



Inspiring Trust, Assuring Safe & Nutritious Food



FOOD SAFETY AND STANDARDS  
AUTHORITY OF INDIA



भातृअनुषु  
ICAR



रा अं अनुके  
NRCG

## **“TRAINING OF TRAINERS” (TOT)**

### **Program on Analysis of pesticide residues**

*May 2017 at ICAR-NRCG, Pune*

# **TRAINING MANUAL**

### **Organized by**

***Food Safety and Standards Authority of India (FSSAI), New Delhi***  
(Ministry of Health & Family Welfare, Government of India)

In collaboration with

***ICAR-National Research Center for Grapes (NRCG), Pune***  
(Under Indian Council of Agricultural Research, New Delhi)

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Food Safety and Standards Authority of India  
Ministry of Health and Family Welfare, Government of India

**Training Manual**  
**For**  
**Analysis of Pesticide Residues**

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## 1. Sampling, transport, traceability and Storage of laboratory samples

**Sampling:** Sampling is a very important activity. A sample has to be representative of the population, whether it is carried out at pre- or post-harvest level. Sampling should be done in a randomized way in accordance with international guidelines, e.g. European Commission Directive 2002/63/EC or similar.

**Transport:** Samples must be transported under appropriate conditions to the laboratory in clean containers and robust packaging. Polythene or polypropylene bags, ventilated if appropriate, are acceptable for most samples but low-permeability bags (e.g. nylon film) should be used for samples to be analyzed for residues of fumigants. Samples of commodities pre-packed for retail sale should not be removed from their packaging before transport. Very fragile or perishable products (e.g. ripe raspberries) may have to be frozen to avoid spoilage and then transported in “dry ice” or similar, to avoid thawing in transit. Samples that are frozen at the time of collection must be transported without thawing. Samples that may be damaged by chilling (e.g. bananas) must be protected from both high and low temperatures. Rapid transport to the laboratory, preferably within one day, is essential for samples of most fresh products. The condition of samples delivered to the laboratory should approximate to that which would be acceptable to a discerning purchaser; otherwise samples should be considered as unfit for analysis.

**Traceability:** Samples must be identified clearly and indelibly, in a way to ensure traceability. The use of marker pens containing organic solvents should be avoided for labelling bags containing samples to be analyzed for fumigant residues, especially if an electron capture detector is to be used. On receipt, each laboratory sample must be allocated a unique code by the laboratory.

**Storage:** Laboratory samples which are not analyzed immediately should be stored under conditions that minimize decay. Fresh products should be stored in the refrigerator, but typically no longer than 5 days. Dried products may be stored at room temperature, but if storage time is expected to exceed two weeks, they should be sub-sampled and stored in the freezer.

**Reference: Sante/11945/2015**

## 2. Preparation of standards

### Preparation of Stock Solution

- Choose a clean container (glass/polypropylene bottle as required) of appropriate capacity for reference standard stock solution preparation.
- Ensure the precision balance is clean and calibrated.
- Put the container on precision balance weighing pan.
- Adjust the zero reading on the balance.
- Take the reference standard and note down description (e.g. Purity and expiry date).
- Weigh the standard (approx 10mg) in standard preparation bottle.
- Weigh suitable solvent (e.g. approx 5 to 10 g ethyl acetate, acetonitrile, methanol or water) to dissolve the weighed amount of standard.
- Specific gravity for ethyl acetate (0.89 g/mL), acetonitrile (0.786 g/mL), methanol (0.79 g/mL) and water (1.0 g/mL)
- Sonicate/ vortex the standard for proper solubility.
- Calculate the concentration of the standard in  $\mu\text{g/mL}$ .
- Weight of standard (corrected, a) =  $[\text{weight of standard} \times \text{Purity}] / 100$
- Volume of solvent (mL, b) =  $[\text{weight of solvent} / \text{Specific gravity}]$
- Calculated conc. ( $\mu\text{g/mL}$ ) =  $[\text{Corrected weight of standard (a)} / \text{Volume of solvent (b)}] \times 1000$
- Label the container with lab code, name of chemical, concentration in  $\mu\text{g/mL}$ , name of solvent, date of preparation, date of expiry.
- Enter the data in the respective logbook and registers.

## Flow chart of preparation - standard solutions

(w/w method –based on weight of solvent)

Ensure that the balance is calibrated



Put empty vial with cap on balance



Tare the balance



Transfer about 10 mg of standard powder/liquid in vial



Note down weight of standard



Tare the balance



Transfer about 10 mL of solvent in vial



Note down weight of solvent



Calculate concentration of stock solution

**Calculate the concentration of the standard in  $\mu\text{g/mL}$**

Weight of standard (corrected, a) = [weight of standard x Purity]/ 100

Volume of solvent (mL, b) = [weight of solvent / Specific gravity]

Calculated conc. ( $\mu\text{g/mL}$ ) = [Corrected weight of standard (a) / Volume of solvent (b)]\*1000

## Flow chart of preparation - standard solutions

(w/v method – based on volume of solvent)

Ensure that the balance is calibrated



Put empty 10 mL volumetric flask on balance



Tare the balance



Transfer about 10 mg of standard powder/liquid in volumetric flask



Note down weight of standard



Transfer solvent and make up the volume up to mark (10 mL)



Calculate concentration of stock solution

**Calculate the concentration of the standard in  $\mu\text{g/mL}$ .**

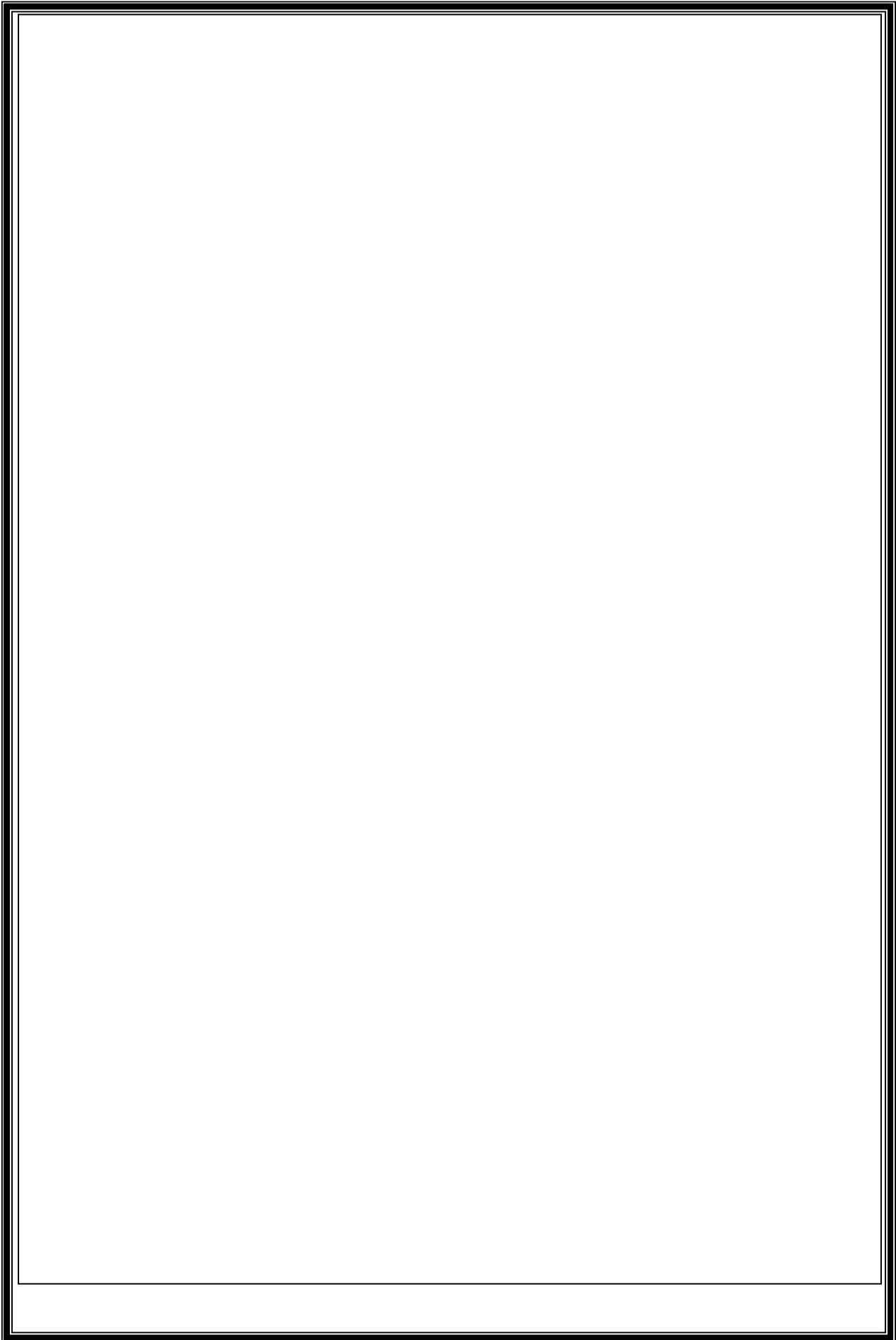
Weight of standard (corrected, a) = [weight of standard x Purity]/ 100

Solvent (mL, b) = Volume of solvent in mL

Calculated conc. ( $\mu\text{g/mL}$ ) = [Corrected weight of standard (a) / Volume of solvent (b)] \* 1000

**Preparation of Pesticide CRM stock, intermediate and calibration standards**

**Lab work notes**



### 3. Pesticide residue analysis in fruits & vegetables using QuEChERS method

#### Introduction

The analysis of pesticide residues in food samples, particularly in fruits and vegetables is increasingly becoming an important activity. Pesticides residue laboratories are required to undertake analyses of an ever increasing number of samples. The analyses typically involve use of multi-residue methods (both GC-MS and LC-MS) to test for over 500 pesticides residues. Extraction is a fundamental process in pesticide residue analysis. Extraction may be defined as the procedure or step adopted to isolate the pesticides from the samples. This is accomplished by employing suitable solvents which remain in contact with the sample for a specific duration. Extraction and clean-up methods vary greatly with the matrix used. It largely depends on the moisture, fat and sugar contents of the commodity. The quantity of sample to be taken for extraction will depend upon the expected levels of residues as well as the sensitivity of technique or tool employed in the subsequent step of identification and quantification.

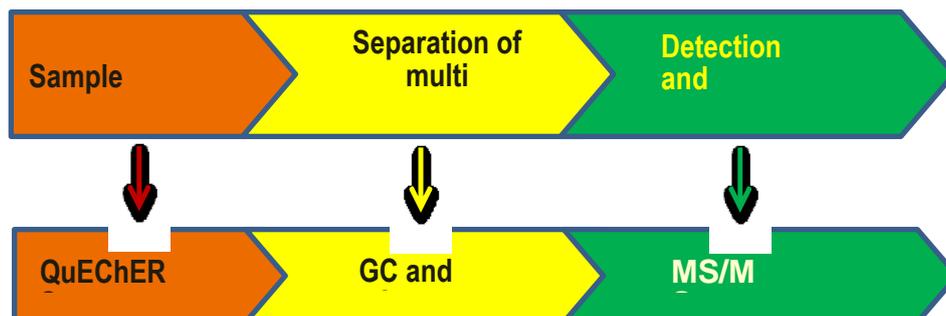
An ideal extraction procedure should be capable of extracting 100% of pesticides only, leaving behind other materials. However, this is not the actual practice. An extraction method should satisfy the following requirements:

- Rapid
- Small solvent usage
- Good recovery of the target pesticides: 70 to 120%.
- Robust: RSD (relative standard deviation) of the recoveries less than 20% at LOQ.

Due to its ease of use and proven robustness, the QuEChERS extraction has become the method of choice for pesticide multi-residue analyses in wide range of matrices.

This course is designed to introduce the concept of method development, and we will start by showing the existing method of pesticide sample preparation. Fortified and blank samples will be utilized to validate the analytical method. Liquid and gas chromatography, coupled with triple quadrupole (TQD) mass spectrometry are used to analyze pesticides.

#### The three steps in Pesticide residue analysis method



## **Sample preparation: QuEChERS(AOAC Official Method)**

The **QuEChERS** is a simple sample preparation technique suitable for multi-pesticide residue analysis in a diverse variety of food and agricultural products. This method is a streamlined approach that makes it easier and less expensive for analytical chemists to examine pesticide residues in food. Once the samples are extracted into the solvent they will be further processed for separation by Gas chromatography (GC) or Liquid chromatography (LC). The GC and LC separation methods are then linked with mass spectrometric detection.

The procedure steps are summarized below:

**Objective:** Participants will review and practice sample preparation using the **QuEChERS** method

### **Materials requirement for sample preparation**

1. Fruits and vegetables are the target food products for this session.
2. **Equipment:**
  - Blender or Homogenizer (High volume)
  - Homogenizer (High volume)
  - Centrifuge: Rotor head with holding capacity: 2 mL, 10 -15 mL and 50 mL centrifuge tubes
  - GC-MS/MS and LC-MS/MS
  - Electronic Balance: Capacities 3-5 kg & 0.01 to 100g
  - Vortex shaker
  - Solvent evaporator
  - Muffle furnace: Capable of 500°C operation
3. **Apparatus and Reagents:**
  - Acetonitrile (MeCN): Quality of sufficient purity.
  - Anhydrous sodium acetate (NaOAc): Powder form (NaOAc.3H<sub>2</sub>O) may be substituted, but 0.17 g per g of sample must be used rather than 0.1 g anhydrous NaOAc per g of sample.
  - Anhydrous magnesium sulfate (MgSO<sub>4</sub>): Powder form; purity > 98%; heated to 500 °C for > 5 hr to remove phthalates and residual water.

- Acetic acid (HOAc): Glacial, quality of sufficient purity.
- TPP: Triphenylphosphine
- Primary Secondary Amine: 40 µm particle size.
- Methanol (MeOH): Quality of sufficient purity for LC-MS/MS
- Water: Quality of sufficient purity for LC-MS/MS
- Formic acid: Quality of sufficient purity
- Ammonium acetate: Quality of sufficient purity
- Ethyl acetate: Quality of sufficient purity
- N-Hexane: Quality of sufficient purity
- Distilled Water
- Measuring Cylinder (50mL)
- Vials (2 & 4 mL)
- Micropipette (1mL)
- Centrifuge bottle (15 & 50mL)

### **Sample preparation and processing prior to analysis**

On receipt, each laboratory sample must be allocated a unique reference code by the laboratory. Sample preparation, sample processing and sub-sampling to obtain analytical portion should take place before visible deterioration occurs.

Grape and tomato samples were prepared following the “AOAC Official Method 2007.01 Pesticide Residues in Foods by Acetonitrile Extraction and Partitioning with Magnesium Sulfate”. The fresh samples were stored at room temperature. Once homogenized, the samples were stored in the refrigerator.

### **Sample preparation**

- The sample is homogenized or blended to obtain a uniform matrix.

### **Sample Extraction**

Sample Extraction is defined as the procedure or step adopted to isolate the pesticides from the samples. This is accomplished by employing suitable solvents which remain in contact with the sample for a specific duration.

### **Procedure**

1. Comminute >1 Kg sample with vertical cutter. Homogenize about 200 g subsample with probe blender.
2. Weigh 15 g of crushed sample in 50 mL Centrifuge tube.
3. Add 15 mL of 1% acetic acid in acetonitrile, vortex for 30 sec. and add 6 g anhydrous  $\text{MgSO}_4$ , 1.5 g of anhydrous sodium acetate and 75  $\mu\text{L}$  I. S. solution
4. Shake vigorously for 1 min.  
Centrifuge at 4000 rpm for 1 min.
5. Transfer 1 mL supernatant into 15 mL centrifuge tube containing 50 mg PSA & 150 mg anhydrous  $\text{MgSO}_4$  and shake for 30 s.
6. Centrifuge 4000 rpm for 1 min.
7. Transfer 0.6 mL extract to GC vial and add TPP.  
Transfer 0.25 mL extract and 0.75 mL water to LC vial
8. Analysis by GC-MS/MS and LC-MS/MS

### **Laboratory Session: Sample Extraction for LC and GC**

#### **Extraction procedure for LC**

- Label ten 50 mL disposable screwcap centrifuge tubes as
  1. Blank1
  2. Blank2
  3. Blank3
  4. Blank4
  5. Spike1
  6. Spike2
  7. Spike3
  8. Test1
  9. Test2
  10. Test3
- Weigh 15 g of sample in each tube and note down the weight
- Spike the sample in the spike labelled tubes with known concentration.
- Start the extraction process as given in the extraction procedure earlier.
- After the filtration of the sample prepare Matrix match standard by using blank extracted matrix
- Now the samples in the vials are analyzed by LC-MS/MS and GC-MS/MS

## Sample Preparation, Extraction & Clean -up



**Crush/Grind**

**Weigh 15 g in 50 mL centrifuge tube**



15 mL (1%)  
Acetic acid in  
acetonitrile  
+ 6 g MgSO<sub>4</sub>  
+ 1.5 g  
Sodium Acetate



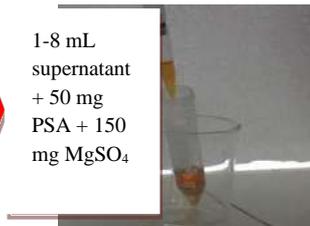
**Centrifuge**

**Homogenize**

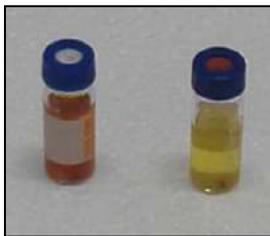
**Vortex (30 sec)**



1-8 mL  
supernatant  
+ 50 mg  
PSA + 150  
mg MgSO<sub>4</sub>



**Vortex (30 sec)**



Transfer 0.6 mL  
supernatant in  
GC vial.  
  
Transfer 0.25  
mL extract and  
0.75 mL water  
to LC vial



**Sample filtration**

**4000 rpm for 1 min**

**Discussion**

1. *What is the role of different salts –  $MgSO_4$ ,  $Na_2SO_4$*
2. *What is the role of acetic acid?*
3. *What is the role of PSA and  $C_{18}$ ?*
4. *Why do we use diethylene glycol?*
5. *Why do we exchange acetonitrile with other solvents?*
6. *Why do we filter the samples?*
7. *Why do we use refrigerated centrifuge?*
8. *Can we use methanol, acetone or ethyl acetate as extraction solvents?*

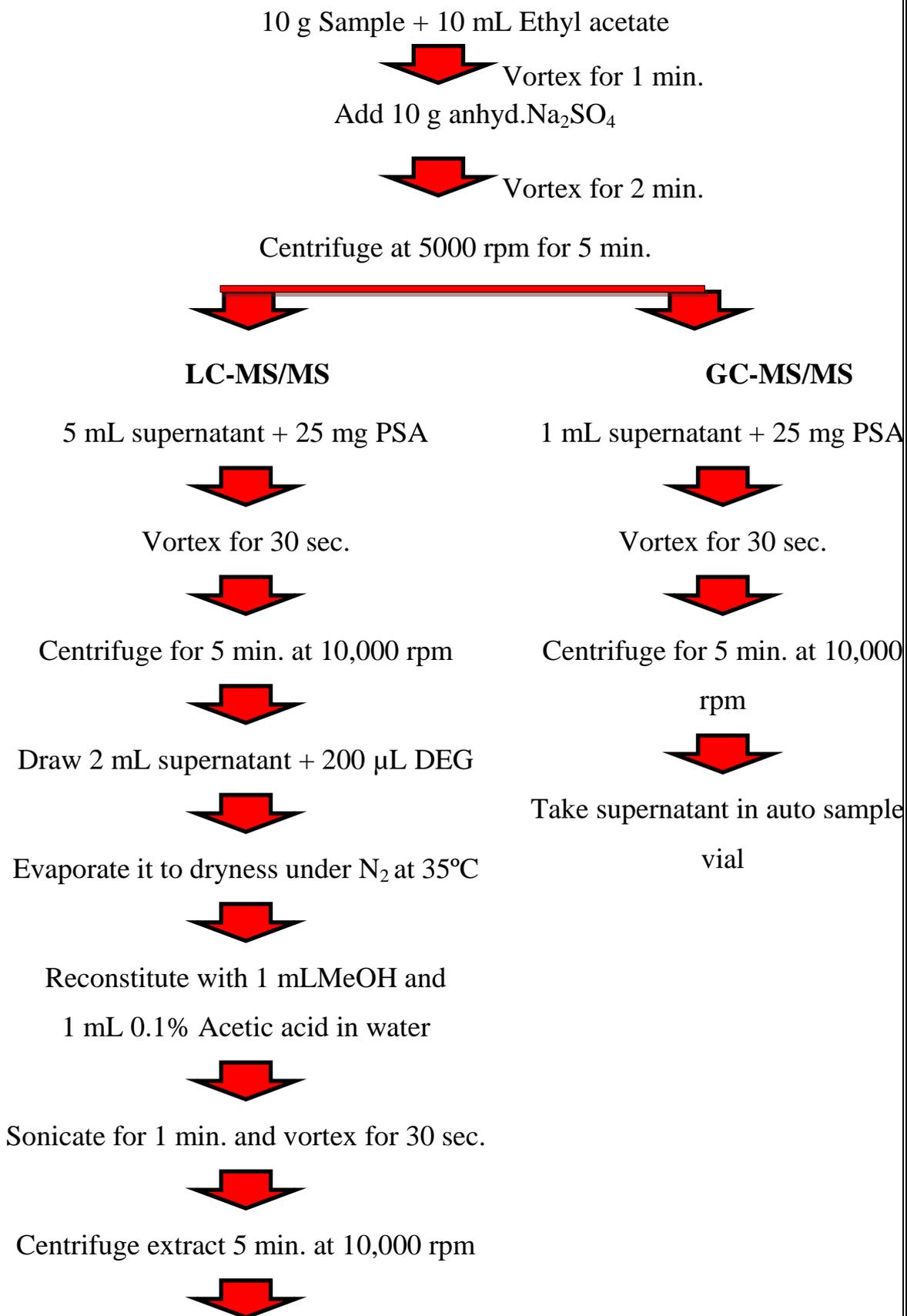
**Lab work notes**

#### 4. Multiresidue analysis by ethyl acetate method by LC-MS/MS and GC-MS/MS

##### Procedure:

- Weigh 10g homogenized sample in a 50 mL polypropylene centrifuge tube.
- Add 10 mL ethyl acetate and vortex for 1 min, add 10 g anhydrous sodium sulfate and homogenize it at 15000 rpm for 2 minute.
- Centrifuge at 5000 rpm for 5 minutes.
- For LC-MS/MS amenable compounds, take 5 mL supernatant into 15 mL polypropylene.
- Centrifuge tubes containing 25 mg primary secondary amine (PSA), shake for 30 seconds and centrifuge it for 5 minutes at 10000 rpm.
- Draw 2 mL supernatant in the test tube containing 200  $\mu$ L of 10% diethylene glycol (DEG) solution.
- Evaporate it to dryness under nitrogen at 35°C.
- Reconstitute with 1 mL methanol and 1 mL 0.1 % acetic acid in water (do not add these solvents initially, first add methanol and then 0.1% acetic acid in water). Sonicate for 1 min and vortex for 30 seconds.
- Centrifuge the extract at 10000 rpm for 5 min and filter through 0.2  $\mu$ m Nylon 6, 6 membrane filter into a LC vial.
- Inject 5 - 20  $\mu$ L from the extract into the LC-MS/MS.
- For GC-MS/MS, take 1 mL supernatant in the Eppendorf tube containing 25 mg PSA and shake vigorously for 30 s.
- Centrifuge at 10000 rpm for 5 min. Take supernatant extract in GC auto sampler vial.
- Inject the cleaned extract into the GC-MS/MS.

## Flow chart of ethyl acetate method extraction



Filter extract 0.2  $\mu$ m Nylon 66 into LC vial

## 5. Analysis of plant growth regulators by LC-MS/MS

### Procedure:

- Weigh 10 g homogenized sample in 50 mL polypropylene centrifuge tube.
- Add an internal standard (CCC d<sub>4</sub>), mix thoroughly by vortexing for 1 min.
- Add 20 mL methanol (1% formic acid).
- Homogenize/vortex the sample for 2 min and centrifuge at 5000 rpm for 5 minute.
- Draw 0.5 mL supernatant and dilute with 0.5 mL water (for C18 column analysis).
- Draw 0.5 mL supernatant and dilute with 0.5 mL methanol (for HILIC analysis).
- Inject into LC-MS/MS.
- Analytes for C<sub>18</sub>: 6-Benzyl adenine, Forchlorfenuron, Homobrassinolide, Gibberellic acid, (GA-3), 2,4-D, 4-Chlorophenoxy acetic acid, and 1-Naphthylacetic acid.
- Analytes for HILIC: Chlormequat, Mepiquat, Ethephon, Uracil and Fosetyl Al.

## **Flow chart of plant growth regulators extraction method**

10 g Sample + 20 mL (with 1 % Formic acid) MeOH and add internal standard (CCC d<sub>4</sub>)



Vortex for 1 min.

Centrifuge at 5000 rpm for 5 min.



0.5 mL extract + 0.5 mL H<sub>2</sub>O [For C<sub>18</sub> Column]

0.5 mL extract + 0.5 mL MeOH [For HILIC column]



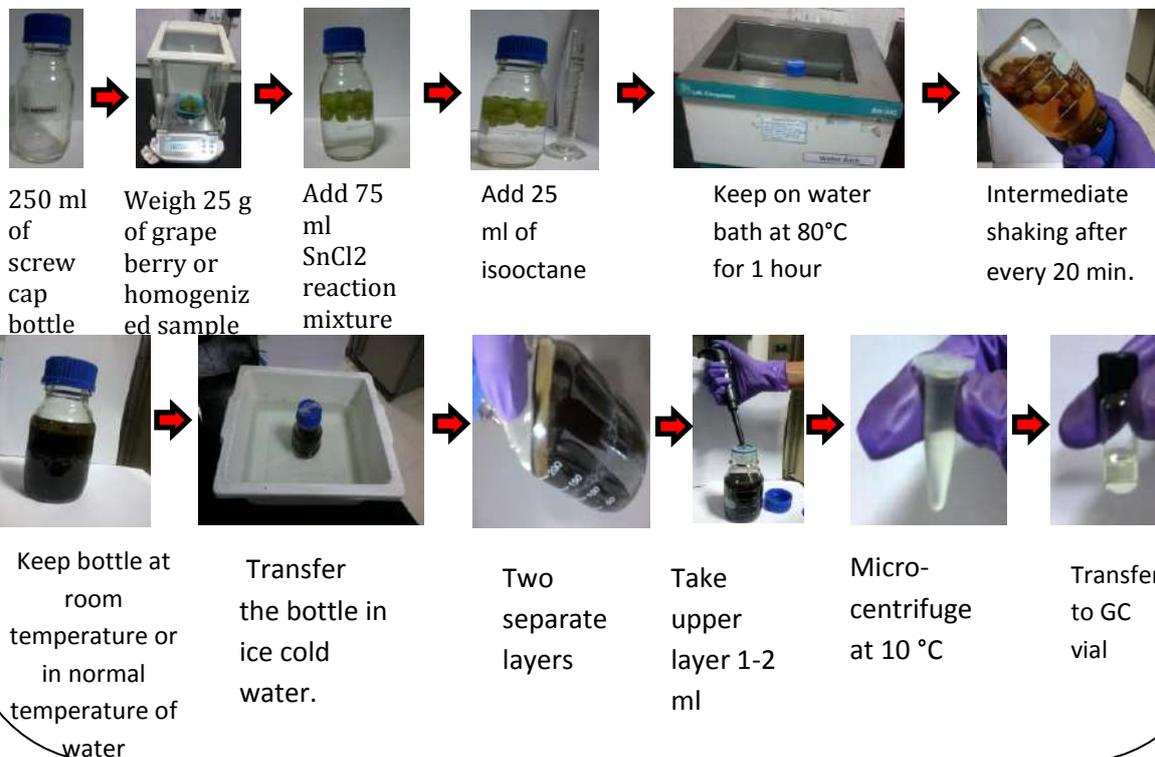
Vortex vial and inject to LC-MS/MS

## 6. Analysis of Dithiocarbamate residues in sample by GC-MS

### Procedure for sample preparation

- a) Weigh 25 g sample in 250mL in screw capped bottle.
- b) Add 75 mL of reaction mixture. [Weigh 30 g of Tin (II) Chloride and dissolve it in the 1000 mL of HCl]
- c) Add 25 mL of isooctane and immediately close the bottle with screw cap.
- d) Place the bottle in water bath at 80 ( $\pm 5$ ) °C.
- e) Mix the content of the bottle by inversion after approximately 20 min.
- f) Repeat step 'e' after every 20 min.
- g) After the total reaction time of 60 min, remove the bottle from the water bath and mix the content of bottle.
- h) Transfer the bottle in cool ice water bath to cool down the temperature quickly.
- i) After cooling the reaction mixture to about 10-20°C, transfer 1.8 mL of isooctane layer in 2 mL micro centrifuge tube.
- j) Centrifuge at 5000 rpm for 5 min at 10°C.
- k) Transfer the supernatant (1 mL) to an auto sampler vial.
- l) Inject in to GC-MS.

## Flow chart for analysis of dithiocarbamate residues in sample



### GC-MS parameters

Sr. No. Parameter Specification	Sr. No. Parameter Specification	Sr. No. Parameter Specification
1.	Column	DB-5 ms (5 % diphenyl, 95 % dimethyl polysiloxane, 30 m x 0.25 mm ID, 0.1 µm film thickness) capillary column OR Rxi-624 30 m x 0.32 mm id, 1.8 µ film thickness having 6% cyanopropyl / 94% dimethyl polysiloxane
2.	Oven Temperature	40 °C for 5 min hold time and then ramp it from 40 °C to 200 °C with 5 min hold time.
3.	Injector program	Programmable Temperature Vaporization with Large Volume Injection volume: 10 µl Injection Program: start temperature 40°C with 0.1 min hold, then

		ramp @10°C/s up to 80°C with 0.3 min hold, then ramp up to 110°C at rate of 10°C/s. For cleaning purpose of liner, the temperature was increased up to 290°C for 4 min. OR Splitless 2 µL at 200 °C
4.	Detector	EI - 70 eV Ion source temperature: 200°C Scan Range: In full scan mode, scanning range = 50-100 amu. Inselected ion monitoring mode, select the m/z 76 and m/z 78.

#### Preparation of Reaction Mixture:

- a) Weigh 30 g of Tin (II) Chloride and dissolve it in the 1000 mL of HCl.
- b) Take a plastic can having capacity 2500-3000 mL.
- c) Add 1000 mL water in it.
- d) Then gradually add 1000 mL of Tin (II) chloride and dissolve in HCl with stirring. Finally a clear solution is obtained.

#### Standards

##### Carbon disulphide standard solution:

- a) Take 20 mL bottle with screw cap (air tight) and tare the weight.
- b) Fill the bottle with isooctane, put cap on the bottle and note down the weight in grams.

This is the weight of the solvent in gram. By using following formula convert weight in gram to mL.

*Weight of the solvent in mL (A)*

*= (weight of the solvent in gram/ density of the solvent in g/mL)*

- c) Change the unit on the balance gram to milligram (mg)
- d) Add pure CS<sub>2</sub> about 20 µL, cap the bottle and note down the weight in mg. This is the weight of the CS<sub>2</sub> standard.

*Actual weight of the Standard (B) = (Weight of standard × Purity of standard)/100*

*Actual concentration of CS<sub>2</sub> stock std solution (C<sub>1</sub>) = (Actual weight of the Std (B)/mL of the*

*solvent (A)*

e) Prepare the working standard of 100 µg/ mL ( $C_2$ ) of 5 mL ( $V_2$ ) in isooctane by using following formula

$$C_1 V_1 = C_2 V_2$$

$$V_1 = C_2 V_2 / C_1$$

**Standard solution of Thiram:**

a) Weigh accurately 10 mg of Thiram in closed standard bottle.

b) Note down the weight of the Thiram in the mg unit.

c) Check the purity of Thiram standard

d) Calculate the actual weight of the Thiram by using following formula

$$e) \text{ Actual weight of the Thiram Standard } (x) = (\text{Weight of standard} \times \text{Purity of standard}) / 100$$

f) Cap the bottle with screw cap and tare it.

g) Change the unit of balance mg to g.

h) Add ethyl acetate in bottle and closed the bottle with screw cap.

i) Note down the weight of the ethyl acetate which has been taken in to the glass bottle in the gram.

j) Convert gram weight of the ethyl acetate in the mL weight. By using following formula.

$$\text{Weight of the Solvent in mL } (y) =$$

$$(\text{Weight of the solvent in gram} / \text{density of the ethyl acetate solvent in (g/mL)})$$

k) Calculate the actual concentration of the standard (mother stock) by using following formula.

$$\text{Actual concentration of thiram mother (stock) solution } (C_1) =$$

$$(\text{Actual weight of the Std } (x) / \text{mL of the solvent } (y))$$

l) Use isooctane for further dilution. Prepare working standard ( $C_2$ ) 1 µg/mL of 5 mL ( $V_2$ ) in isooctane by using following formula.

$$C_1 V_1 = C_2 V_2$$

- m) Store mother stock solution at - 20°C.
- n) Store the working standard solution at 0°C.
- o) Perform further dilutions in isooctane.
- p) 1 g of Thiram liberates 0.6333 g of CS<sub>2</sub>

**External calibration solvent standards for GC-MS**

- a) Freshly prepare fivepoints calibration standards at 0.01, 0.02, 0.05, 0.1 and 0.2µg/mL freshly every day from the working standard of 1µg/mL of CS<sub>2</sub>.
- b) Prepare matrix matched standard at the same concentrations by extracting fresh sample using the procedure and spiking the extract with appropriate volumes of working standards (1µg/mL)

**Recovery sample preparation**

For recovery experiment, spike calculated amount of thiram in the aqueous layer,

Immediately close the bottle and follow the same extraction procedure.

*For recovery level 1 ppm, spike quantity from working standard = 0.4 mL*

*For recovery level 0.5 ppm spike quantity from working standard = 0.2 mL*

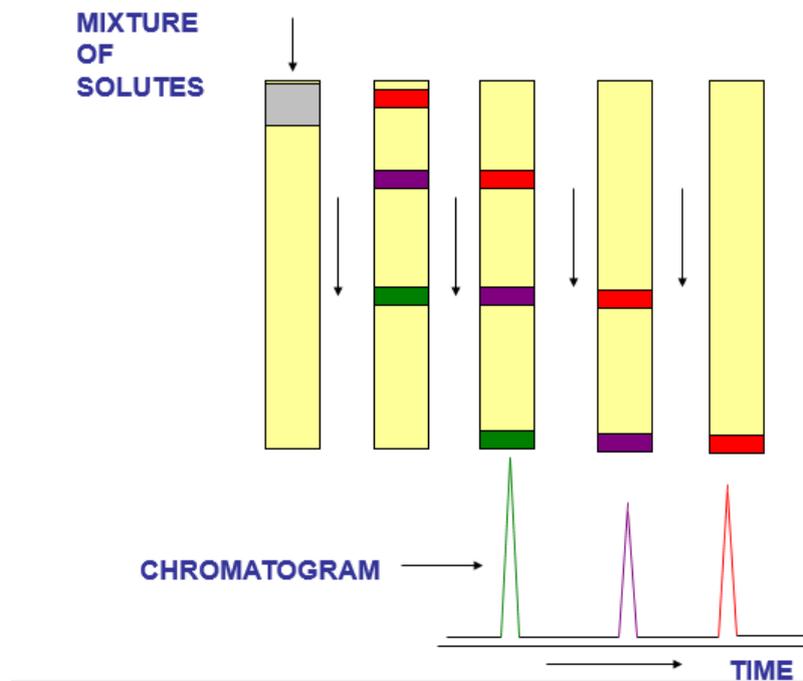
**Lab work notes**

## 7. Chromatographic separation and detection

Chromatography is a separation technique. The components of a mixture are distributed between two phases, the stationary phase and the mobile phase. The mobile phase moves through or over the surface of the fixed (stationary) phase. The components of the mixture have different affinities for each phase, hence some are retained longer on the stationary phase than others causing separation. High performance liquid chromatography (HPLC) and Gas chromatography (GC) are two widely used separation techniques in analytical world.

Principle: Analogous to extraction except that in chromatography one phase is held in place while the other phase is moving. The phase which is moving is called mobile phase and the one that is stationary phase is called stationary phase

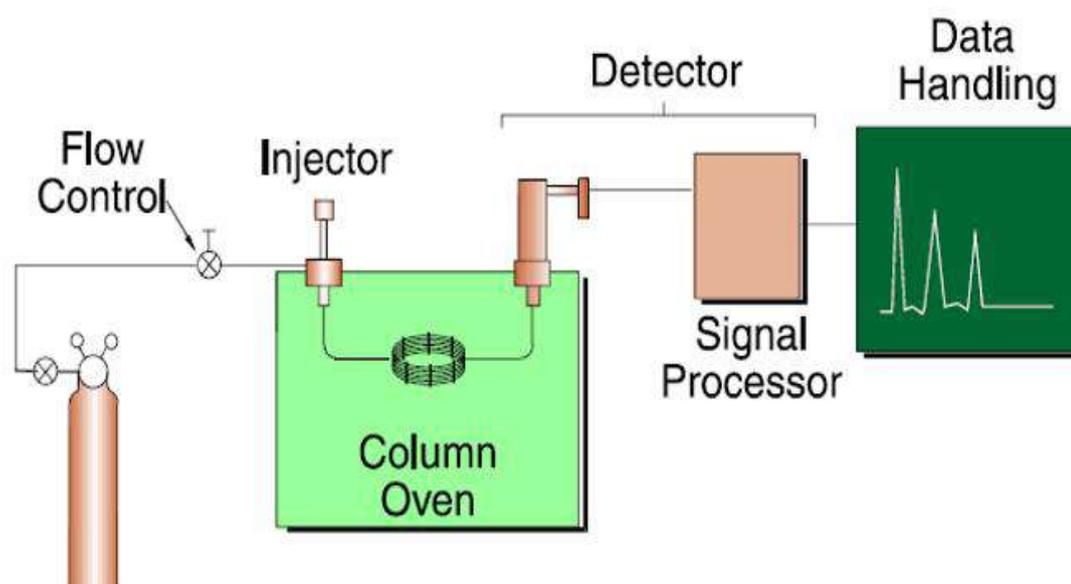
### Separation of solute molecules



**Fig 1:** Schematic representation of principle of chromatography

## Parts of GC

- Carrier gas
- Injector
- Column
- Oven
- Detector
- Computer
- Other important accessories - Gas purification system, Septa, Ferrules, Liners, Syringes etc.

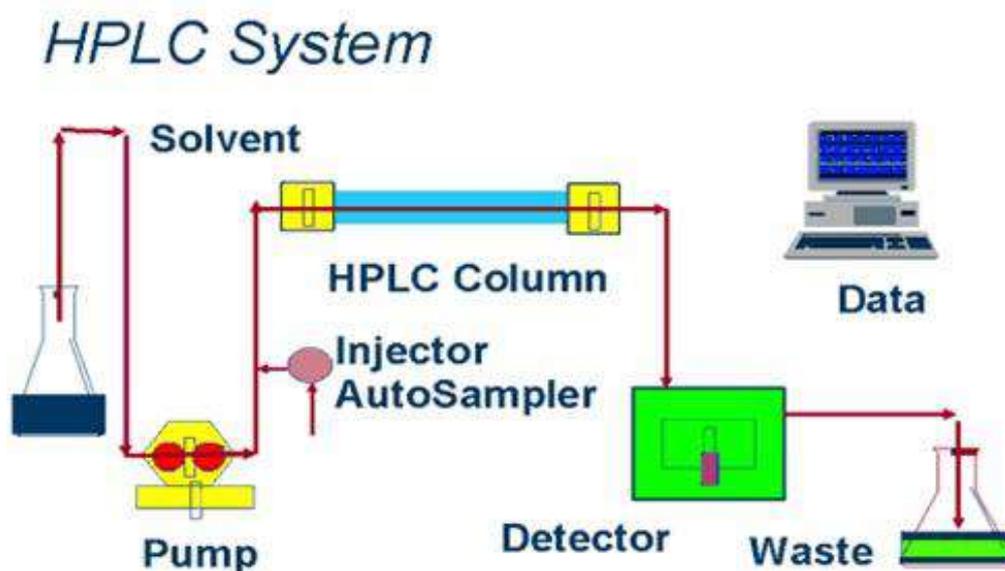


**Fig 2:** Schematic representation of Gas chromatography

**Lab work notes**

**HPLC (High performance liquid chromatography):** HPLC on first appearance appears to be a complex system comprising of modular boxes and an intricate bunch of tubings though in modern systems most tubings are concealed inside the modules. If you take a careful look the mystery of the modules will start unfolding and you will be able to resolve them into simple functional units each having a defined role.

- Mobile phase – acts as a carrier stream for the sample
- Injector – injects fixed volume of sample into the mobile phase stream
- Pump – delivers mobile phase at constant flow rate
- Column – separates the sample components
- Column oven – maintains constant column temperature
- Detector – detects individual eluting sample components
- Computer and integrator



**Fig 3:** Schematic representation of High Performance Liquid Chromatography

**Mobile phase:** Mobile phase requires to be freshly prepared and it should be free of suspended matter. Always adopt same mixing procedure to eliminate errors due to heat of mixing.

**Sample Injectors:** Manual injection requires filling a fixed volume loop with the sample. The loop needs to be flushed repeatedly with the mobile phase so that traces of earlier sample are eliminated. Wait for baseline to stabilize before proceeding with your sample analysis. Auto injectors are manufactured to deliver precise volumes every time and sufficient wash cycles should be allowed to ensure clean injections. Injector calibration involves confirmation of injector accuracy, precision, linearity and carryover tests.

**Pumps:** A pump comprises of several parts that are precisely designed to deliver consistent flow rates. However, due to regular usage wear and tear results in flow rate deviations. Timely replacement of worn out parts improves the precision of flow delivery but still regular calibration is necessary. Pump calibration involves establishing flow rate accuracy and gradient composition accuracy.

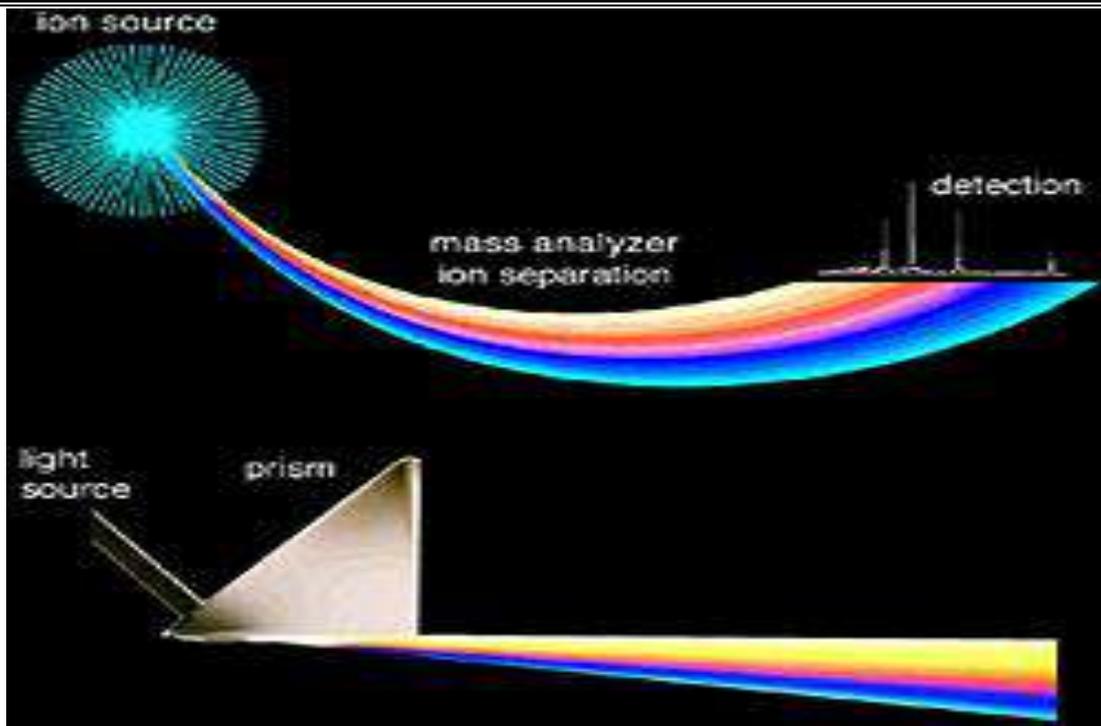
**Columns:** A column is the most critical component of the HPLC system. If a column is not well maintained it loses its separation efficiency. Using working standards you should establish that the system suitability parameters remain within the prescribed limits. In case of deviations outside the specified limits the column should be replaced

**Column oven:** The retention times of the compounds are dependent on mobile phase viscosity which in turn is temperature dependent. The column should be maintained within specified temperature limits to get consistency of retention times. It becomes necessary to calibrate the temperature reproducibility of the column oven from time to time.

**Detector:** A detector gives absorbance signals that are dependent on wavelength of absorption by the sample components. The absorption signals can drop due to lamp life, residual impurities or other contributing factors. Timely replacement of lamps and regular cleaning of detector eliminates such problems but detector requires regular calibration using specified reference compounds which are injected into the mobile phase stream. The two main parameters requiring calibration are linearity of response and wavelength accuracy.

**Mass Spectrometry:** A mass spectrometer determines the mass of a molecule by measuring the mass-to-charge ratio ( $m/z$ ) of its ion. Ions are generated by inducing either the loss or gain of a charge from a neutral species. Once formed, ions are electrostatically directed into a mass analyzer where they are separated according to  $m/z$  and finally detected. The result of molecular ionization, ion separation, and ion detection is a spectrum that can provide molecular mass and even structural information. An analogy can be drawn between a mass spectrometer and a prism, as shown in Figure. In the prism, light is separated into its component wavelengths which are then detected with an optical receptor, such as visualization. Similarly, in a mass spectrometer the generated ions are separated in the mass analyzer, digitized and detected by an ion detector

Mass spectrometry is the art of measuring atoms and molecules to determine their molecular weight. Such mass or weight information is sometimes sufficient, frequently necessary, and always useful in determining the identity of a species. To practice this art one puts charge on the molecules of interest, i.e., the analyte, then measures how the trajectories of the resulting ions respond in vacuum to various combinations of electric and magnetic fields.



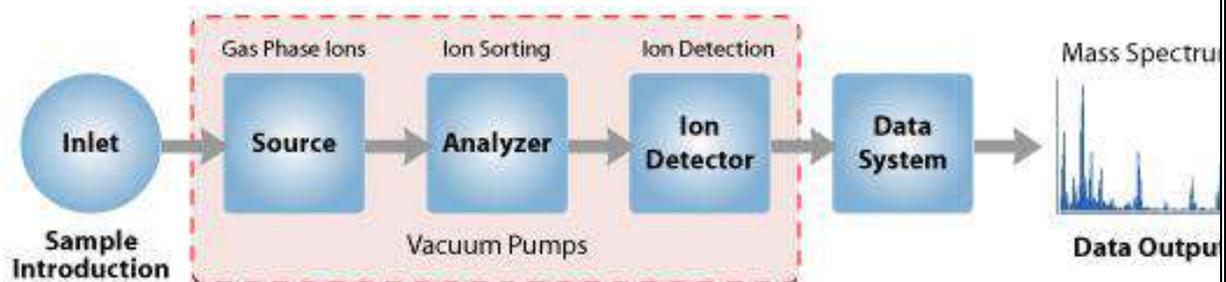
**Fig 4:** The mass analysis process as compared to the dispersion of light by a prism.

### Mass Spectrometer as detector for LC and GC

A mass spectrum is a graph of ion intensity as a function of mass-to-charge ratio

Components of Mass spectrometer are

1. Producing ions from the sample
2. Separating ions of differing masses
3. Detecting the number of ions of each mass produced
4. Collecting the data and generating the mass spectrum



**Fig 5:** Schematic representation of Mass Spectrometer

## **Ionization sources**

Electrospray ionization (ESI), Atmospheric pressure chemical ionization (APCI), Matrix associated laser desorption ionization (MALDI) etc. are various ionization techniques used in LC-MS/MS. However ESI is the most popular ionization source.

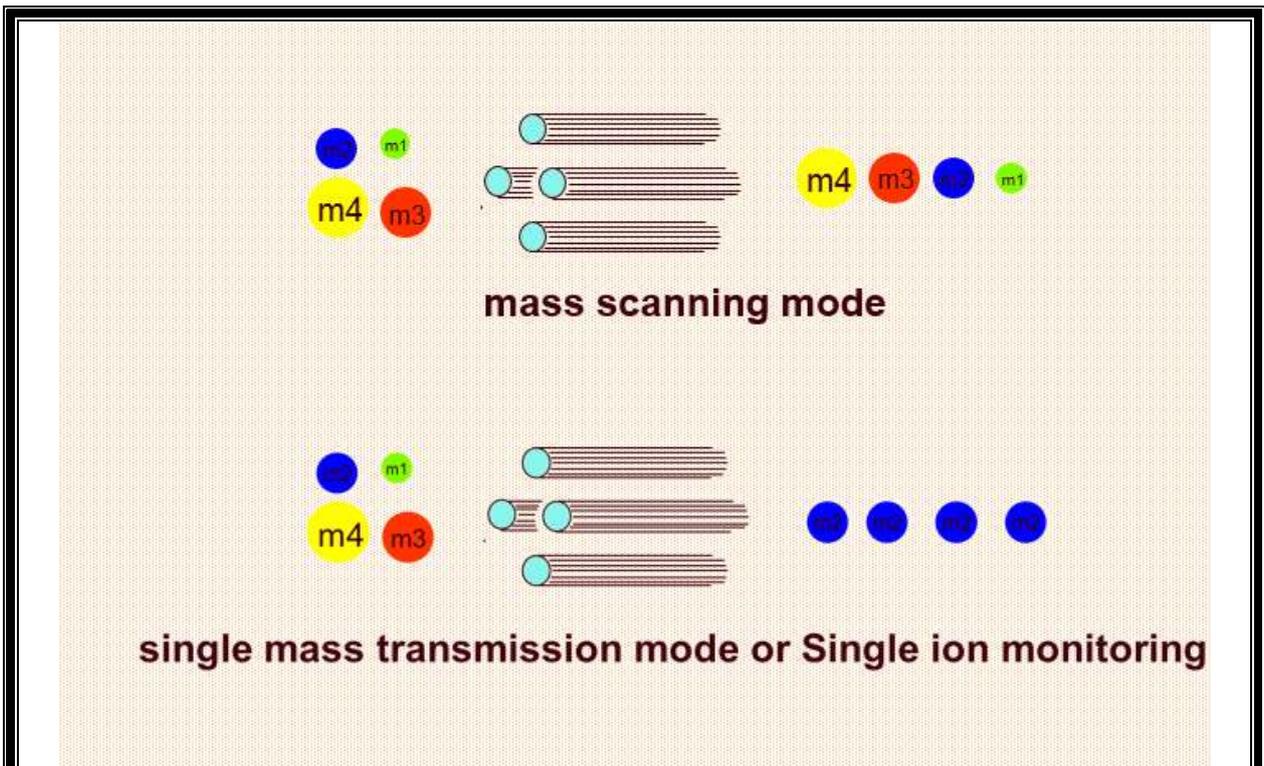
## **Mass Analyzer**

### **Triple quadrupole**

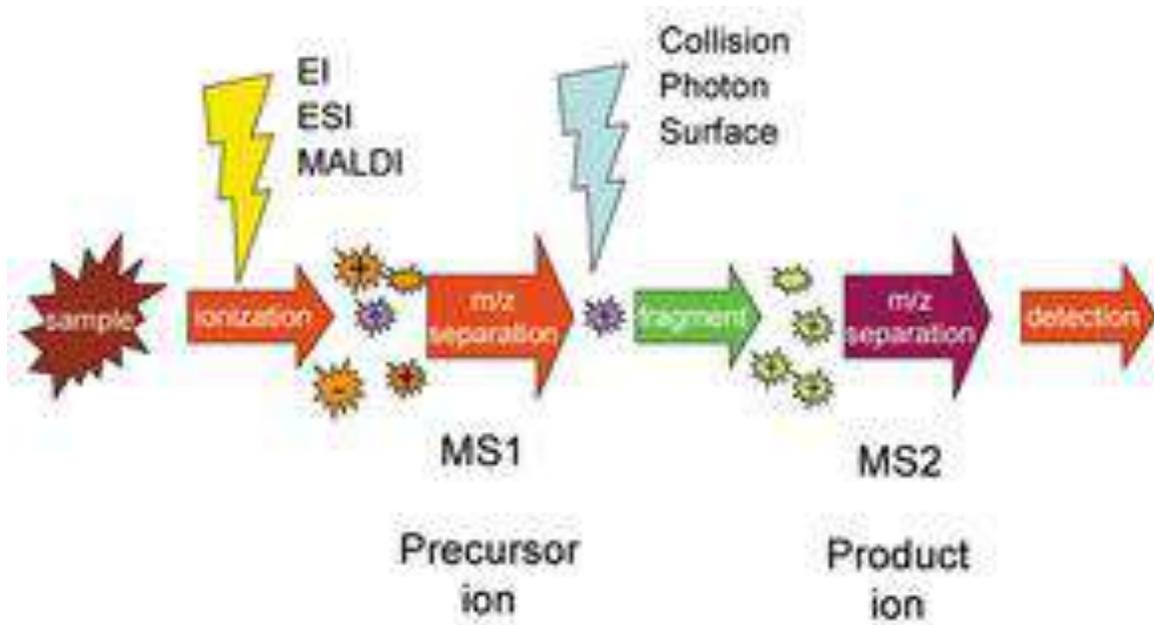
A triple-quadrupole mass spectrometer, also known as QqQ, is a tandem MS method in which the first (Q1) and third (Q3) quadrupoles act as mass filters and the second causes fragmentation of the analyte through interaction with a collision gas. It is a radiofrequency-only quadrupole, and can be used in either SIM or scan mode. Each of the two mass filters (Q1 and Q3) contains four parallel, cylindrical metal rods. Both Q1 and Q3 are controlled by direct current (DC) and radio-frequency (rf) potentials, while the collision cell (q) is only subjected to RF potential. The RF potential associated with the collision cell (q) allows all ions that were selected for to pass through it. In some instruments, the normal quadrupole collision cell has been replaced by hexapole or octopole collision cells which improve efficiency.

SRM - Selected reaction monitoring (SRM) is a method used in tandem mass spectrometry in which an ion of a particular mass is selected in the first stage of a tandem mass spectrometer and an ion product of a fragmentation reaction of the precursor ion is selected in the second mass spectrometer stage for detection.

MRM - Multiple SRM transitions can be measured within the same experiment on the chromatographic time scale by rapidly toggling between the different precursor/fragment pairs (multiple reaction monitoring, MRM)



**Fig 6:** Schematic representation of Triple Quadrupole Mass Spectrometer with Scan and SIM modes



**Fig 7:** Schematic representation of Triple Quadrupole Mass Spectrometer with SRM mode

**Lab work notes**

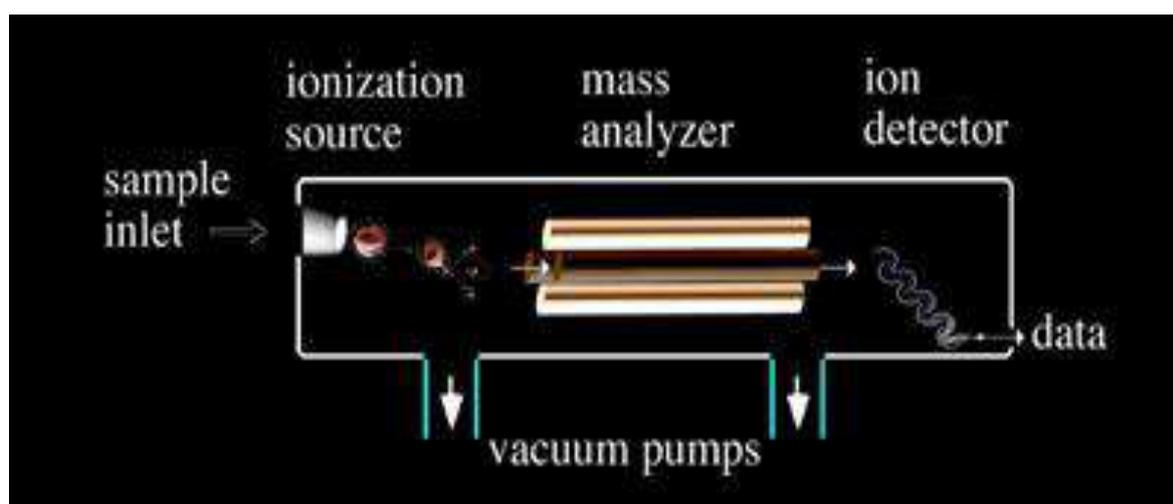
## Operation of Liquid chromatography with Mass Spectrometer (LC-MS/MS):

### Some Basics:

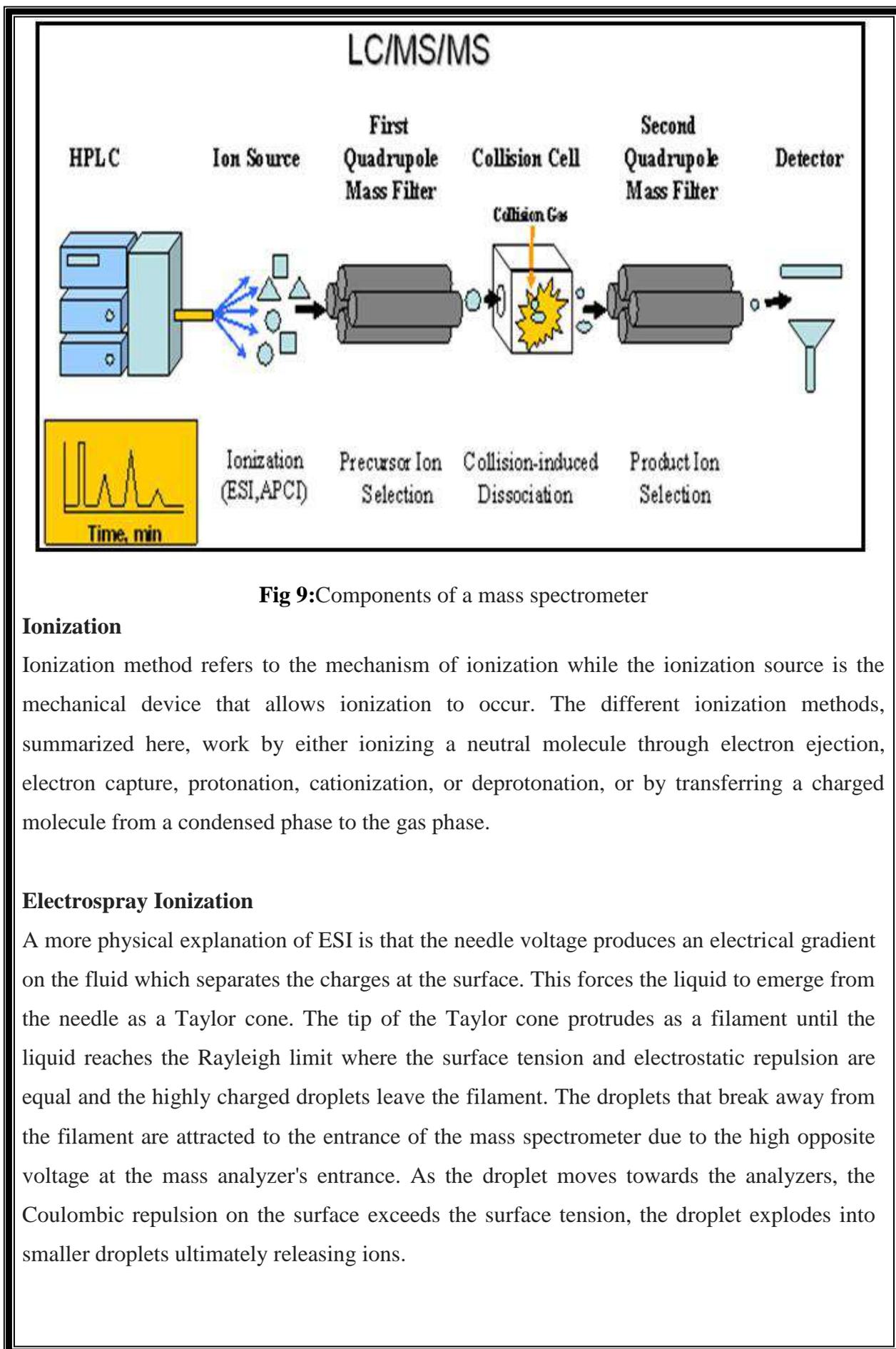
Four basic components are, for the most part, standard in all mass spectrometers: a sample inlet, an ionization source, a mass analyzer and an ion detector. Some instruments combine the sample inlet and the ionization source, while others combine the mass analyzer and the detector. However, all sample molecules undergo the same processes regardless of instrument configuration. Sample molecules are introduced into the instrument through a sample inlet. Once inside the instrument, the sample molecules are converted to ions in the ionization source, before being electrostatically propelled into the mass analyzer. Ions are then separated according to their  $m/z$  within the mass analyzer. The detector converts the ion energy into electrical signals, which are then transmitted to a computer.

### Sample Introduction Techniques

Sample introduction was an early challenge in mass spectrometry. In order to perform mass analysis on a sample, which is initially at atmospheric pressure (760 torr), it must be introduced into the instrument in such a way that the vacuum inside the instrument remains relatively unchanged ( $\sim 10^{-6}$  torr). The most common methods of sample introduction are direct insertion with a probe or injection into the ionization source such as ESI-MS.



**Fig 8:**Mass spectrometer



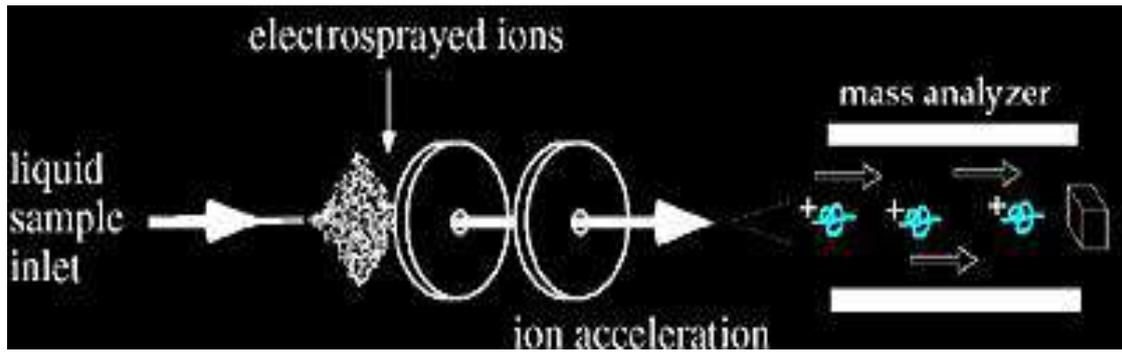
**Fig 9:**Components of a mass spectrometer

**Ionization**

Ionization method refers to the mechanism of ionization while the ionization source is the mechanical device that allows ionization to occur. The different ionization methods, summarized here, work by either ionizing a neutral molecule through electron ejection, electron capture, protonation, cationization, or deprotonation, or by transferring a charged molecule from a condensed phase to the gas phase.

**Electrospray Ionization**

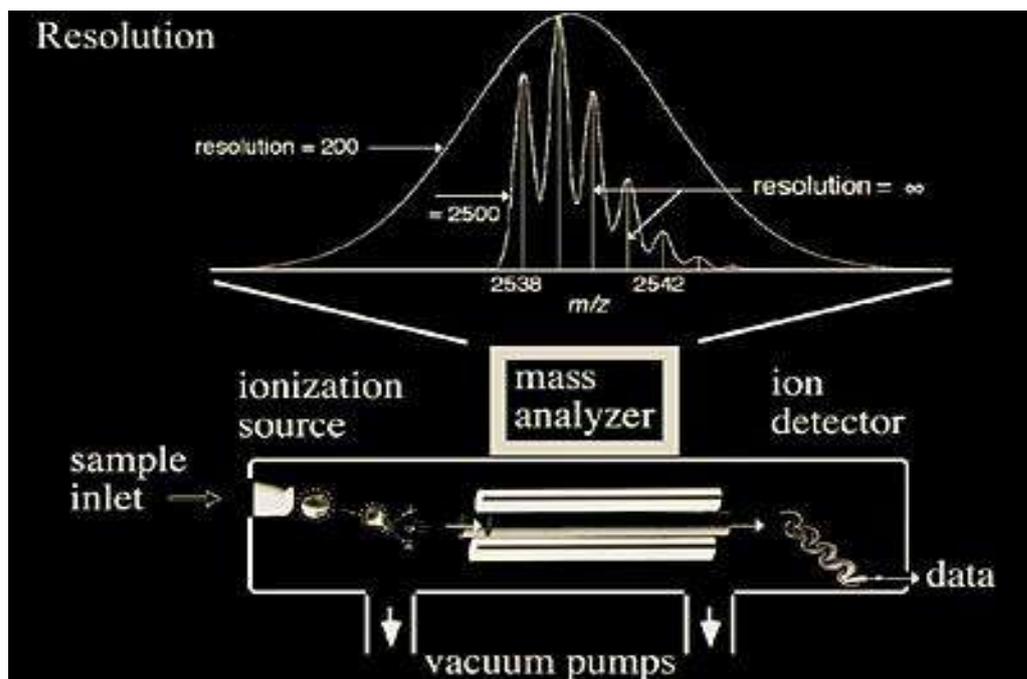
A more physical explanation of ESI is that the needle voltage produces an electrical gradient on the fluid which separates the charges at the surface. This forces the liquid to emerge from the needle as a Taylor cone. The tip of the Taylor cone protrudes as a filament until the liquid reaches the Rayleigh limit where the surface tension and electrostatic repulsion are equal and the highly charged droplets leave the filament. The droplets that break away from the filament are attracted to the entrance of the mass spectrometer due to the high opposite voltage at the mass analyzer's entrance. As the droplet moves towards the analyzers, the Coulombic repulsion on the surface exceeds the surface tension, the droplet explodes into smaller droplets ultimately releasing ions.



**Fig 10:**Electro spray ionization (ESI) mass spectrometry

### Mass Analysis

Analytical instruments in general have variations in their capabilities as a result of their individual design and intended purpose. This is also true for mass spectrometers. While all mass spectrometers rely on a mass analyzer, not all analyzers operate in the same way; some separate ions in space while others separate ions by time. In the most general terms, a mass analyzer measures gas phase ions with respect to their mass-to-charge ratio ( $m/z$ ), where the charge is produced by the addition or loss of a proton(s), cation(s), anion(s) or electron(s). The addition of charge allows the molecule to be affected by electric fields thus allowing its mass measurement. This is an important aspect to remember about mass analyzers they measure the  $m/z$  ratio, not the mass. It is often a point of confusion because if an ion has multiple charges, the  $m/z$  will be significantly less than the actual mass.



**Fig 11:** The effect of resolution up on mass accuracy

## Tuning the mass spectrometer

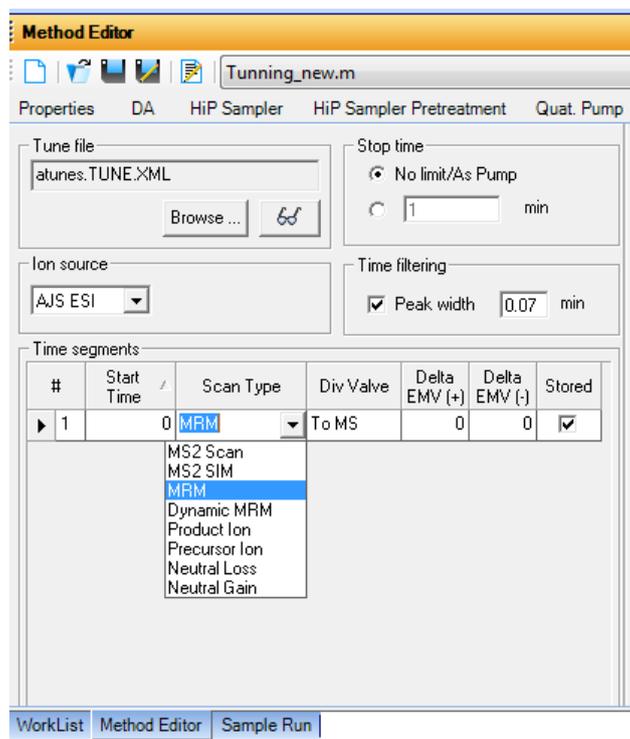
Tuning is the process of selecting all the parameters that will be used in MS acquisition method. These include

- Ion mode
- Instrument parameters
- ESI and collision gases

During the tuning the parent and product ions of each pesticide molecule are identified and optimized with respect to the fragmentor voltage and collision energy.

### Introduction to mass spectrometer set up for tuning

#### Method editor – QQQ parameters – Source



#### Let us take an example of spinetoram.

Prepare a 1 ppm solution of spinetoram in a suitable solvent and add it to the tuning vial.

Its molecular weight is 748. As per the literature survey, it is analysed in the positive mode. Therefore M+1 i.e. 749 mass needs to be subjected for MS analysis.

In the MS2 scan, choose the mass range between 700 to 800 m/z. 749 will be shown as a peak.

The next step is to decide its product ions and optimize their response. For this activity, mass of 749 needs to be fragmented.

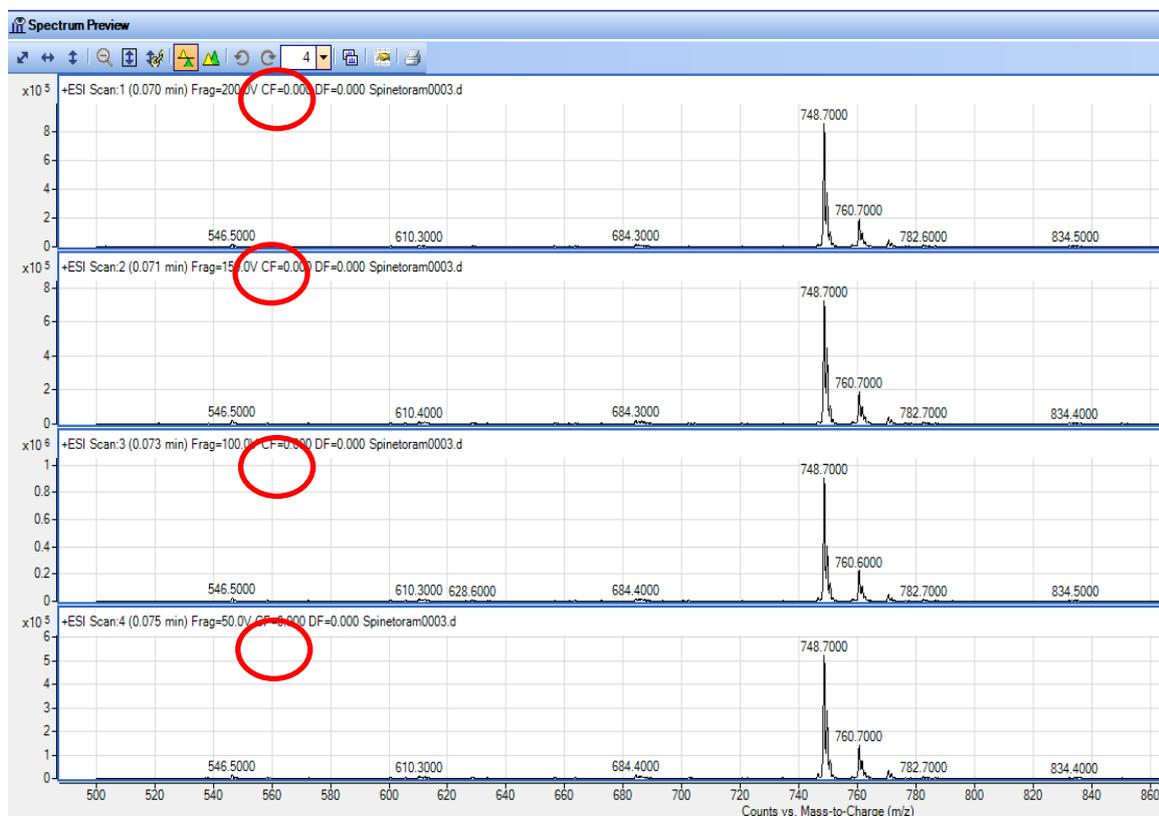
To begin with choose an intermediate voltage from the ranges available and subject mass 224 to fragmentation.

This will produce various product ions. While selecting the product ions, following points need to be kept in mind

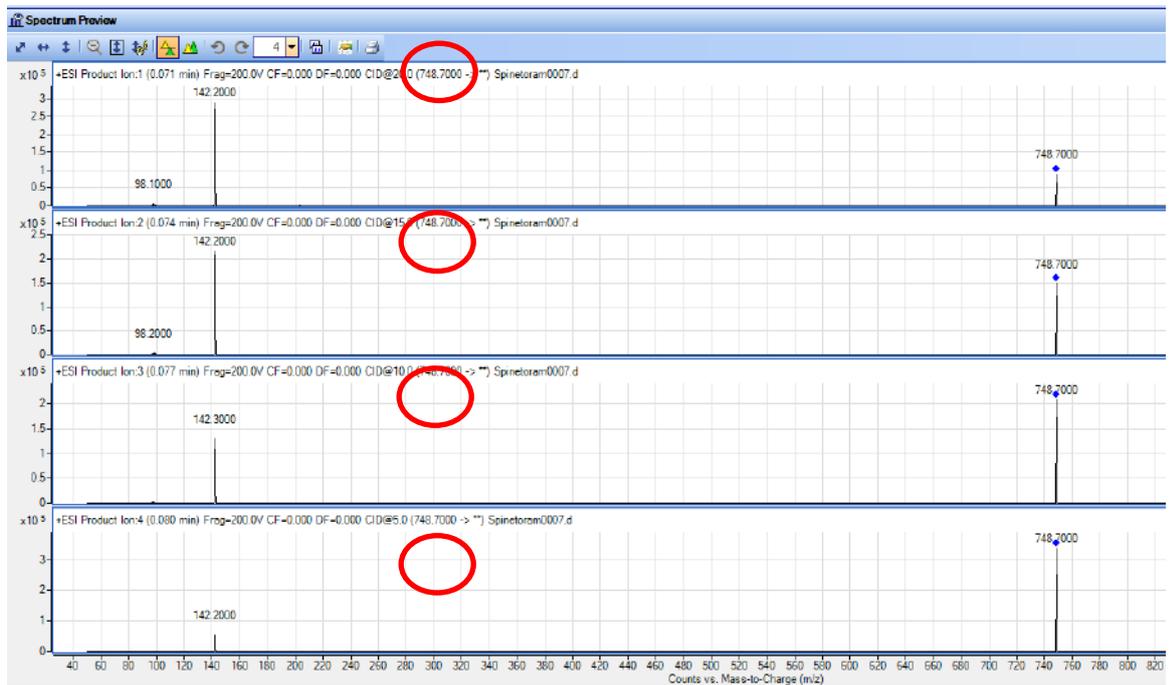
- Select a higher mass product ion over a very low mass product ion
- A small intensity of the molecular ion also should be visible, which proves that the product ions are formed, from this molecular ion.

For spinetoram, intensity of product ions 148 and 92 is found to be optimum and therefore further optimization with respect to fragmentor voltage and collision energy needs to be carried out.

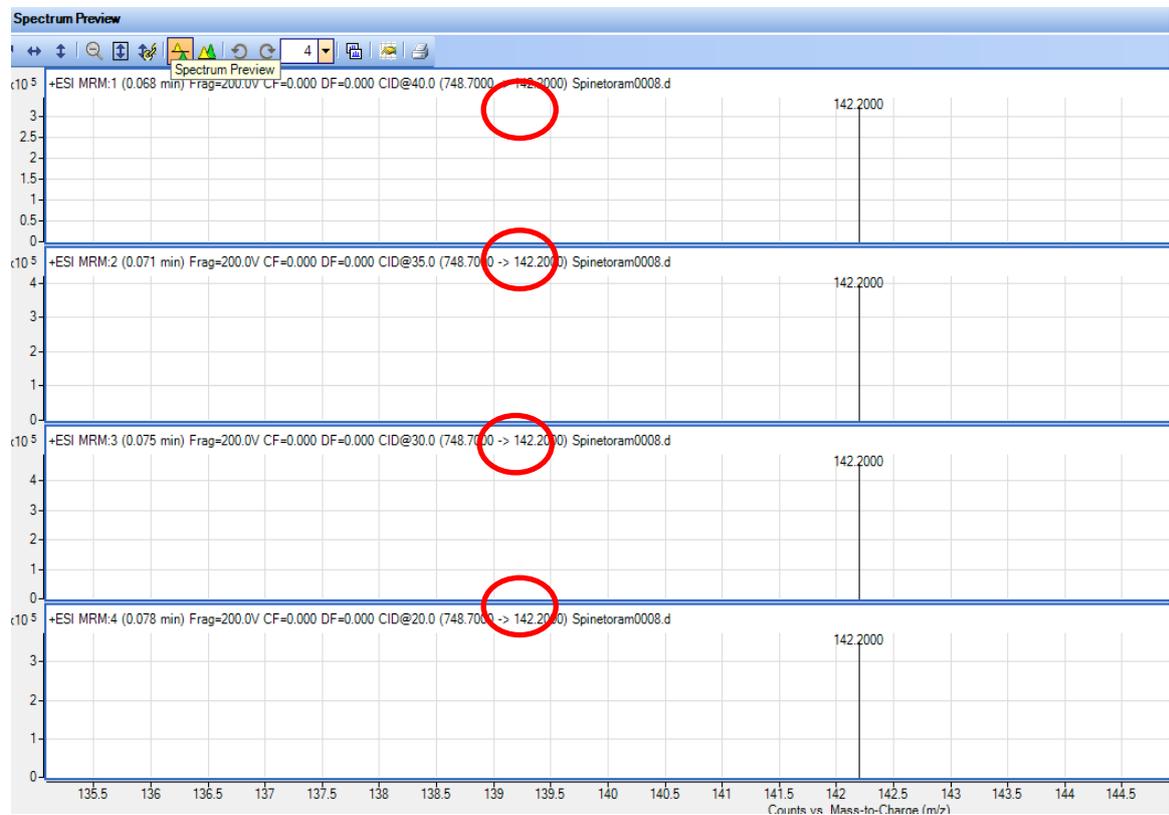
### MS2 Scan Spinetoram



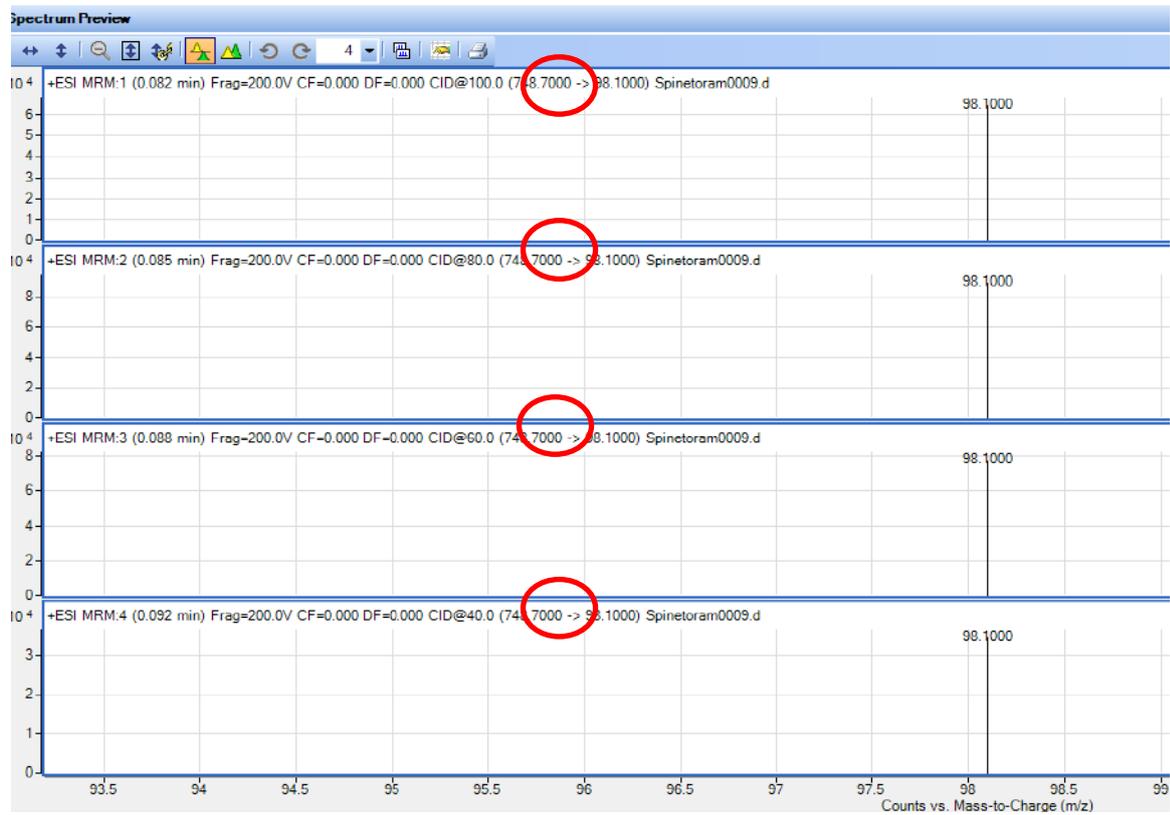
## Fragmentation of molecular ion (749) into Product Ions



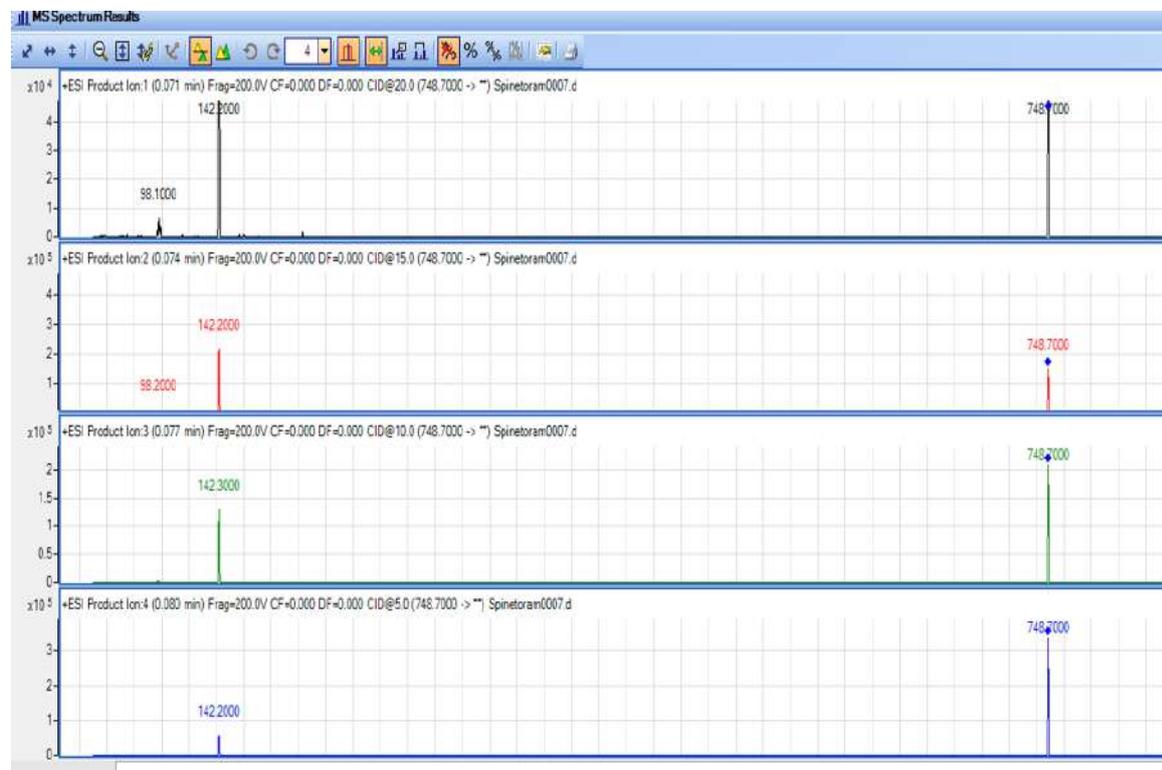
## Optimization of Product Ion 749 → 142



## Optimization of Product Ion 749 → 98



## Confirmation of MRM



Apart from parent and product ions, Source parameters have to be optimized for highest response of analytes. These parameters include gas flow, nebulizer temperature and sheath gas temperature, sheath gas flow, capillary and nebulizer voltages etc.

## Mass Parameters

The screenshot shows the 'Method Editor' window with the 'Source' tab selected. The 'Source parameters' section is visible, showing various settings for the mass spectrometer source. The 'Time segments' table shows a scan at 7.13 minutes.

#	Start Time	Scan Type	Div Valve	Delta EHV (+)	Delta EHV (-)	Stored
1	0	MS2 Scan	To MS	0	0	<input checked="" type="checkbox"/>

Source parameters:

- Gas Temp: 225 °C
- Gas Flow: 10 l/min
- Nebulizer: 30 psi
- Sheath Gas Temp: 280 °C
- Sheath Gas Flow: 11 l/min
- Capillary: 3000 V (Positive), 3000 V (Negative), 2388 nA
- Nozzle Voltage: 500 V
- Chamber Current: 0.47 µA

Once all the above parameters are optimized, the LC-MS (GC-MS) acquisition method can be set up

## Acquisition method

The screenshot shows the 'Acquisition' window with the 'Scan segments' table and 'Dynamic MRM Parameters' section.

Compound Group	Compound Name	ISTD?	Precursor Ion	MS1 Res	Product Ion	MS2 Res	Ret Time (min)	Delta Ret Time	Fragmentor	Collision Energy	Cell Accelerator Voltage	Polarity
	Simazine	<input type="checkbox"/>	202.2	Unit	124.2	Unit	5.06	1	100	15	7	Positive
	S-metolachlor	<input type="checkbox"/>	284.1	Unit	252.1	Unit	6.69	1	100	15	7	Positive
	S-metolachlor	<input type="checkbox"/>	284.1	Unit	176.3	Unit	6.69	1	100	25	7	Positive
	Spinetoram	<input checked="" type="checkbox"/>	748.7	Unit	142.2	Unit	7.13	1	200	30	7	Positive
	Spinetoram	<input checked="" type="checkbox"/>	748.7	Unit	98.1	Unit	7.13	1	200	82	7	Positive
	Spinosad A	<input type="checkbox"/>	732.3	Unit	142.1	Unit	7.1	1	160	30	7	Positive
	Spinosad A	<input type="checkbox"/>	732.3	Unit	98.1	Unit	7.1	1	160	75	7	Positive
	Spinosad D	<input type="checkbox"/>	746.3	Unit	142.2	Unit	7.29	1	140	30	7	Positive
	Spinosad D	<input type="checkbox"/>	746.3	Unit	98.1	Unit	7.29	1	140	75	7	Positive
	Spirodiclofen	<input type="checkbox"/>	411	Unit	313	Unit	8.21	1	75	5	7	Positive
	Spirodiclofen	<input type="checkbox"/>	411	Unit	71.1	Unit	8.21	1	75	10	7	Positive
	Spiromesifen	<input type="checkbox"/>	371	Unit	273.2	Unit	7.95	1	70	5	7	Positive
	Spiromesifen	<input type="checkbox"/>	371	Unit	255.1	Unit	7.95	1	70	20	7	Positive
	Spirotetramat	<input type="checkbox"/>	373.4	Unit	330.3	Unit	6.43	1	100	10	7	Positive
	Spirotetramat	<input type="checkbox"/>	373.4	Unit	302.23	Unit	6.43	1	100	10	7	Positive

Dynamic MRM Parameters:

- Cycle Time: 1000 ms
- Triggered MRM:  Triggered, Repeats: 3

The calibration standards and samples are analysed using this acquisition method. For quantification of samples, a processing method needs to be set up. This processing method address following points - Name of the pesticides, their retention times, their MRMs, Calibration levels, quantification ion, qualifier, ratio of quantifier to qualifier ion, acceptable % deviation for this ratio. For every single pesticide, these parameters have to be filled in.

### Processing method for Quantification

Quantifier										
Name	TS	Transition	Scan	Type	Precursor Ion	Product Ion	Uncertainty	RT	Dil. High Conc.	
Milbemectin-A4	1	565.3 -> 239.8	MRM	Target	565.3	239.8	Relative	4.000	100.0000	
Monocrotophos	1	224.0 -> 127.0	MRM	Target	224.0	127.0	Relative	3.482	100.0000	

Qualifier						
Precursor Ion	Product Ion	Transition	Rel. Resp.	Uncertainty	Area Sum	
224.0	127.0	224.0 -> 193.0	72.0	30.0		

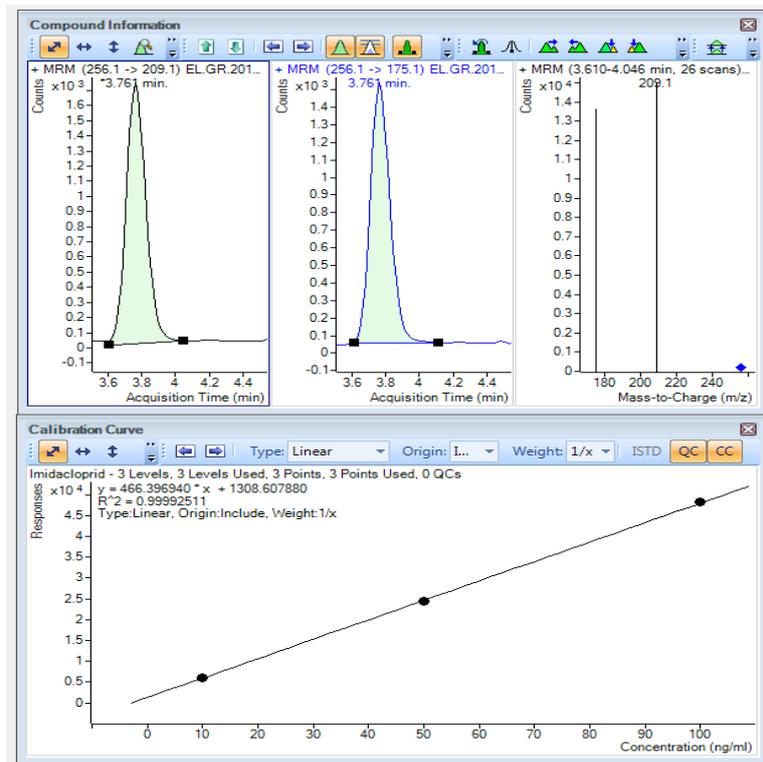
Quantifier										
Name	TS	Transition	Scan	Type	Precursor Ion	Product Ion	Uncertainty	RT	Dil. High Conc.	
Myclobutanil	1	289.0 -> 70.0	MRM	Target	289.0	70.0	Relative	6.205	100.0000	

Qualifier						
Precursor Ion	Product Ion	Transition	Rel. Resp.	Uncertainty	Area Sum	

Once the processing method is ready, calibration standards and samples are processed using it and a quantification file is obtained. The quantification file include matrix linearity and results of samples.

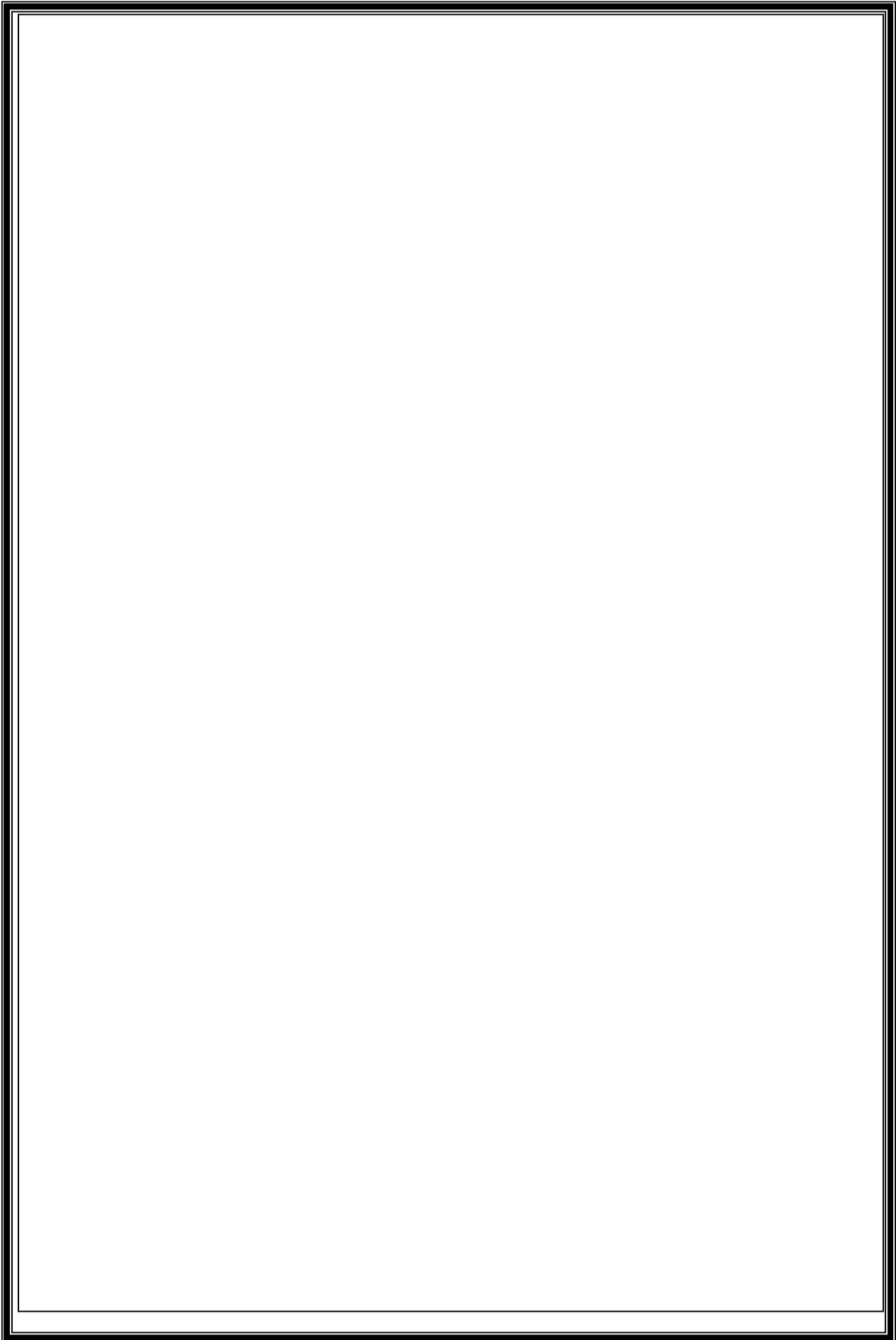
### Matrix Linearity





Lab Work Notes – Enter the MRM parameters

**Lab work notes**



## 8. Method validation

Method validation is the process used to confirm that the analytical procedure employed for a specific test is suitable for its intended use. Results from method validation can be used to judge the quality, reliability and consistency of analytical results.

### Different Parameters in method validation

- ⊙ Specificity/Selectivity
- ⊙ Linearity
- ⊙ Method Sensitivity: Limit of detection (LOD) & Limit of quantitation(LOQ)
- ⊙ Accuracy:Trueness & Precision, Repeatability &Reproducibility
- ⊙ Ruggedness
- ⊙ Uncertainty

### Standards and Reagent Preparation:

Individual standards should be prepared gravimetrically in ~1000 mg/L concentration by weighing 10 mg from each standard into a 20 mL amber screw cap vial on a five digit analytical balance and dissolving in 10 mL of appropriate solvent (acetone, toluene or acetonitrile depending on the individual compound). Concentrations of each individual standard stock solution can be calculated gravimetrically using weight of added compounds and solvents. All individual standard stocks should be stored in a freezer at -20 °C. Validity of individual standard stock solutions is 6 month. Form this working solutions can be prepared by serial dilution.

### Reagent Blank

Reagent blank should be made to identify sources of interference. To study the signals of “apparent residues” or blank signals appear due to un-removed co-extractives in the extraction process, or impurities in the solvents or reagents or instrument noise.

### Specificity/Selectivity

- Predominantly a function of the measuring technique. Can vary according to class of compound or matrix. At least 3 “processed” blanks have to undergo the whole procedure before being analyzed. Any interfering substances derived from the

analytical process to be identified.

There should be no peak(s) at the same retention time as the analyte of interest.

### **Linearity**

Ability to elicit test results that are directly proportional to the concentration of analytes in samples within a given range. To get the calibration curves standard has to prepare at six levels (matrix matched) and inject in duplicate. The linearity of calibration curves can be assessed in calibration ranges of 10–200, ng/g. Correlation coefficient values should be  $\geq 0.99$ .

Matrix effects can be evaluated by plotting of measured relative peak areas of calibration standards in solvent against the areas in the relevant matrix.

### **Limit of Detection (LOD)**

The lowest concentration that can be identified positively using a particular method in a particular matrix. Samples that do not bear residues at or above the LOD are referred to as “non-detects” (NDs).

### **Limit of Quantitation (LOQ)**

The lowest tested concentration at which an acceptable mean recovery (normally 70–110%) and RSD (normally  $< 20\%$ ) are obtained. Samples that do not bear residues  $\geq$  LOQ are often referred to as “nonquantifiable.”

### **Accuracy**

This can be defined as the closeness of agreement between a test result and the accepted reference value. It is determined by determining trueness and precision. The portion of the compound, recovered in the final determinative step, after processing of the sample (Extraction and Clean-up).

### **Trueness**

Method trueness can be assessed by recovery studies using blank matrices spiked at three concentration levels (L1, L2 and L3) and inject in six individually prepared replicates. Spiking of samples should be prior to sample preparation. The sample concentrations, recovery and relative standard deviation (% RSD) can be calculated. According to SANCO requirements recovery values are deemed acceptable if between 70–120%.

The analytical material is fortified, extracted and analyzed.

$$\text{Recovery (\%)} = (\text{Recovered Concentration} / \text{Added Concentration}) \times 100$$

### **Precision**

Instrument injection precision was tested for both retention time and peak area for all target compounds by subsequent injections ( $n=6$ ) of low concentration level (L1) standard solutions. Instrument injection precision for retention time should be below 2% and for area below 5% for indicating reliable instrument performance.

### **Repeatability**

The precision under conditions where independent test results are obtained with the same

method, identical test material, same laboratory or same operator.

### **Reproducibility**

Precision under reproducibility conditions like same method, identical test items, different laboratories, different operators, and different equipment.

### **Ruggedness**

Defined as: “The susceptibility of an analytical method to changes in experimental conditions which can be expressed as a list of the sample materials, analytes, storage conditions, environmental and / or sample preparation conditions under which the method can be applied as presented or with specified minor modifications.

Conditions which could in practice be subject to fluctuation (e.g. stability of reagents, composition of the sample, pH and temperature) any variations which could affect the analytical result should be indicated.”

### **Uncertainty**

Uncertainty can be estimated by the standard deviation calculated on within laboratory reproducibility.

## **Laboratory Session on Method Validation:**

### **Specificity:**

- To check the retention time of an individual analyte, inject individual analyte solution six times at one concentration.
- Calculate standard deviation and RSD (Relative Standard Deviation) for retention time.
- To check retention Time of Mixture of analytes, inject Mixture of standard solution six times at one concentration.
- Calculate standard deviation and RSD for retention time.
- To study on Area of individual analyte, inject individual analyte solution six times at one concentration.
- Calculate standard deviation and RSD for retention time.
- To study on Area in mixture of analytes, inject Mixture of standard solution six times at one concentration.
- Calculate standard deviation and RSD for retention time.

### **Linearity**

#### **a) Linearity on Standard**

- Individual: Inject 5 different linear concentrations in triplicate. Calculate % RSD for the area under the peak.
- Mixture: Inject mixture of an analytes at 5 different concentrations in triplicate.
- Calculate standard deviation and RSD for the area under the peak.

#### **b) Matrix Match Standard**

- Prepare the extract of sample matrix.
- Prepare required 5 concentrations standard dilutions with sample extract (matrix).
- Inject sample matrix blank in triplicate.

- Inject mixture of standard solution in sample matrix three times at 5 linear concentrations for GC-MS/MS & LC-MS/MS.

**Determination of LOD (Level of Detection):**

- Spike the sample with standards at minimum five levels starting from lowest at which all analyte are detected plot the calibration curve and from the curve calculate LOQ using following formula:
- $LOD = 3 \times (\text{Standard Deviation} / \text{Slope})$ .

**Determination of LOQ (Level of Quantitation):**

- Spike the sample with standards at minimum five levels starting from lowest at which all analyte are detected and plot the calibration curve and from the curve calculate LOQ using following formula:
- $LOQ = 10 \times (\text{Standard Deviation} / \text{Slope})$

**Accuracy**

- Spike the sample at LOQ, 5 times LOQ, and 10 or 50 times LOQ level in 6 replications each.
- Calculate % Recovery and RSD for recovery.

**Repeatability**

- Spike the sample at 5 times LOQ in 3 replication each.
- Calculate % Recovery and RSD for recovery on same day.

**Reproducibility**

- Spike the sample at 5 times LOQ level in 3 replication each.
- Calculate % Recovery and RSD for recovery with other analyst, other day.

**Ruggedness**

Spike the sample at 5 times LOQ. Carry out the analysis it will be repeated by change in analyst or change in technique or change in equipment or change in analytical column (i.e. Different Brand column).

**ACCEPTANCE CRITERIA:**

**For Pesticide Residue Analysis**

Sr. No.	Parameters	Acceptance Limit
1	<b>Linearity</b> – The coefficient of correlation	> 0.99
2	<b>Specificity</b> – RSD of Retention time RSD of Area	$\leq 2.0 \%$ $\leq 5.0 \%$

3	<b>Matrix Match Standard – Matrix Interference</b>	No interference of matrix in the method
4	<b>Accuracy –</b> % Recovery & RSD at LOQ % Recovery & RSD at 5 times LOQ % Recovery & RSD at 10 times	% Recovery should be within 70 – 130% RSD should be $\leq 20\%$ .
5	<b>Repeatability –</b> % Recovery & RSD at 5 times LOQ	% Recovery should be within 70 – 130% RSD should be $\leq 15\%$ .
6	<b>Reproducibility</b> % Recovery & RSD at 5 times LOQ	% Recovery should be within 70 – 130% RSD should be $\leq 15\%$ .
7.	<b>Ruggedness</b> % Recovery & RSD at 5 times LOQ	% Recovery should be within 70 – 130% RSD should be $\leq 15\%$ .

**Laboratory safety:**

Safety is our priority and will enforce all normal laboratory safety procedures applicable  
Please follow the safety precautions

1. Laboratory coat should be worn in the laboratory
2. Use fume hoods whenever handling volatile or hazardous chemicals
3. Safety goggles should be worn at all times in the laboratory
4. Appropriate gloves should be worn as needed
5. Gloves should not be worn outside the laboratory
6. Label all your samples

In the event of an emergency please contact one of the supervising instructors immediately.

## 9. Reference

- SANTE/11945/2015: Guidance document on analytical quality control and method validation procedures for pesticides residues analysis in food and feed.
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- AOAC Official Method 2007.07 Pesticide Residues in Food by Acetonitrile Extraction and Partitioning with Magnesium Sulfate. Gas Chromatography/Mass Spectrometry and Liquid Chromatography/Tandem Mass Spectrometry.
- Multiresidue Analysis of Multiclass Plant Growth Regulators in Grapes by Liquid Chromatography/Tandem Mass Spectrometry  
Dasharath P. Oulkar, Kaushik Banerjee, Manoj S. Ghaste, and Sahadeo D. Ramteke, Journal of AOAC International, 2011, 94, 3.
- Quantitative Screening of Agrochemical Residues in Fruits and Vegetables by Buffered Ethyl Acetate Extraction and LC-MS/MS Analysis  
Manjusha R. Jadhav, Dasharath P. Oulkar, Ahammed Shabeer T. P., and Kaushik Banerjee, J. Agric. Food Chem, DOI: 10.1021/jf505221e.