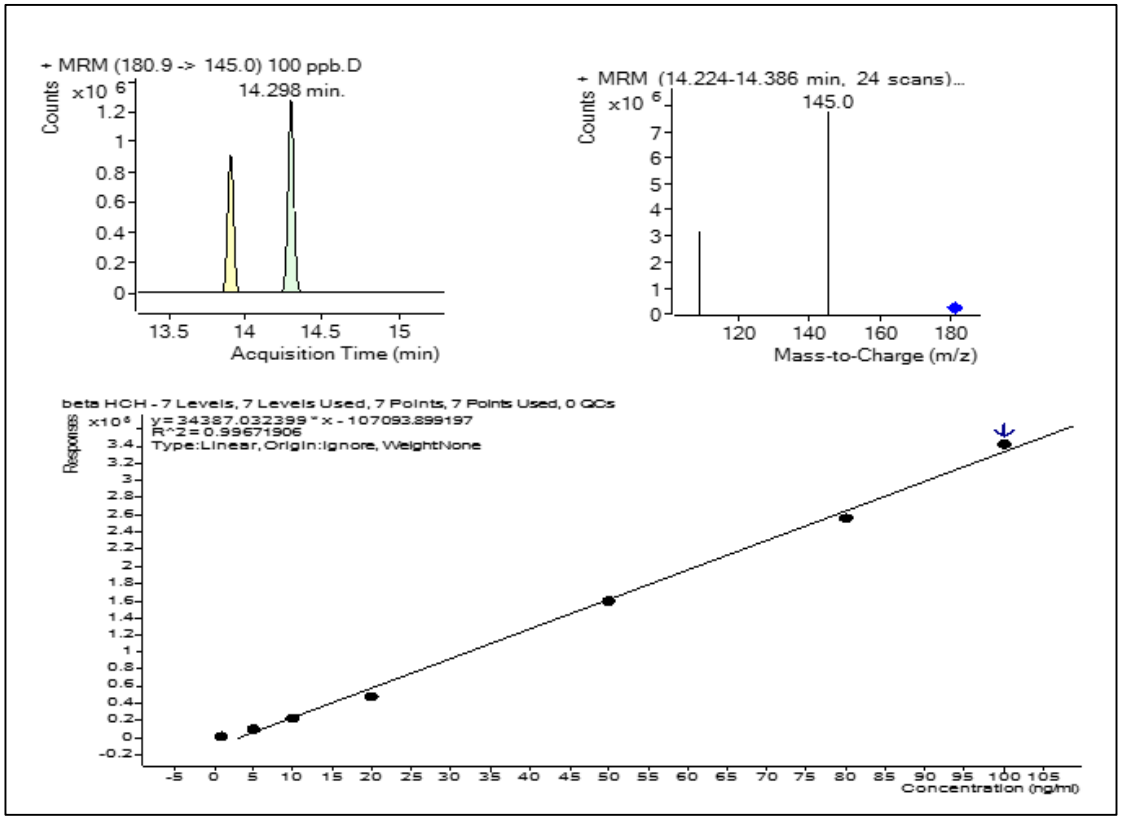


Fig 6: showing the Linearity & Fragmentation pattern for β- HCH by GC-MS/MS QQQ

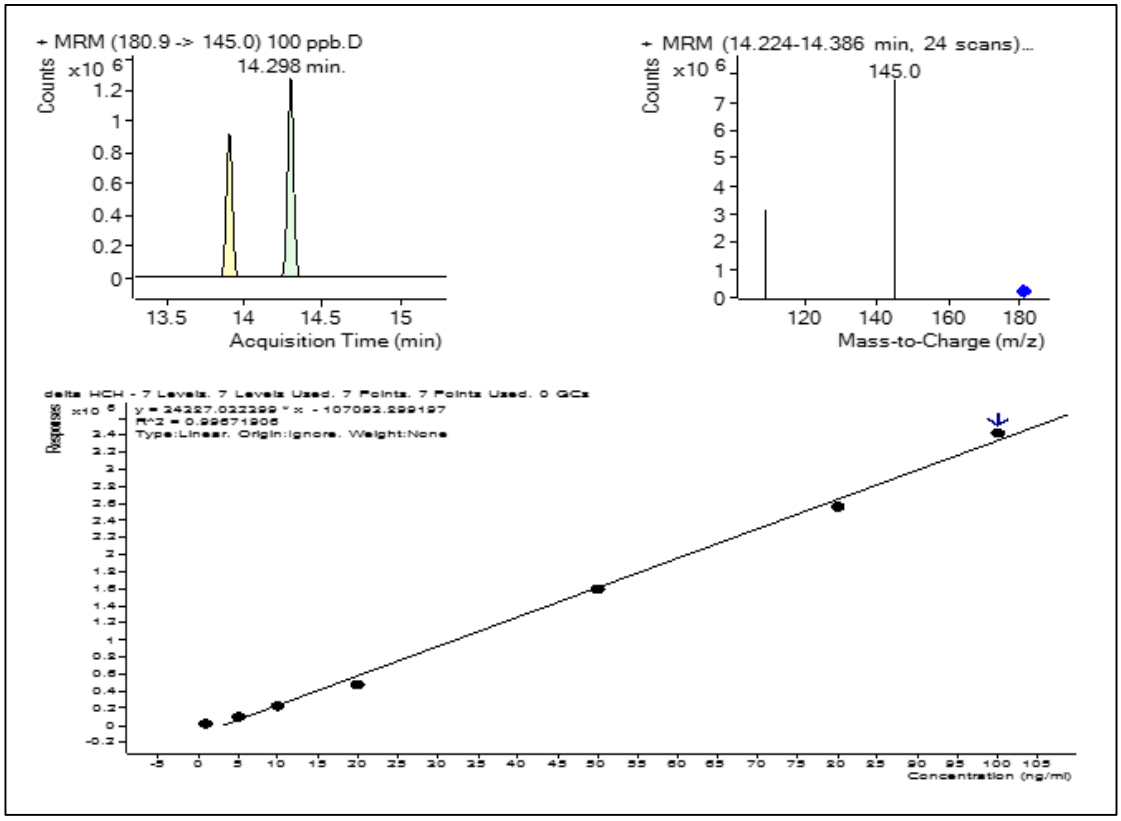


Fig 7: showing the Linearity & Fragmentation pattern for δ- HCH by GC-MS/MS QQQ

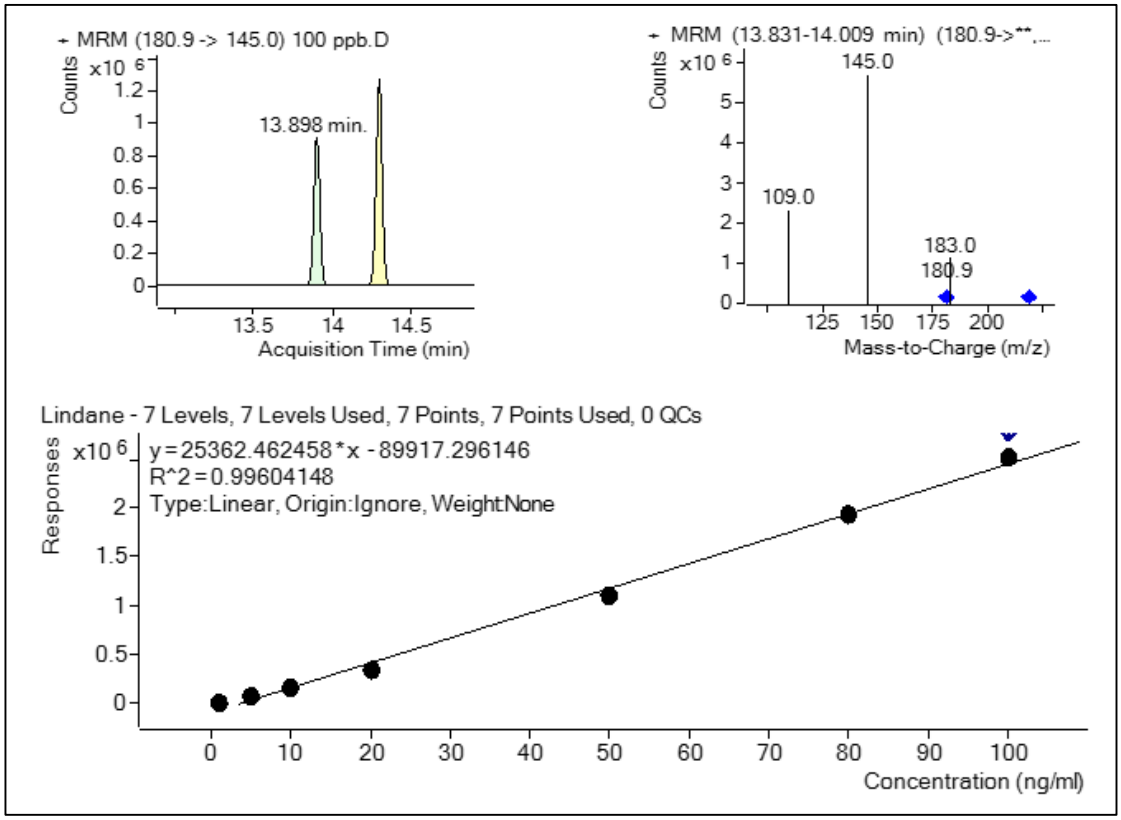


Fig 8: showing the Linearity & Fragmentation pattern for Lindane by GC-MS/MS QQQ

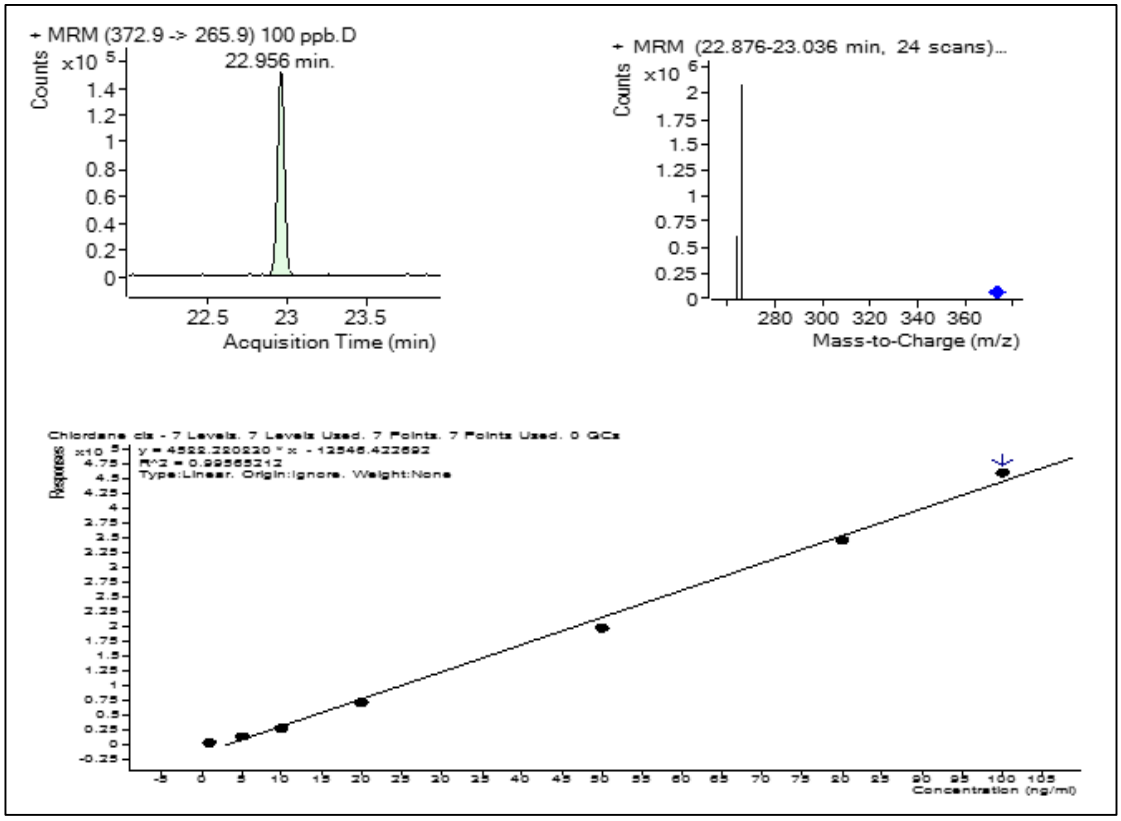


Fig 9: showing the Linearity & Fragmentation pattern for Chlordane –Cis by GC-MS/MS QQQ

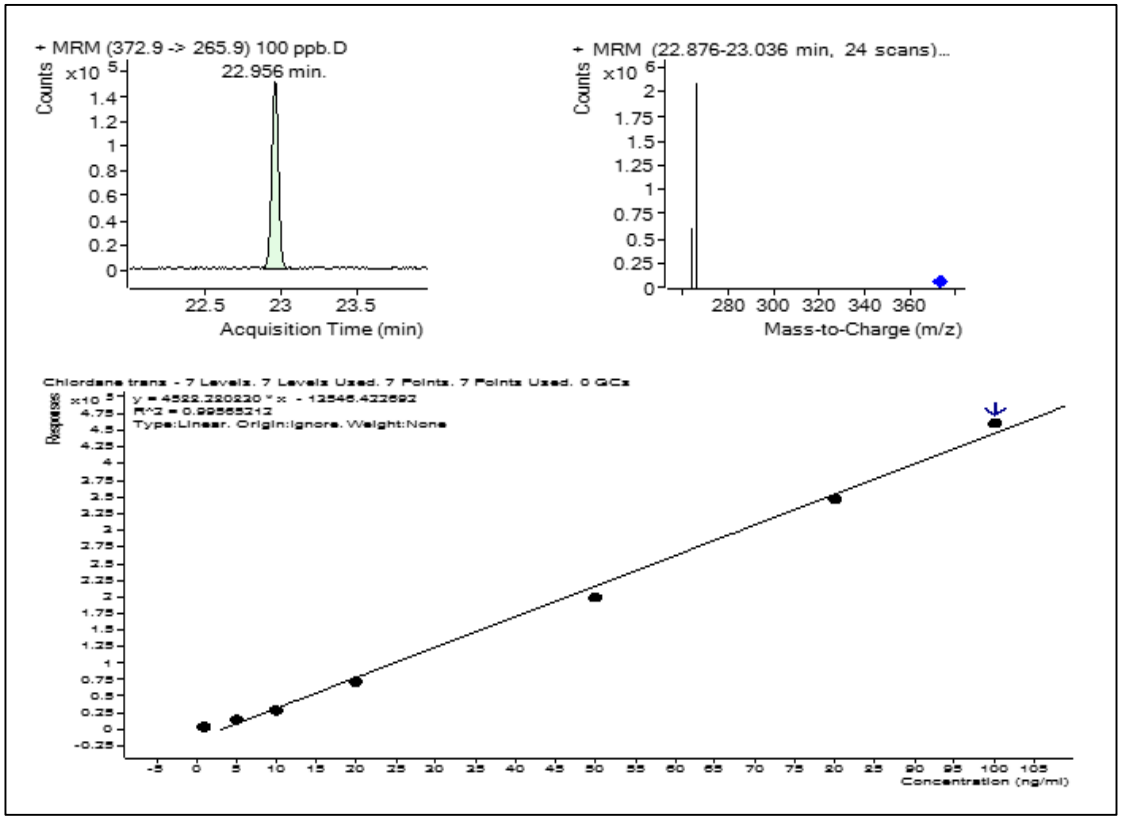


Fig 10: showing the Linearity & Fragmentation pattern for Chlordane –Trans by GC-MS/MS QQQ

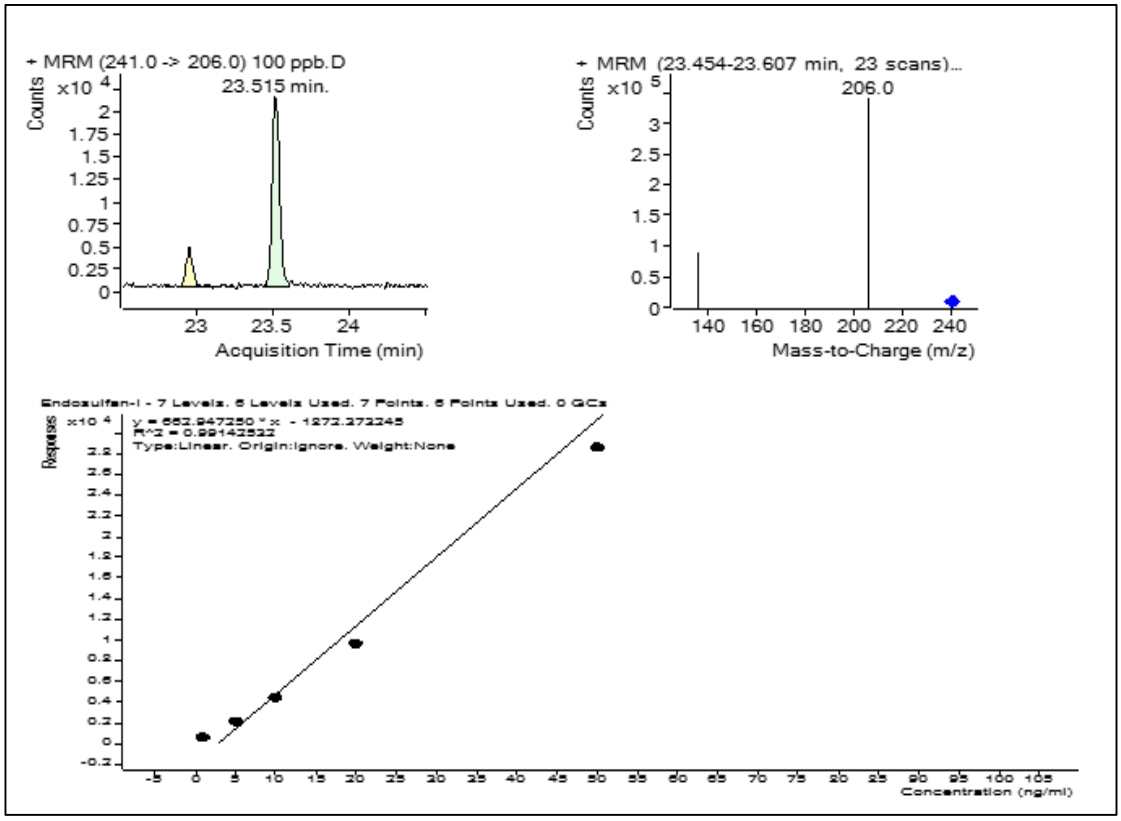


Fig 11: showing the Linearity & Fragmentation pattern for α-Endosulfan by GC-MS/MS QQQ

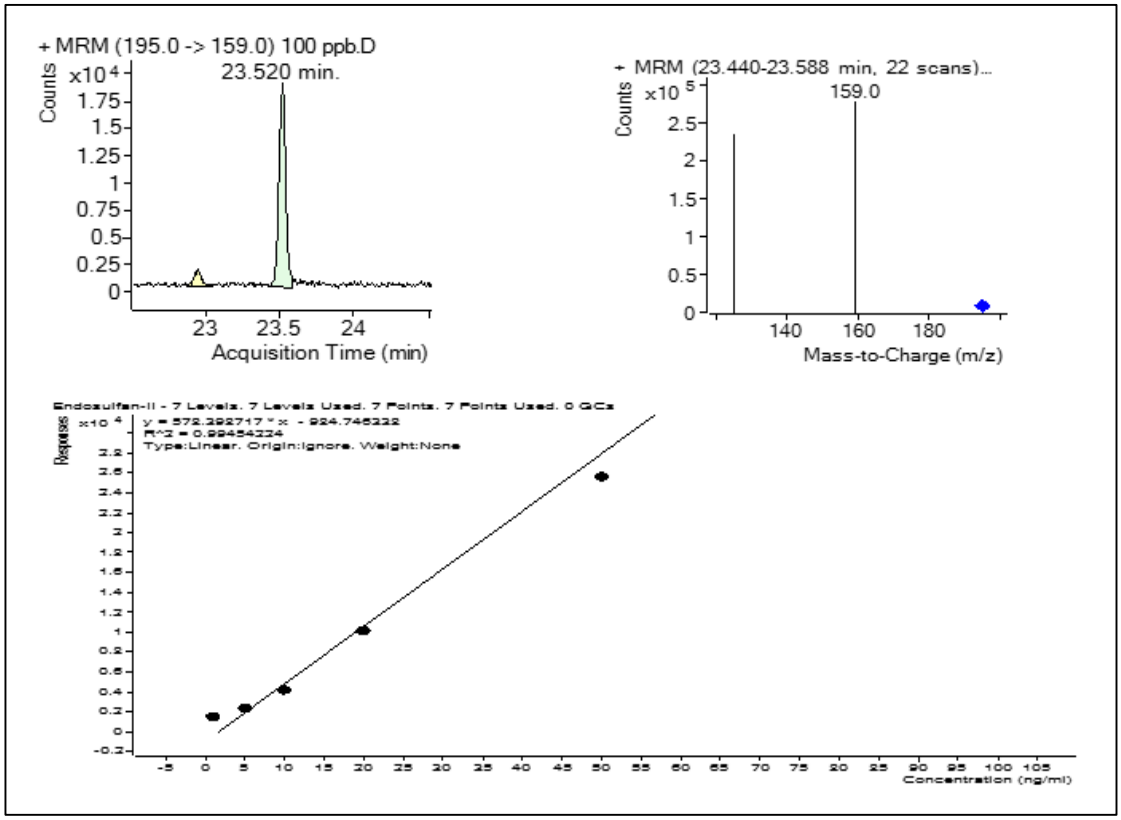


Fig 12: showing the Linearity & Fragmentation pattern for β-Endosulfan by GC-MS/MS QQQ

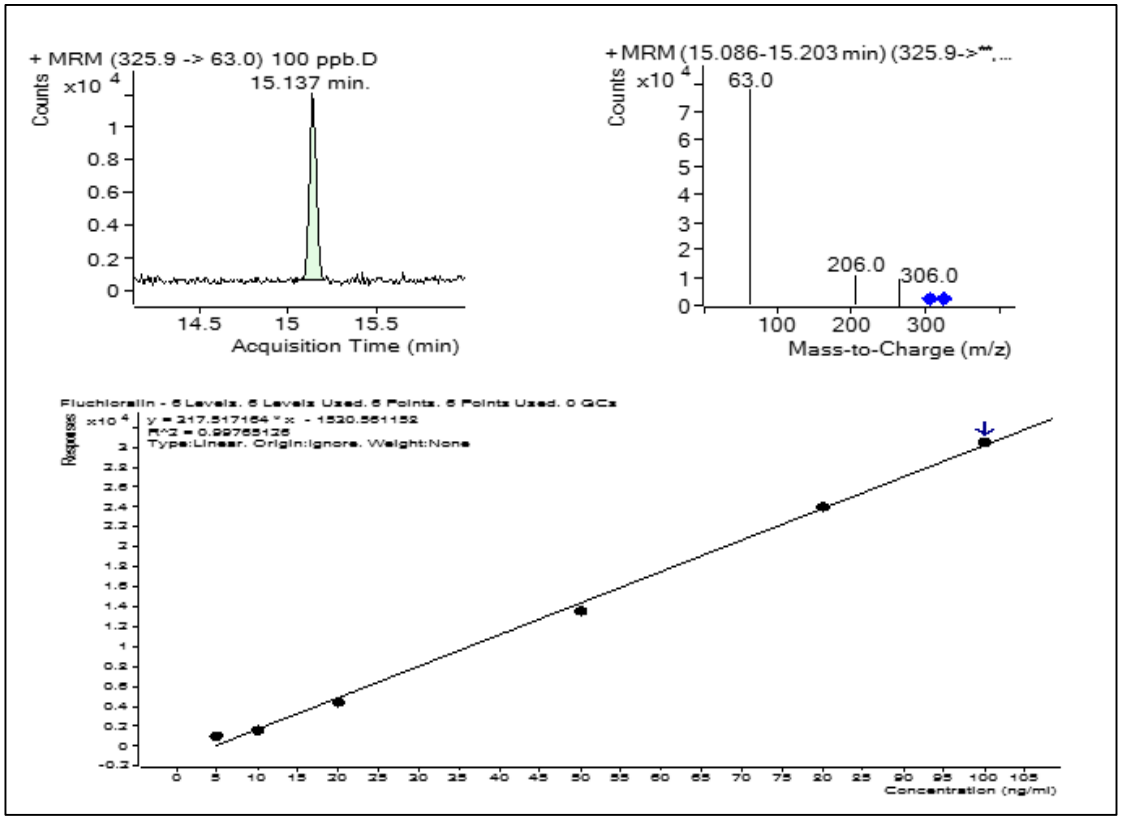


Fig 13: showing the Linearity & Fragmentation pattern for Fluchloralin by GC-MS/MS QQQ

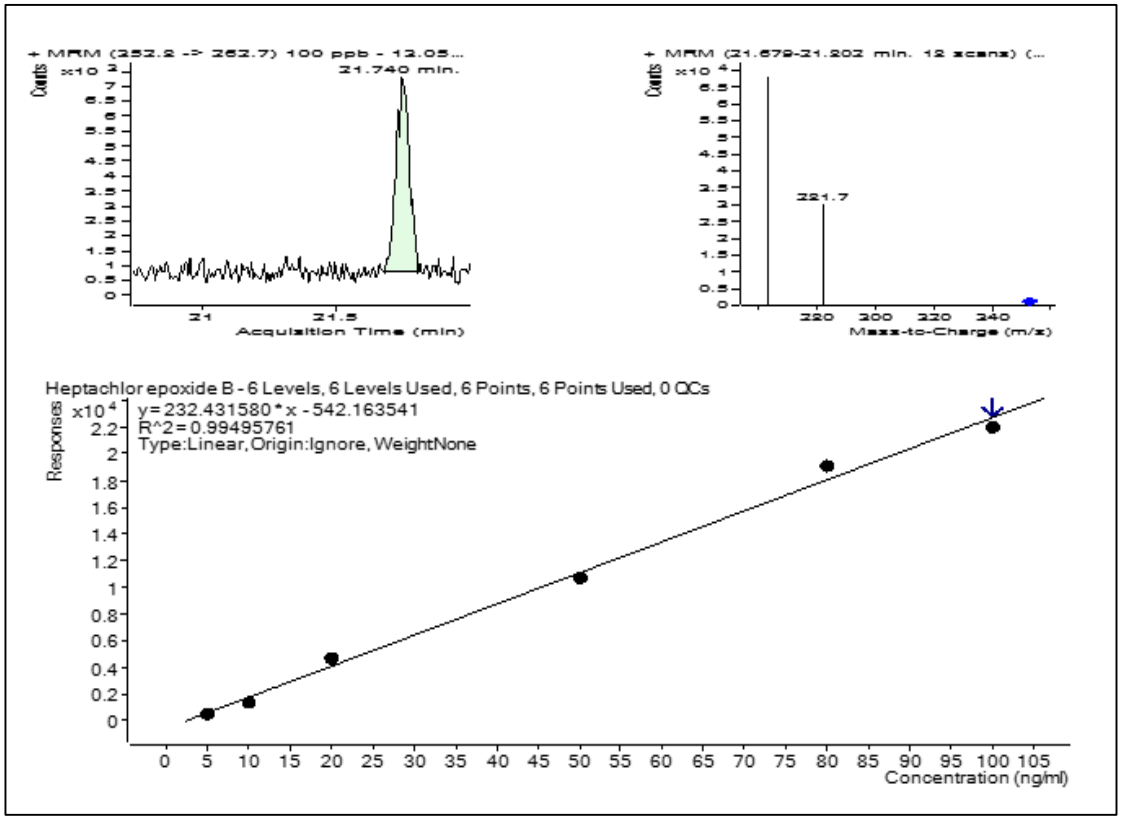


Fig 14: showing the Linearity & Fragmentation pattern for Heptachlor Epoxide Isomer-B by GC-MS/MS QQQ

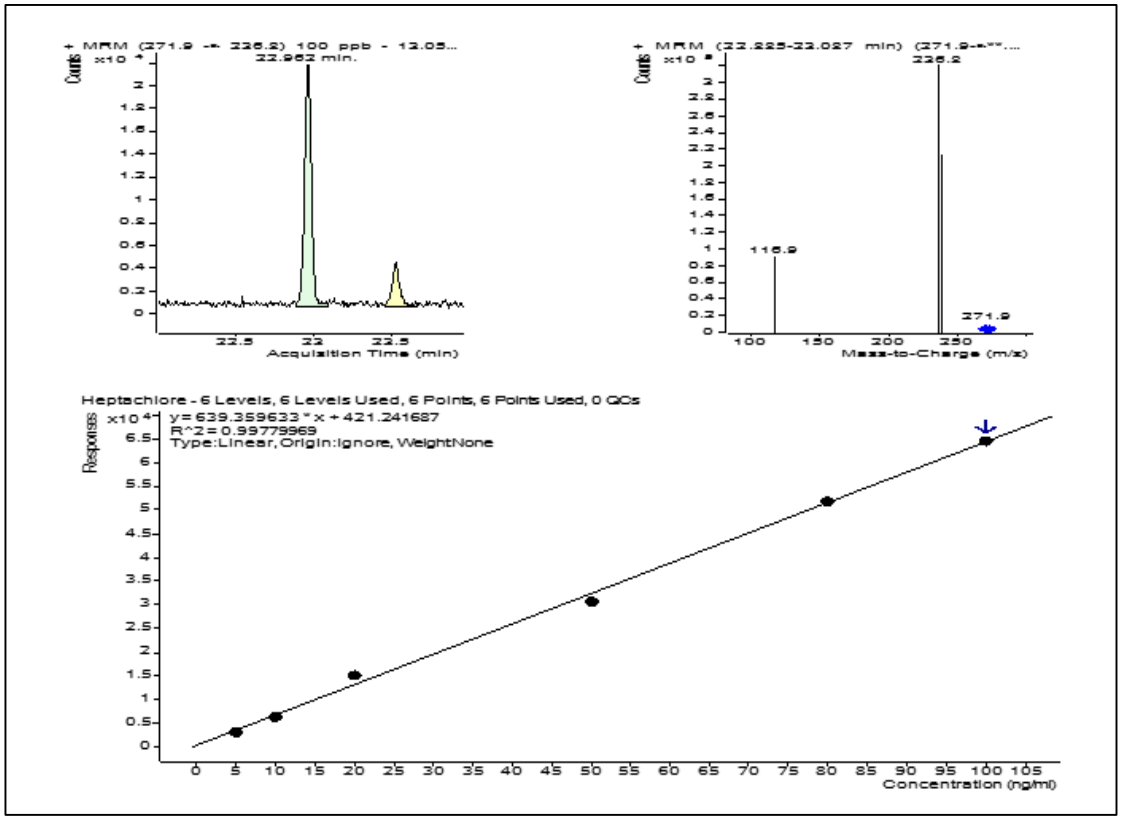


Fig 15: showing the Linearity & Fragmentation pattern for Heptachlor by GC-MS/MS QQQ

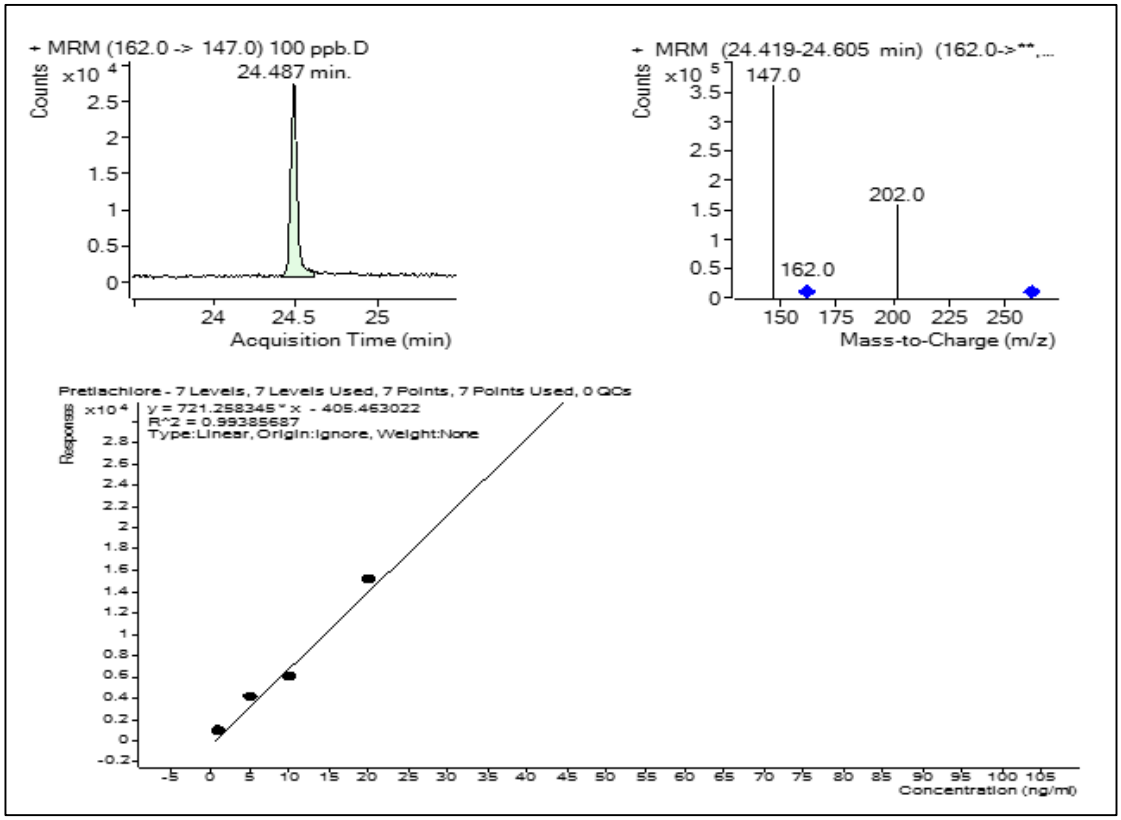


Fig 16: showing the Linearity & Fragmentation pattern for Pretilachlore by GC-MS/MS QQQ

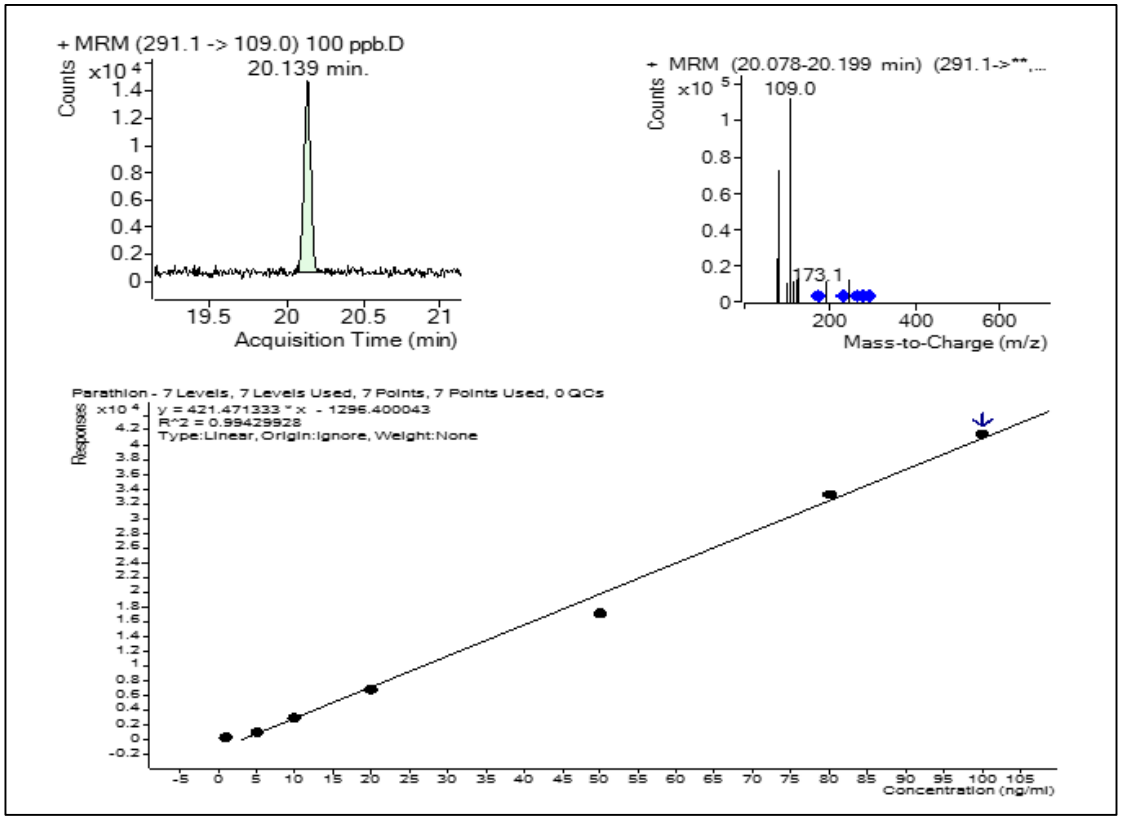


Fig 17: showing the Linearity & Fragmentation pattern for Parathion by GC-MS/MS QQQ

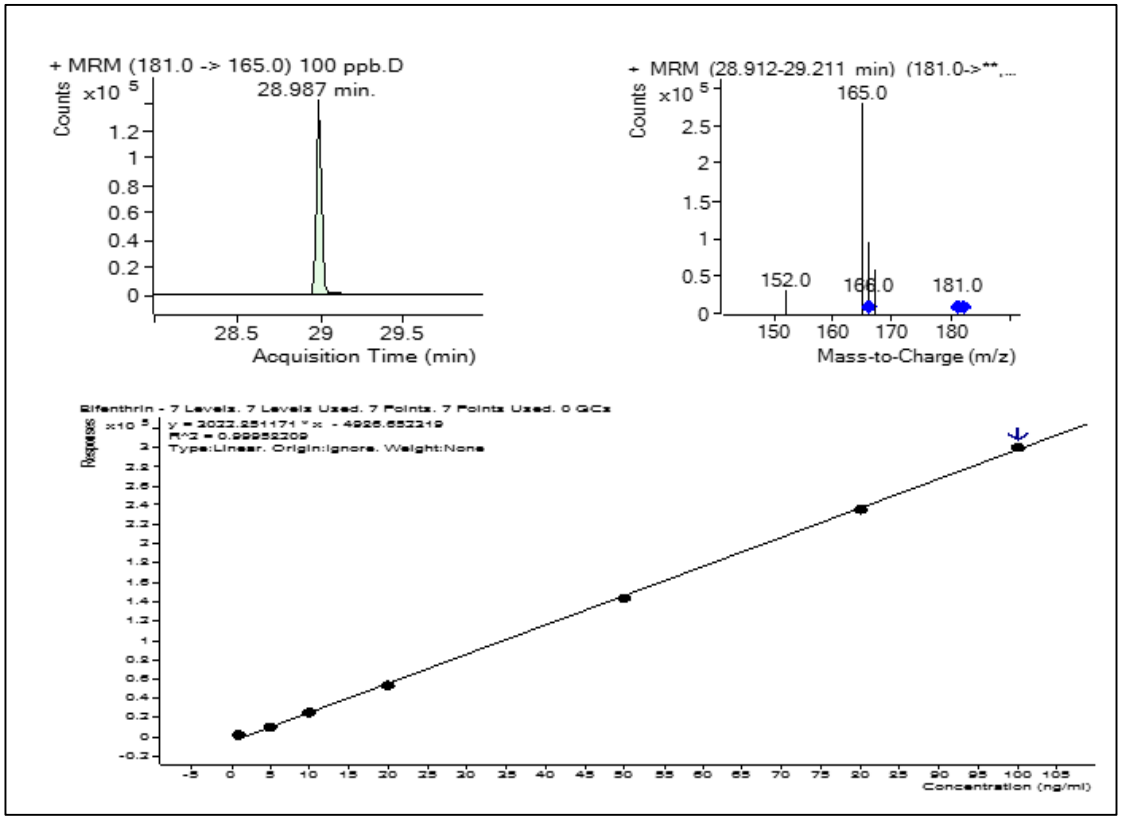


Fig 18: showing the Linearity & Fragmentation pattern for Bifenthrin by GC-MS/MS QQQ

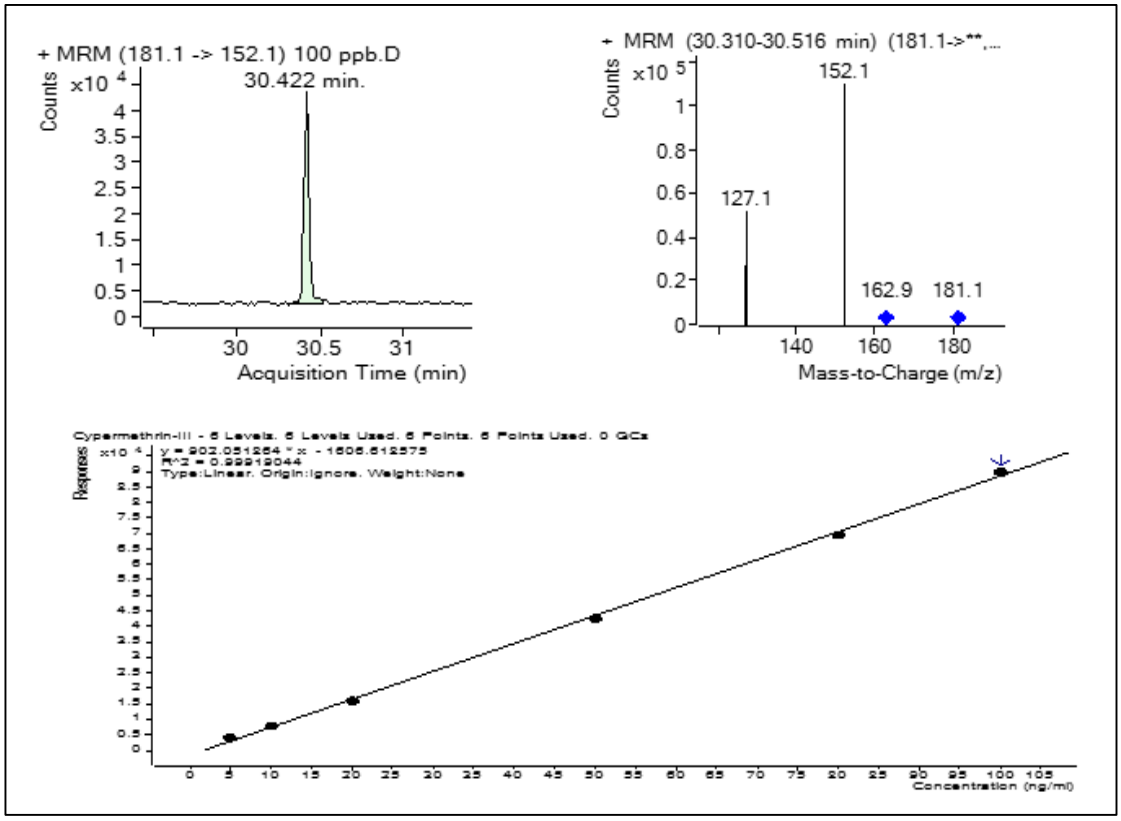


Fig 19: showing the Linearity & Fragmentation pattern for Cypermethrin-III by GC-MS/MS QQQ

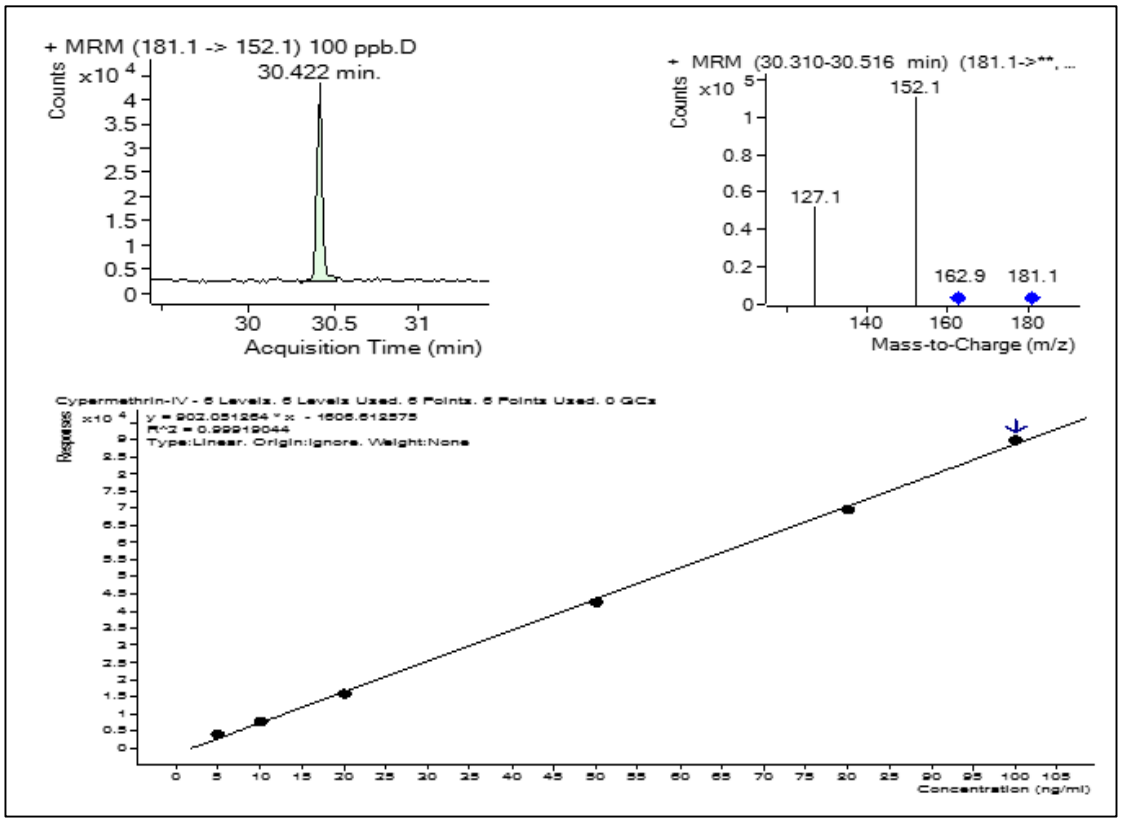


Fig 20: showing the Linearity & Fragmentation pattern for Cypermethrin-IV by GC-MS/MS QQQ

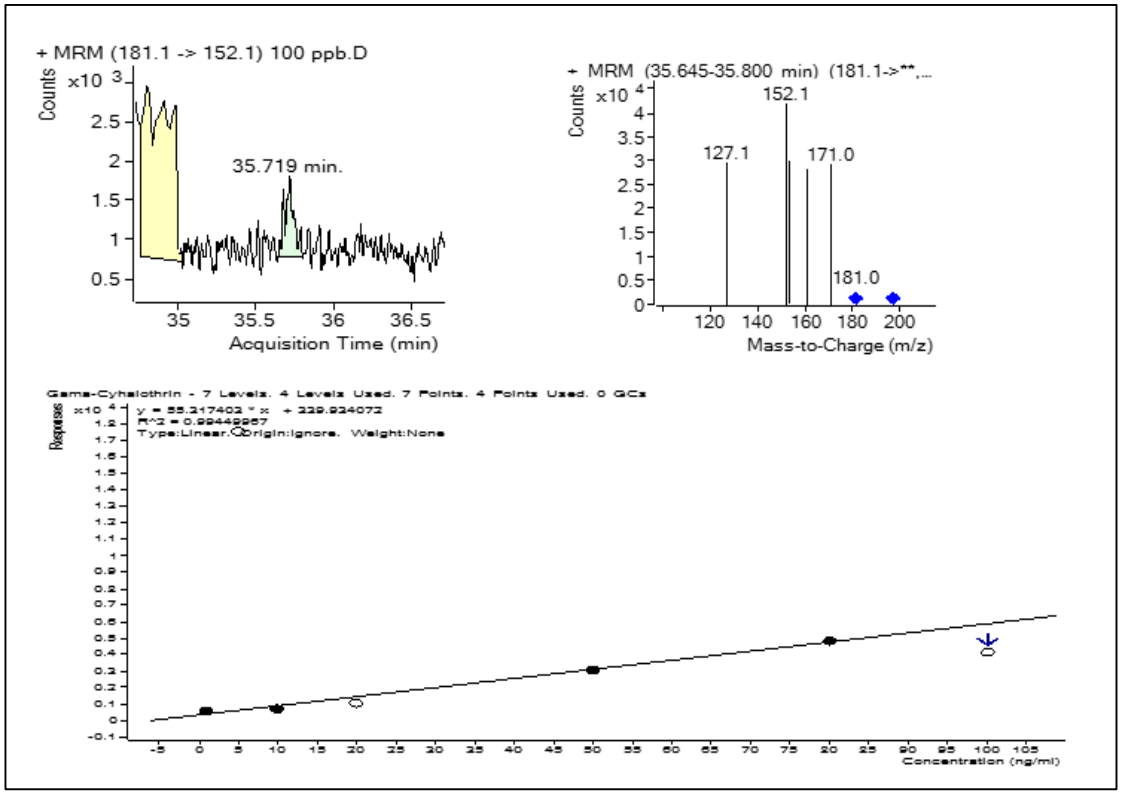


Fig 21: showing the Linearity & Fragmentation pattern for ʎ-Cyhalothrin by GC-MS/MS QQQ

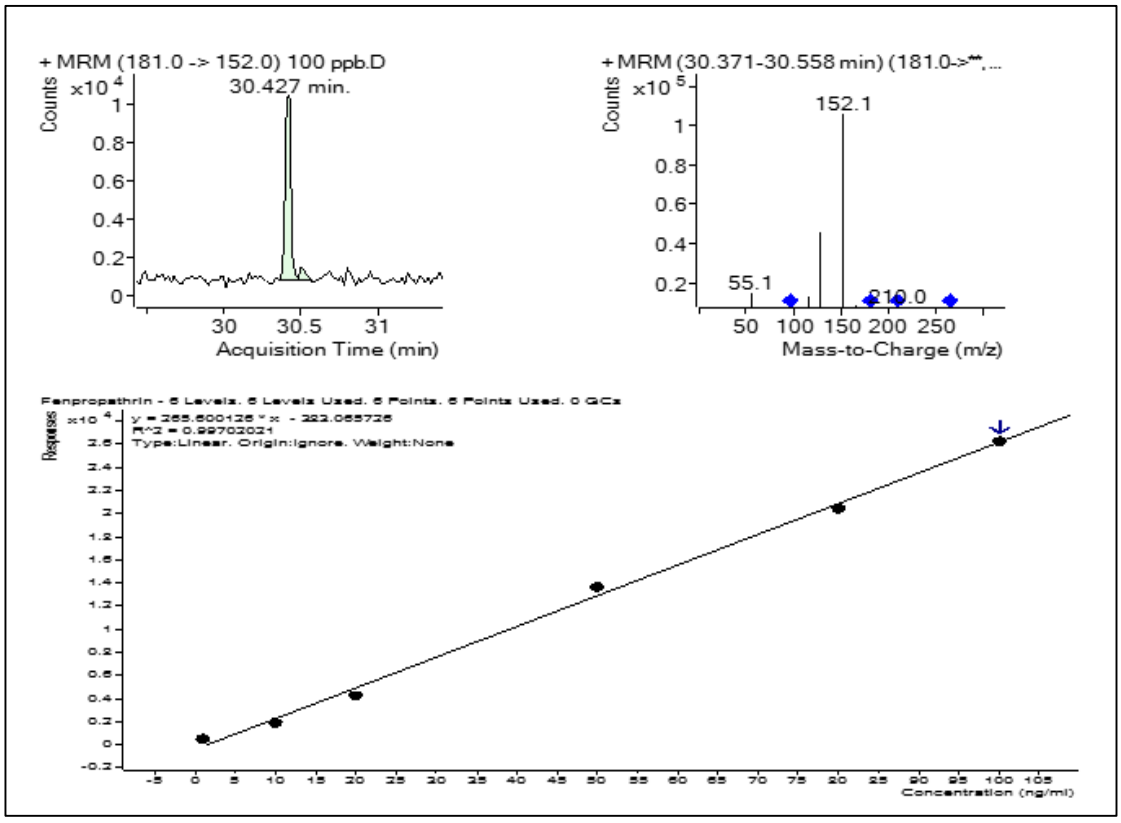


Fig 22: showing the Linearity & Fragmentation pattern for Fenpropathrin by GC-MS/MS QQQ

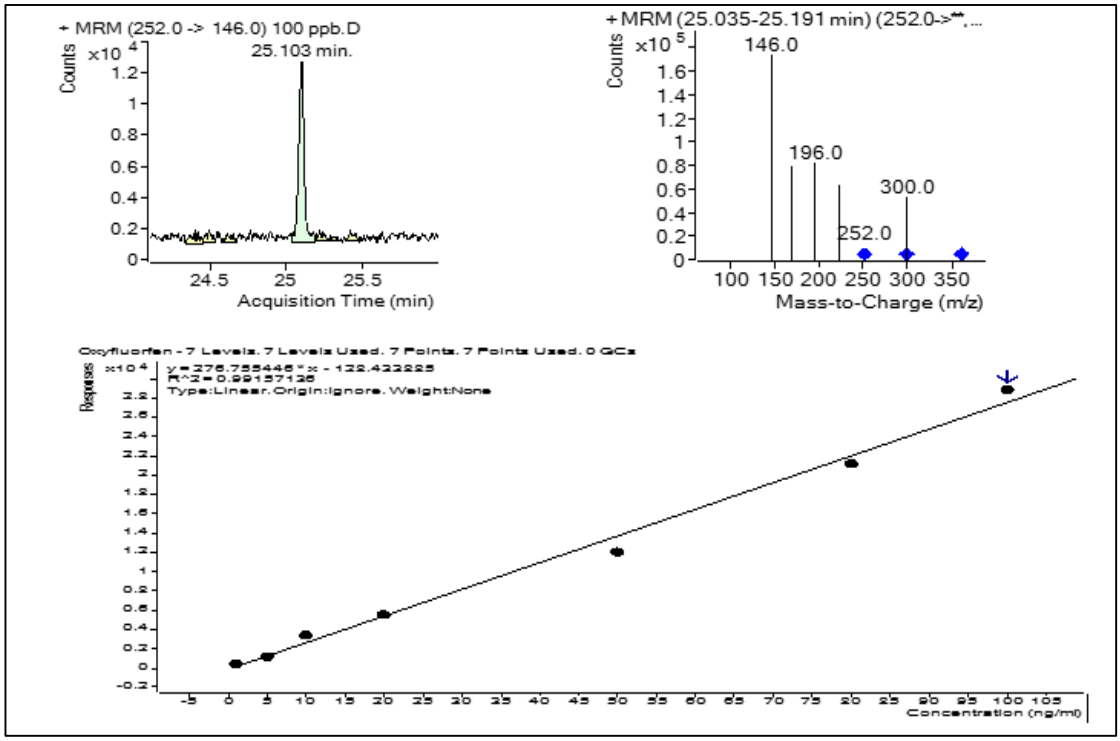


Fig 23: showing the Linearity & Fragmentation pattern for Oxyflorfen by GC-MS/MS QQQ

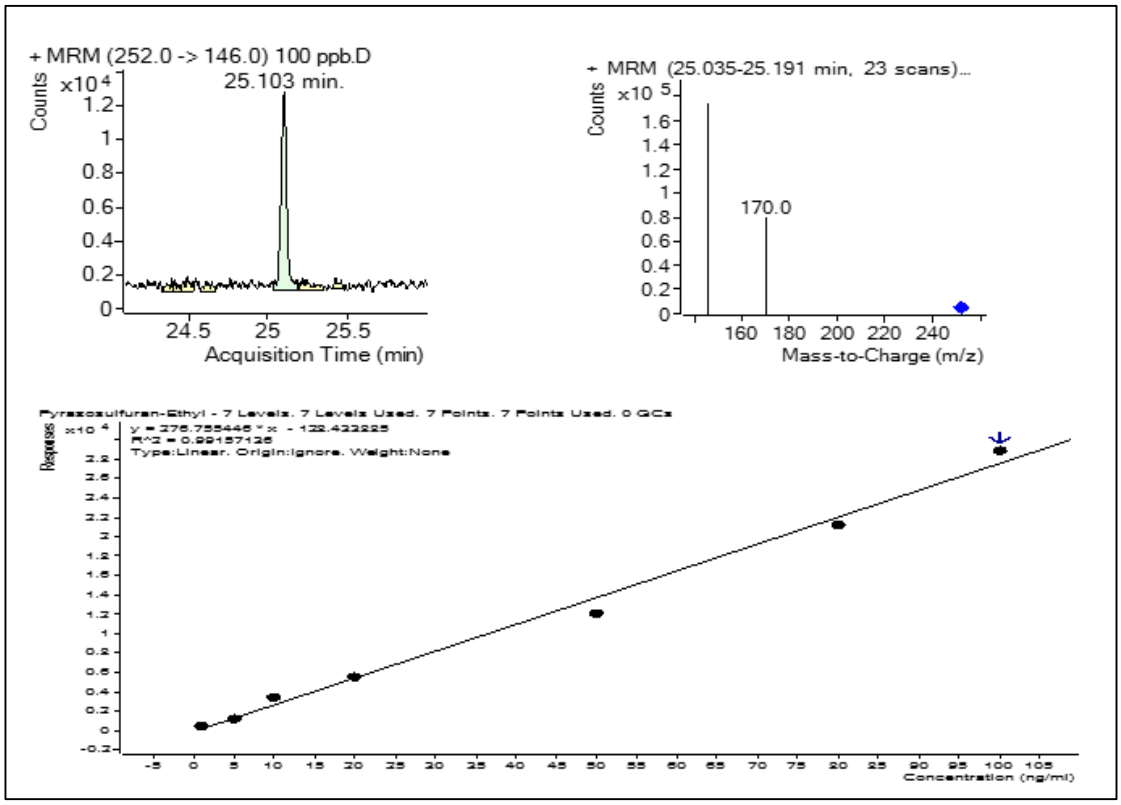


Fig 24: showing the Linearity & Fragmentation pattern for Pyrazosulfron-Ethyl by GC-MS/MS QQQ

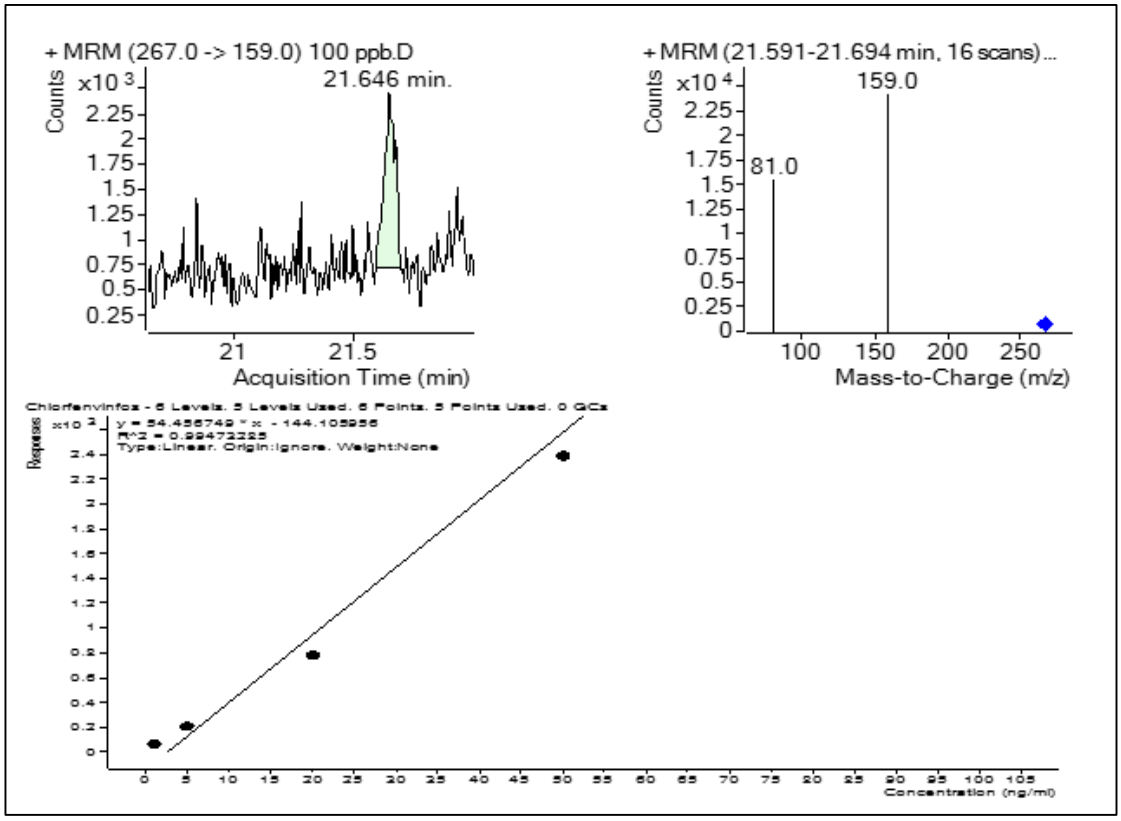


Fig 25: showing the Linearity & Fragmentation pattern for Chlorfenvinphos by GC-MS/MS QQQ

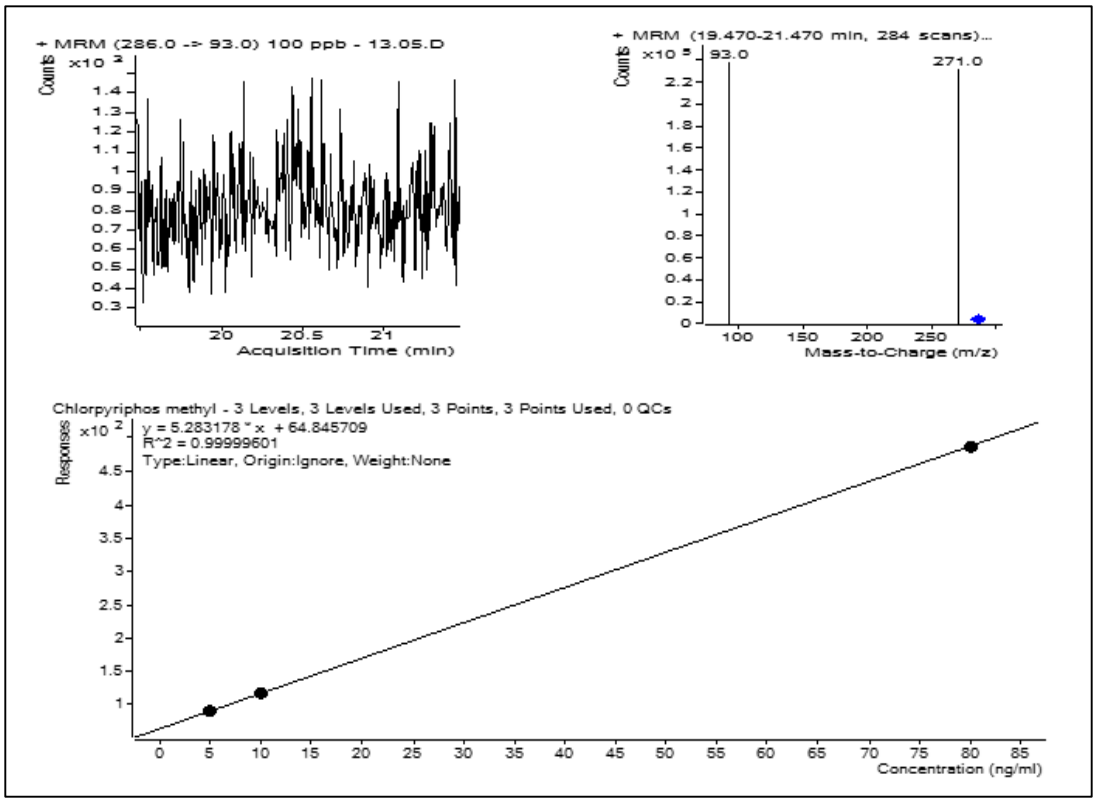


Fig 26: showing the Linearity & Fragmentation pattern for Chlorpyriphos-methyl by GC-MS/MS QQQ

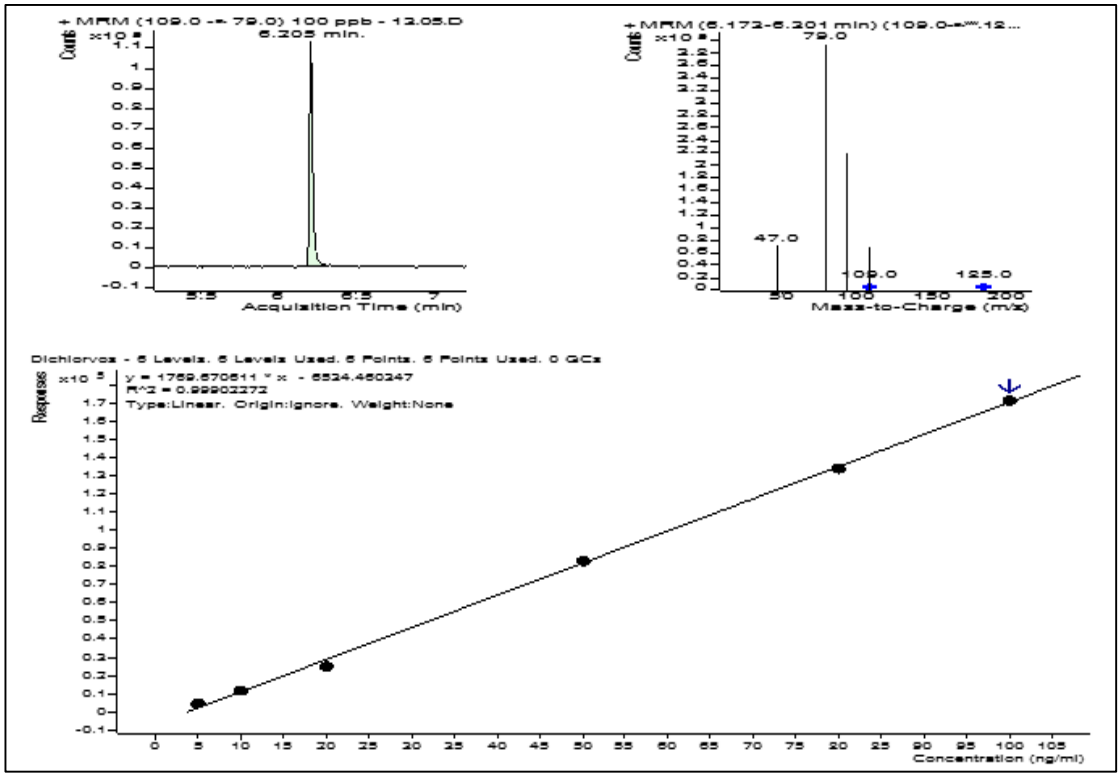


Fig 27: showing the Linearity & Fragmentation pattern for Dichlorvos by GC-MS/MS QQQ

4. Conclusion

This work demonstrates the ability to determine pesticides in fruit juices and vegetable pastes using the validated QuEChERS method for sample preparation and gas chromatograph attached with mass spectrometer in the positive ion mode used for analysis. The QuEChERS sample preparation is suitable for determination of several classes of pesticide residues in matrices with high sugar content, more lipids and steroids contents.This method is considered as a flexible, sample preparation concept based on application of different solvents, salts, buffers and sorbents. The simultaneous analysis can be performed for hundreds of pesticides using GC-MS/MS. None of the pesticides were detected in the samples. All pesticides were found below the tolerance level (0.01 – 1.0 mg/Kg). In this manner it can be assume that there is no apparent risk to the consumers. The analytical procedure provides accurate results and it is applicable for routine analysis of many other fruits and vegetable matrices. The large number of pesticides belonging to different chemical classes can be analyzed.

5. Acknowledgement

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6. References

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