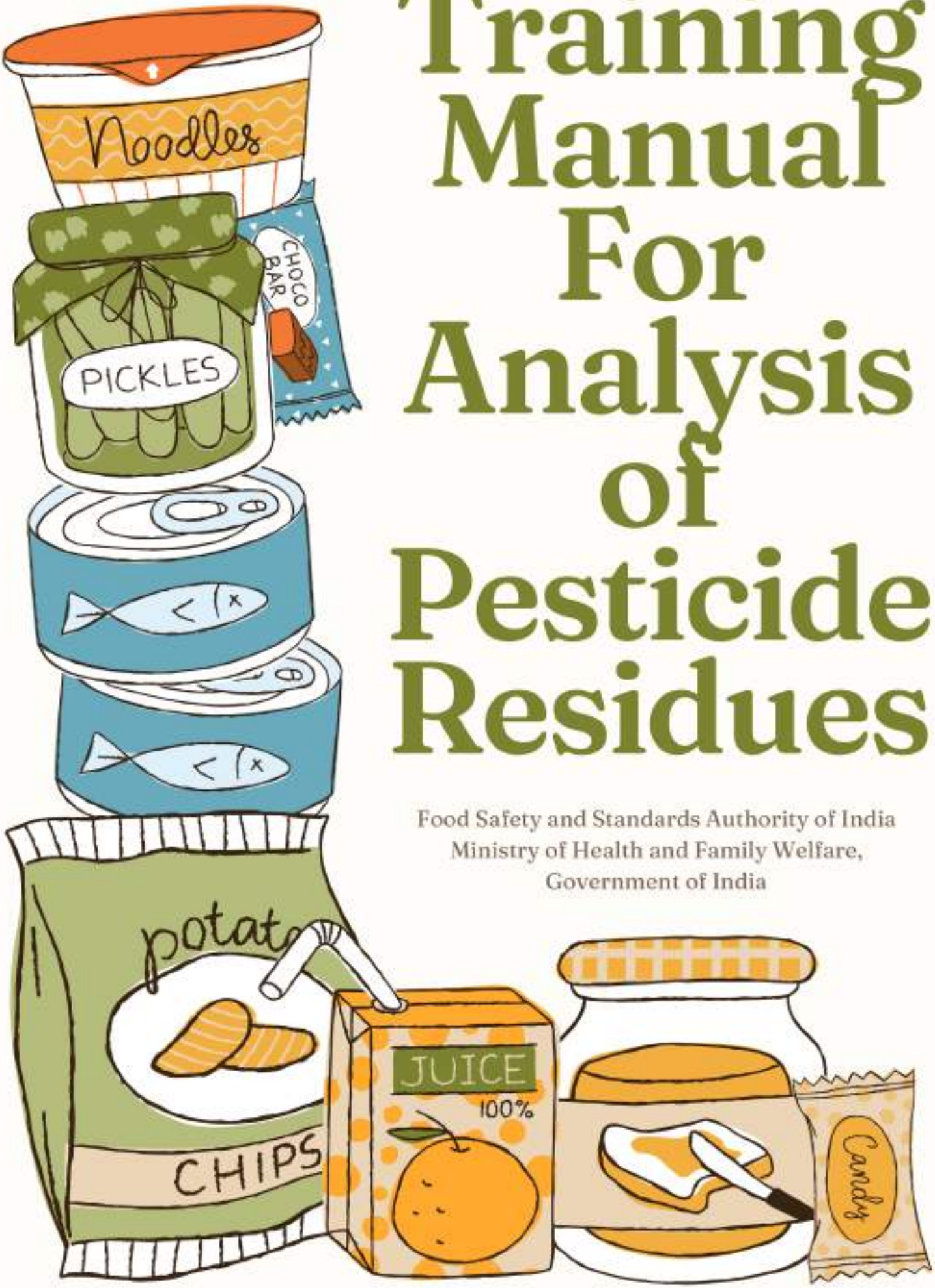
Training Manual For Analysis of Pesticide Residues



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

CONTENTS

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 foranalysis.

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, methanolor 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 µg/mL.
- Weight of standard (corrected, a) = [weight of standard x Purity]/ 100
- Volume of solvent (mL, b) = [weight of solvent / Specific gravity]
- Calculated conc.(µg/mL) = [Corrected weight of standard (a)/ Volume of solvent (b)]*1000
- Label the container with lab code, name of chemical, concentration in µg/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 µg/mL

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

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

Calculated conc.(µg/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 about10 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 µg/mL.

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

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

Calculated conc.(µg/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.

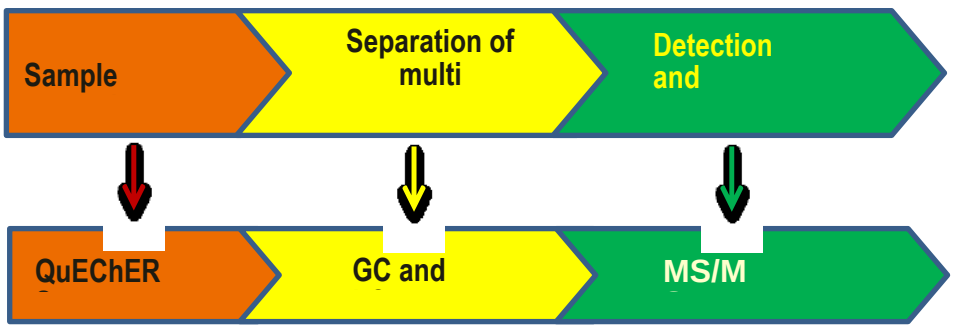
An ideal extraction procedure should be capable of extracting 100% of pesticides only, leaving behind other materials. However, this is not the actual practice. An extraction method should satisfy the following requirements:

- Rapid
- Small solventusage
- Good recovery of the target pesticides: 70 to120%.
- Robust: RSD (relative standard deviation) of the recoveries less than 20% atLOQ.

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 analyzepesticides.

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 vegetablesare the target food products for thissession.
2. **Equipment:**
    - Blender or Homogenizer (Highvolume)
    - Homogenizer (Highvolume)
    - Centrifuge: Rotor head with holding capacity: 2 mL, 10 -15 mL and 50 mL centrifugetubes
    - GC-MS/MS andLC-MS/MS
    - Electronic Balance: Capacities 3-5 kg & 0.01 to 100g
    - Vortexshaker
    - Solvent evaporator
    - Muffle furnace: Capable of 500°Coperation
3. **Apparatus and Reagents:**
    - Acetonitrile (MeCN): Quality of sufficient purity.
    - Anhydrous sodium acetate (NaOAc): Powder form ($NaOAc.3H_2O$) may be substituted, but 0.17 g per g of sample must be used rather than 0.1 g anhydrous NaOAc per gof sample.
    - Anhydrous magnesium sulfate ($MgSO_4$): Powder form; purity > 98%; heated to 500°C for > 5 hr to remove phthalates and residualwater.

- Acetic acid (HOAc): Glacial, quality of sufficientpurity.
- TPP: Triphenylphosphine
- Primary Secondary Amine: 40 µm particle size.
- Methanol (MeOH): Quality of sufficient purity forLC-MS/MS
- Water: Quality of sufficient purity forLC-MS/MS
- Formic acid: Quality of sufficientpurity
- Ammonium acetate: Quality of sufficientpurity
- Ethyl acetate: Quality of sufficientpurity
- N-Hexane: Quality of sufficientpurity
- DistilledWater
- 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 analyticalportion 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 uniformmatrix.

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 withvertical 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 $MgSO_4$, 1.5 g of anhydrous sodium acetate and 75 µL I. S. solution
4.	Shake vigorously for 1 min. Centrifuge at 4000 rpm for 1 min.
5.	Transfer 1mL supernatant into 15 mL centrifuge tube containing 50mg PSA & 150mg anhydrous $MgSO_4$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 0.25mL extract and 0.75 mL water to LC vail
8.	Analysis by GC-MS/MS and LC-MS/MS

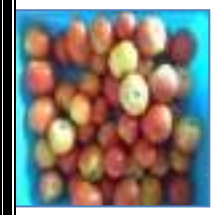
Laboratory Session: Sample Extraction for LC and GC

Extraction procedure for LC

- Label ten 50 mL disposable screwcap centrifuge tubesas
    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 theweight
- Spike the sample in the spike labelled tubes with knownconcentration.
- Start the extraction process as given in the extraction procedureearlier.
- After the filtration of the sample prepare Matrix match standard by using blank extracted matrix
- Now the samples in the vials are analyzed byLC-MS/MS and GC-MS/MS

Sample Preparation, Extraction & Clean -up



Crush/Grind

Weigh 15 g in 50 mL centrifuge tube

Centrifuge

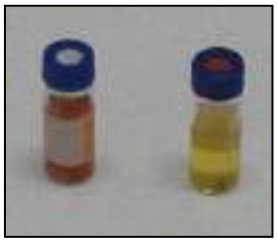
Homogenize

15 mL (1%) Acetic acid in acetonitrile + 6 g $MgSO_4$ + 1.5 g SodiumAcetate

Vortex (30 sec)

1-8 mL supernatant + 50 mg PSA + 150 mg $MgSO_4$

Vortex (30 sec)



Sample filtration

Transfer 0.6 mL supernatant in GC vial.

Transfer 0.25 mL extract and 0.75 mL water to LC vail

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 secondsand centrifuge it for 5 minutes at 10000 rpm.
- Draw 2 mL supernatant in the test tube containing 200 μL 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 thesesolvents 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 μm Nylon 6, 6 membrane filter into a LC vial.
- Inject 5 - 20 μL 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 chartof ethyl acetate method extraction

10 g Sample + 10 mL Ethyl acetate

↓ Vortex for 1 min.

Add 10 g anhyd.Na_2SO_4

↓ Vortex for 2 min.

Centrifuge at 5000 rpm for 5 min.

LC-MS/MS	**GC-MS/MS**
5 mL supernatant + 25 mg PSA	1 mL supernatant + 25 mg PSA
↓	↓
Vortex for 30 sec.	Vortex for 30 sec.
↓	↓
Centrifuge for 5 min. at 10,000 rpm	Centrifuge for 5 min. at 10,000 rpm
↓	↓
Draw 2 mL supernatant + 200 µL DEG	Take supernatant in auto sample vial
↓	
Evaporate it to dryness under N_2 at 35ºC	
↓	
Reconstitute with 1 mLMeOH and 1 mL 0.1% Acetic acid in water	
↓	
Sonicate for 1 min. and vortex for 30 sec.	
↓	
Centrifuge extract 5 min. at 10,000 rpm	
↓	

Filter extract 0.2 μm 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_4), 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_{18}: 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 chartof plant growth regulators extraction method

10 g Sample + 20 mL (with 1 % Formic acid) MeOH and add internal standard (CCC d_4)

Vortex for 1 min.

Centrifuge at 5000 rpm for 5 min.

0.5 mL extract + 0.5 mL H_2O [For C_{18} Column]

0.5 mL extract + 0.5 mLMeOH [For HILICcolumn]

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 (±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 chartfor analysis of dithiocarbamate residues in sample

250 ml of screw cap bottle

Weigh 25 g of grape berry or homogenized sample

Add 75 ml SnCl2 reaction mixture

Add 25 ml of isooctane

Keep on water bath at 80°C for 1 hour

Intermediate shaking after every 20 min.

Keep bottle at room temperature or in normal temperature of water

Transfer the bottle in ice cold water.

Two separate layers

Take upper layer 1-2 ml

Micro-centrifuge at 10 °C

Transfer to GC vial

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 ofHCl.

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 inHCl with stirring. Finally a clear solution isobtained.

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 isthe 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_2 about 20 µL, cap the bottle and note down the weight in mg. This is the weightof the CS_2 standard.

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

Actual concentration of CS_2 stock std solution (C_1*) = (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) Actual weight of the Thiram Standard (x) = (Weight of standard × 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.

Weight of the Solvent in mL (y) =

(Weight of the solvent in gram/ density of the ethyl acetate solvent in (g/mL))

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

Actual concentration of thiram mother (stock) solution (C_1) =

(Actual weight of the Std (x)/ mL of the solvent (y))

l) Use isooctane for further dilution. Prepare working standard (C_2) 1µg/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_2

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_2.

b) Prepare matrix matched standard at the same concentrations by extracting fresh sample usingthe procedure and spiking the extract with appropriate volumesof working standards (1µg/mL)

Recovery sample preparation

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

Immediatelyclose 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 chromatorgraphy (HPLC) and Gas chromattography (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

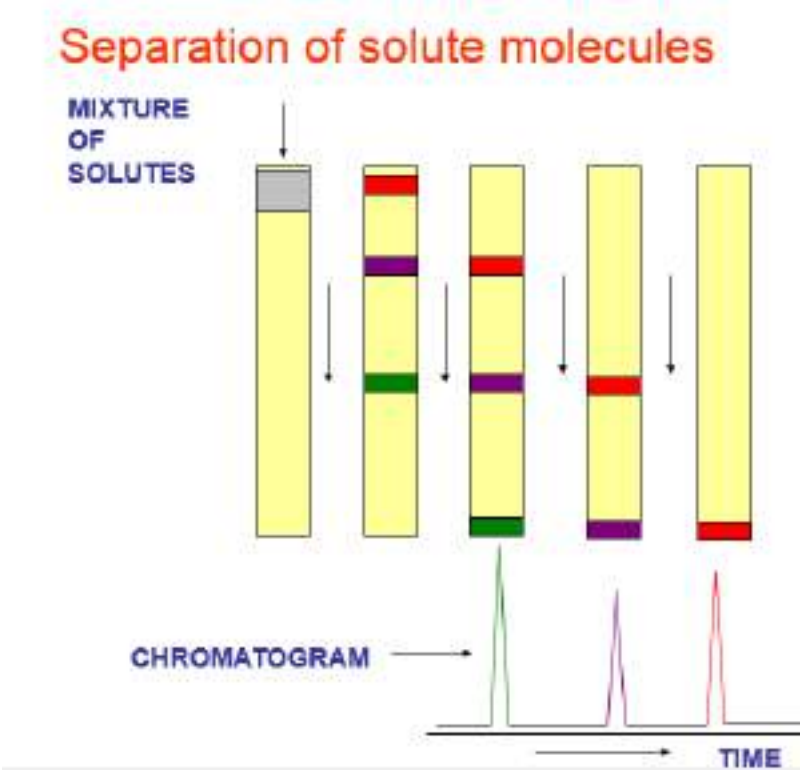


Fig 1: Schematic representation of principle of chromatography

Parts of GC

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

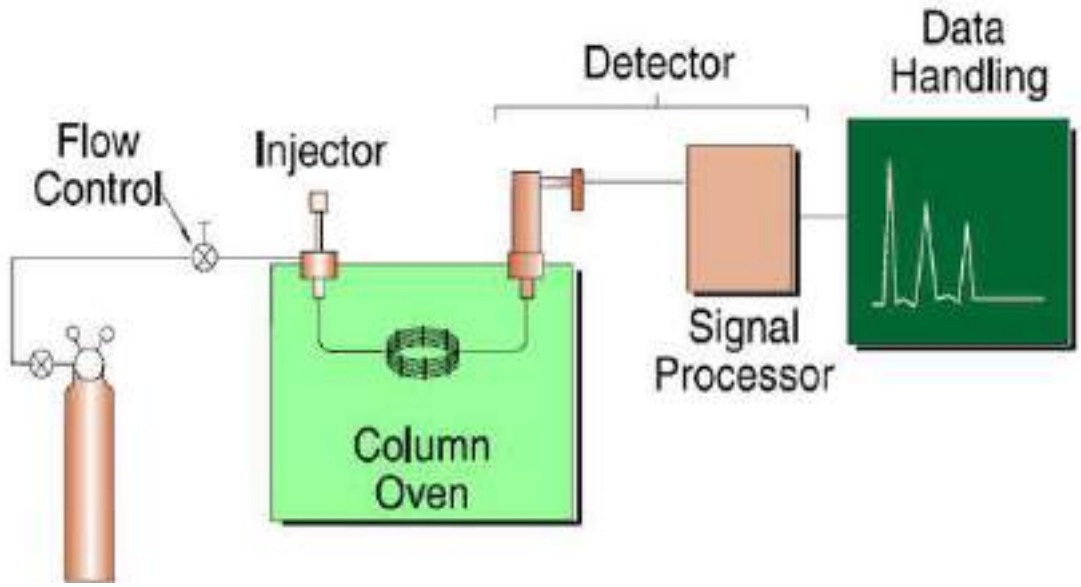


Fig 2: Schematic representation of Gas chromatography

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

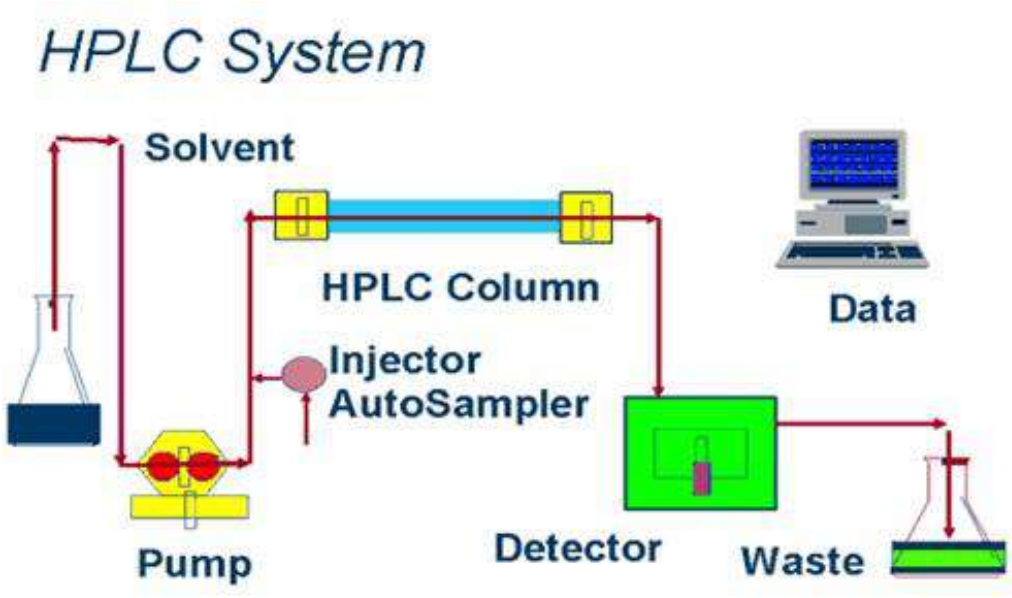


Fig 3: Schematic representation of High Performance Liquid Chromatography

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.

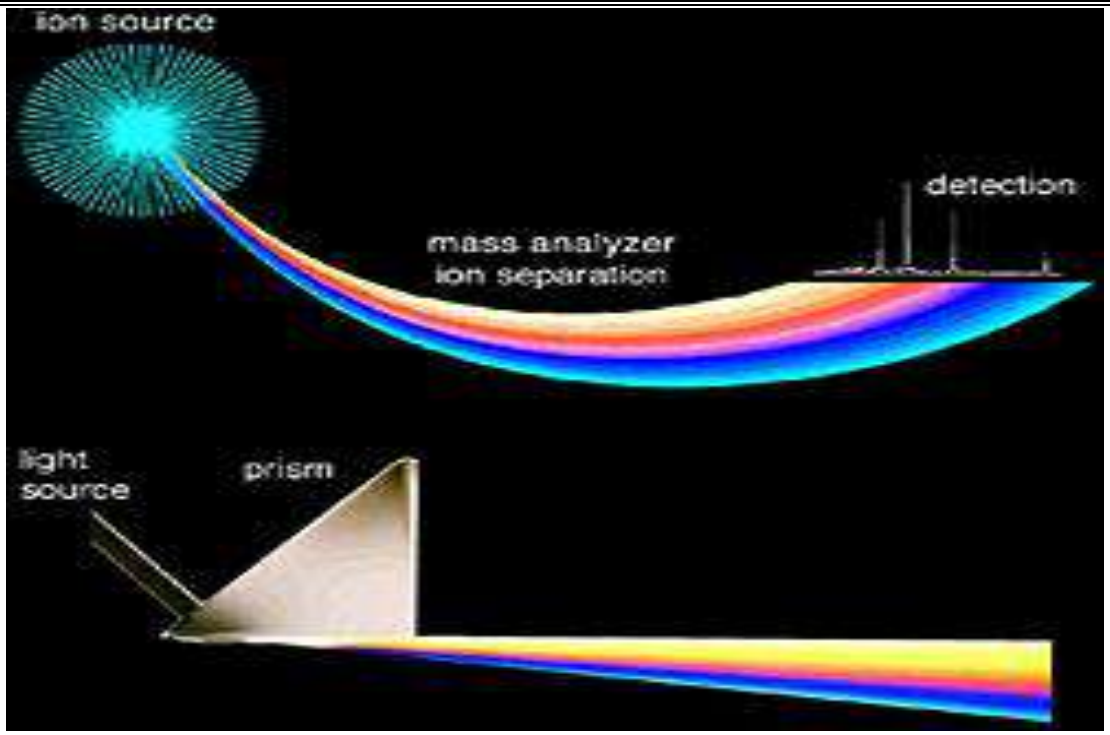


Fig 4: The mass analysis process as compared to the dispersion of light by a prism.

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

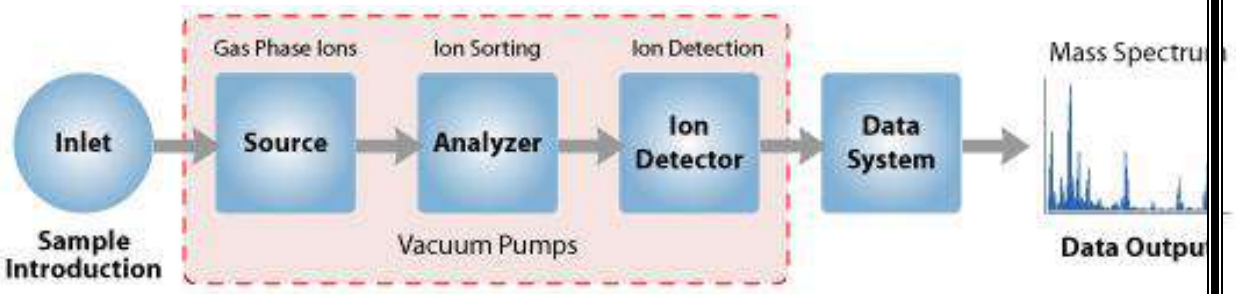


Fig 5: Schematic representation of Mass Spectrometer

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 gasit 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)

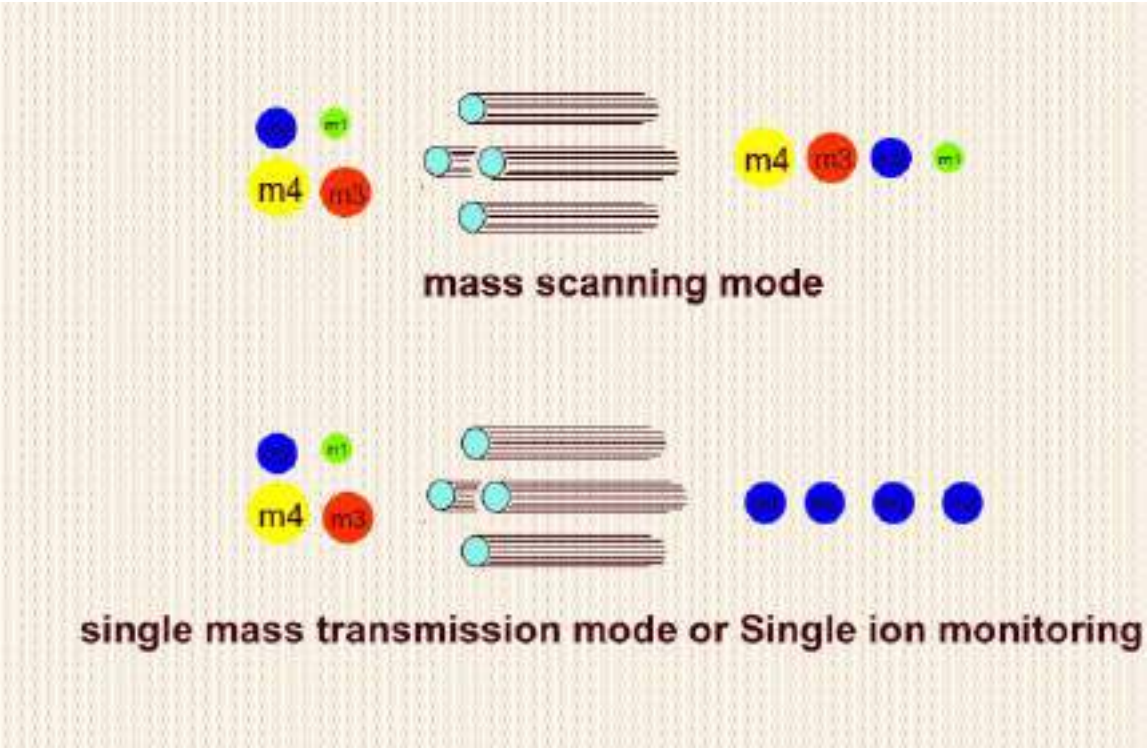


Fig 6: Schematic representation of Triple QuadrupoleMass Spectrometer with Scan and SIM modes

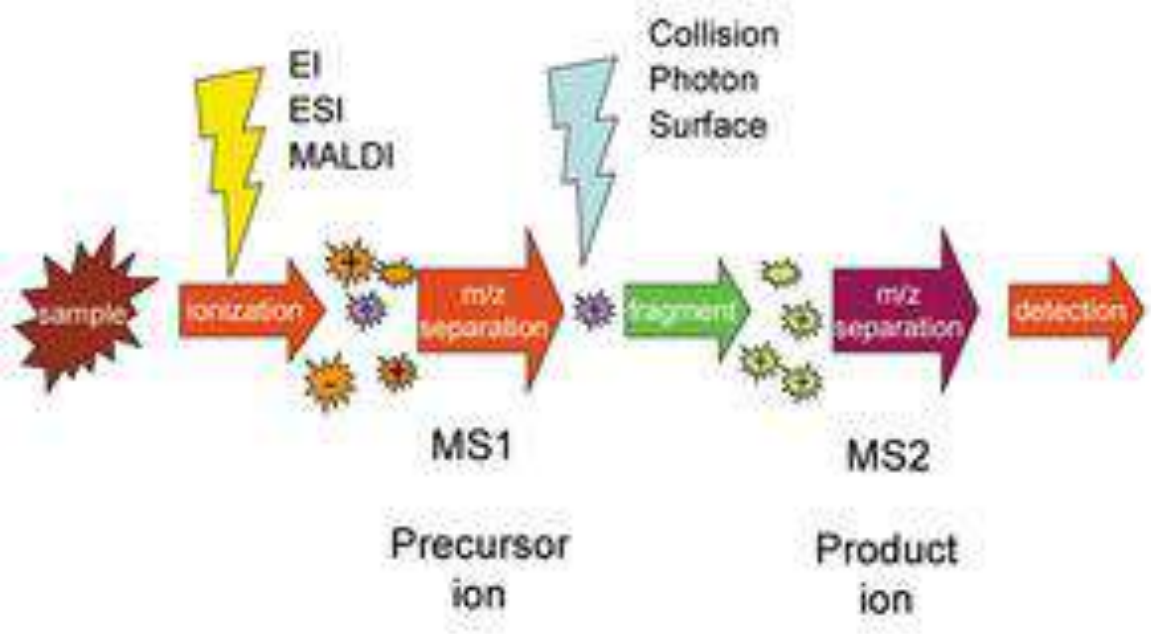


Fig 7: Schematic representation of Triple QuadrupoleMass Spectrometer with SRM mode

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 (~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.

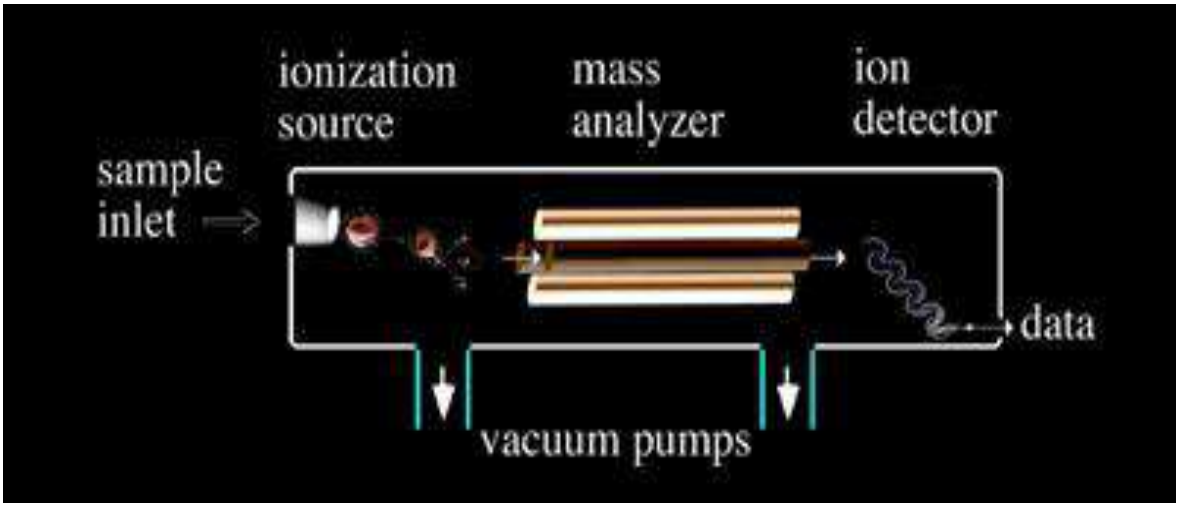


Fig 8: Mass spectrometer

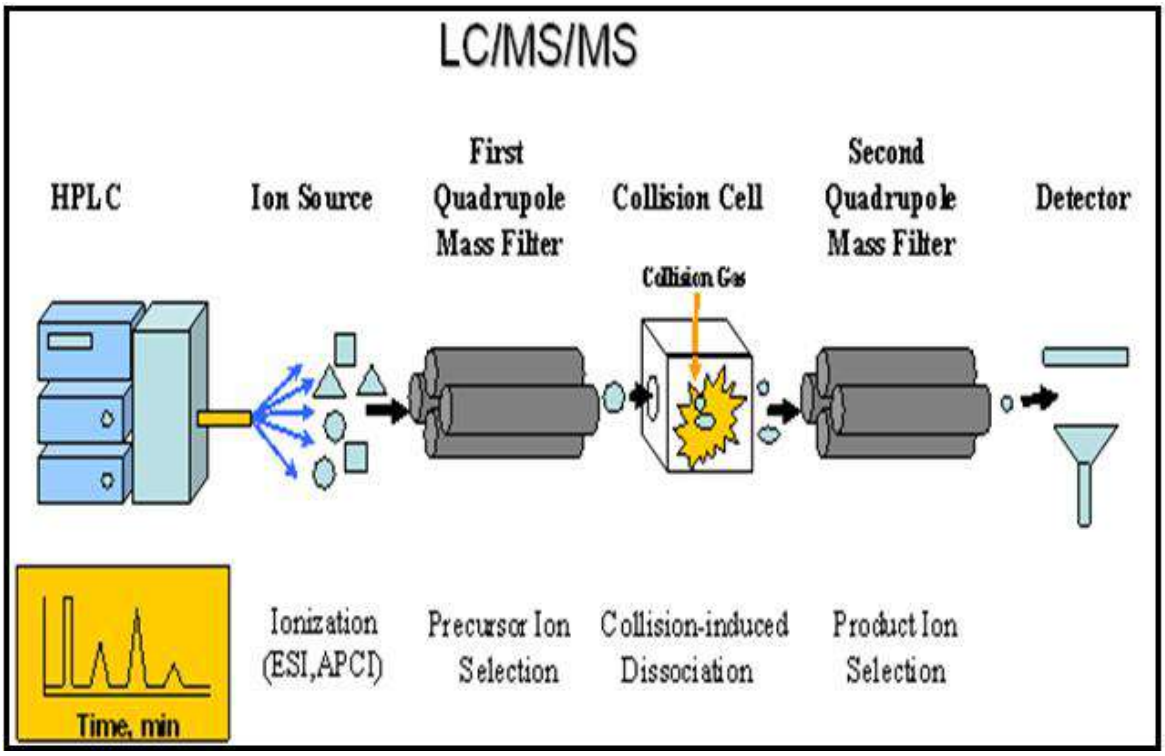


Fig 9: Components of a mass spectrometer

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.

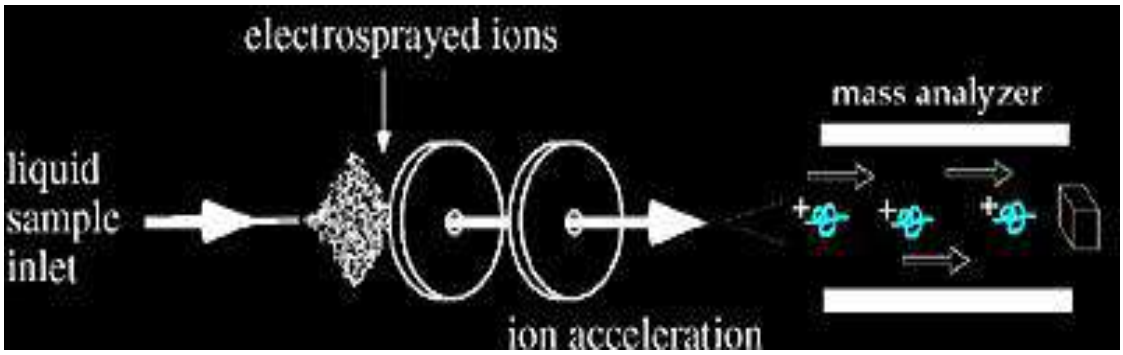


Fig 10: Electro spray ionization (ESI) mass spectrometry

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.

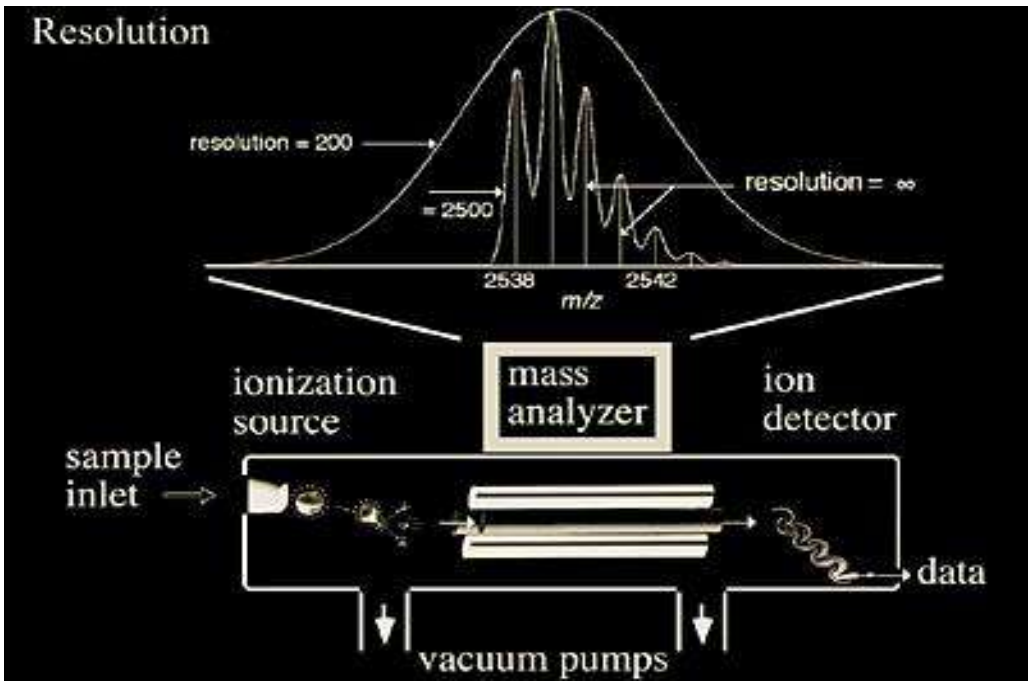


Fig 11: The effect of resolution up on mass accuracy

Tuning the mass spectrometer

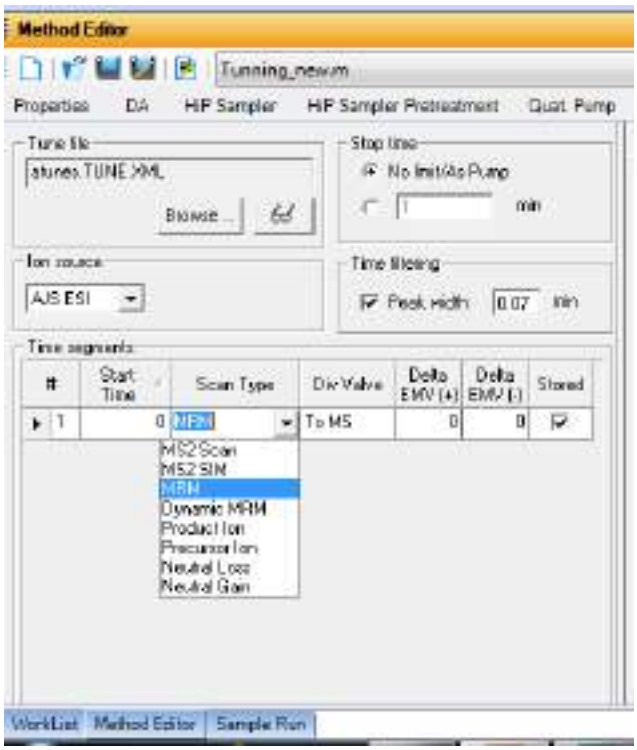
Tuning is the process of selecting all the parameters that will be used in MS acquisition method. These include

- 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 spinetoramin 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 fragemnted.

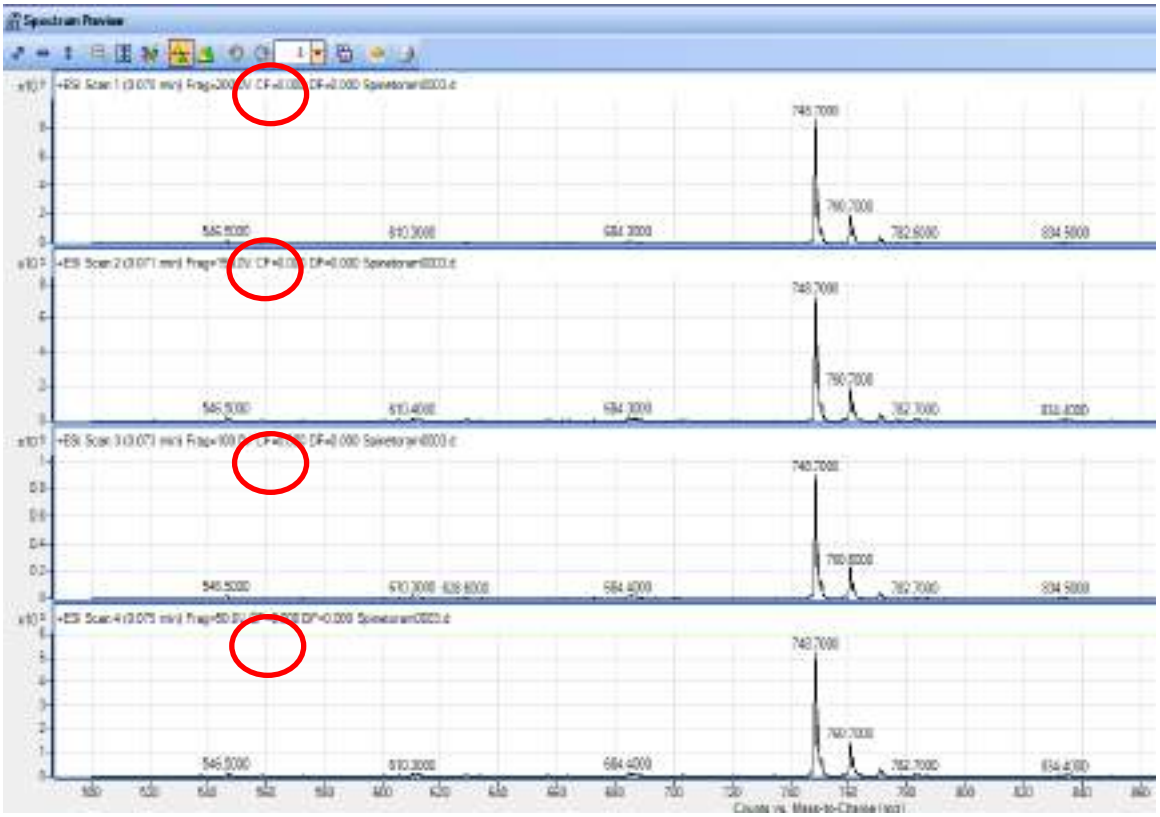
To begin with choose an intermediate voltage from the rages available and subject mass 224 to fragmentation.

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

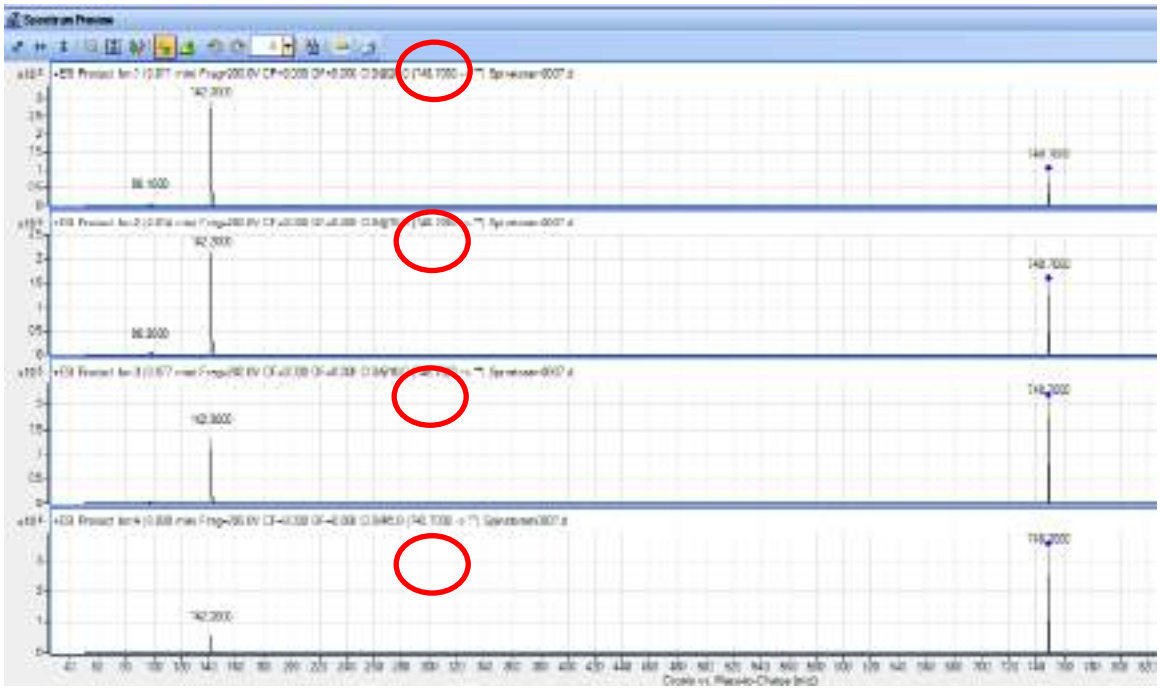
- Select a higher mass product ion over a very low mass product ion
- A small intensity of the molecular ion also should be visible, which proves that the product ions are formed, from this molecular ion.

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

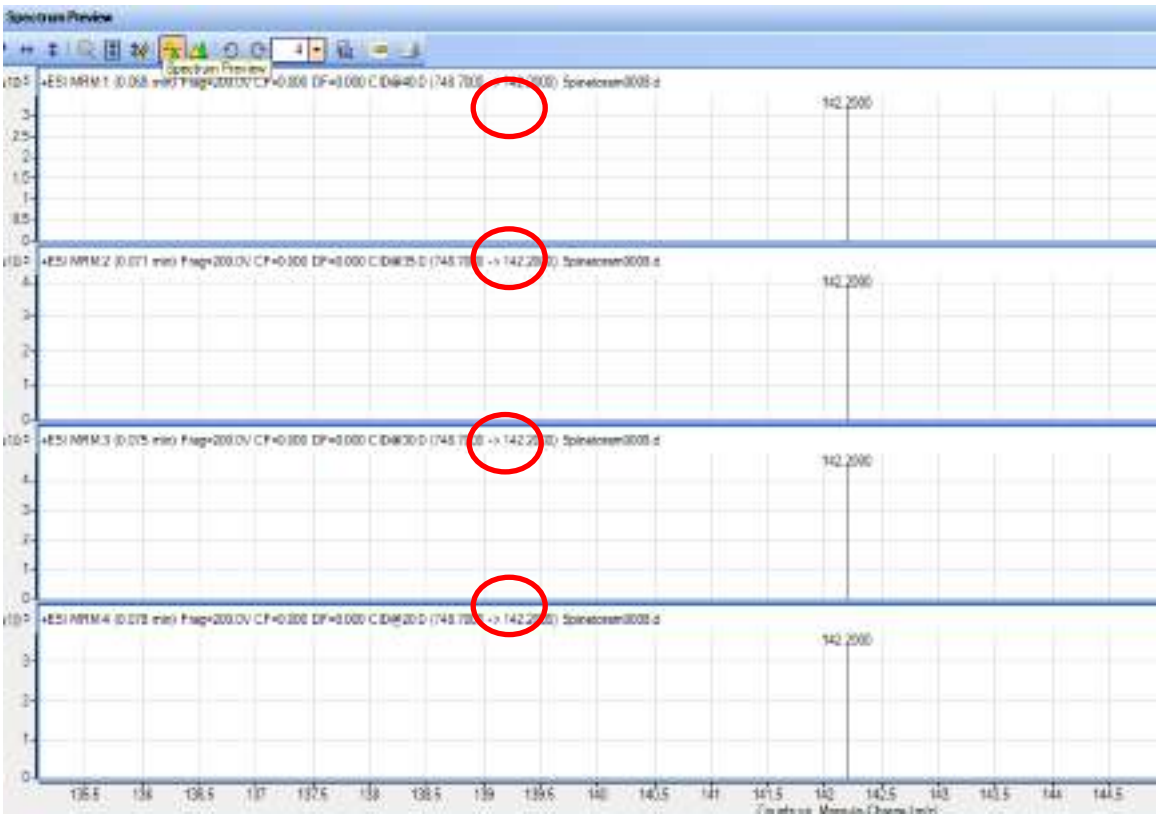
MS2 Scan Spinetoram



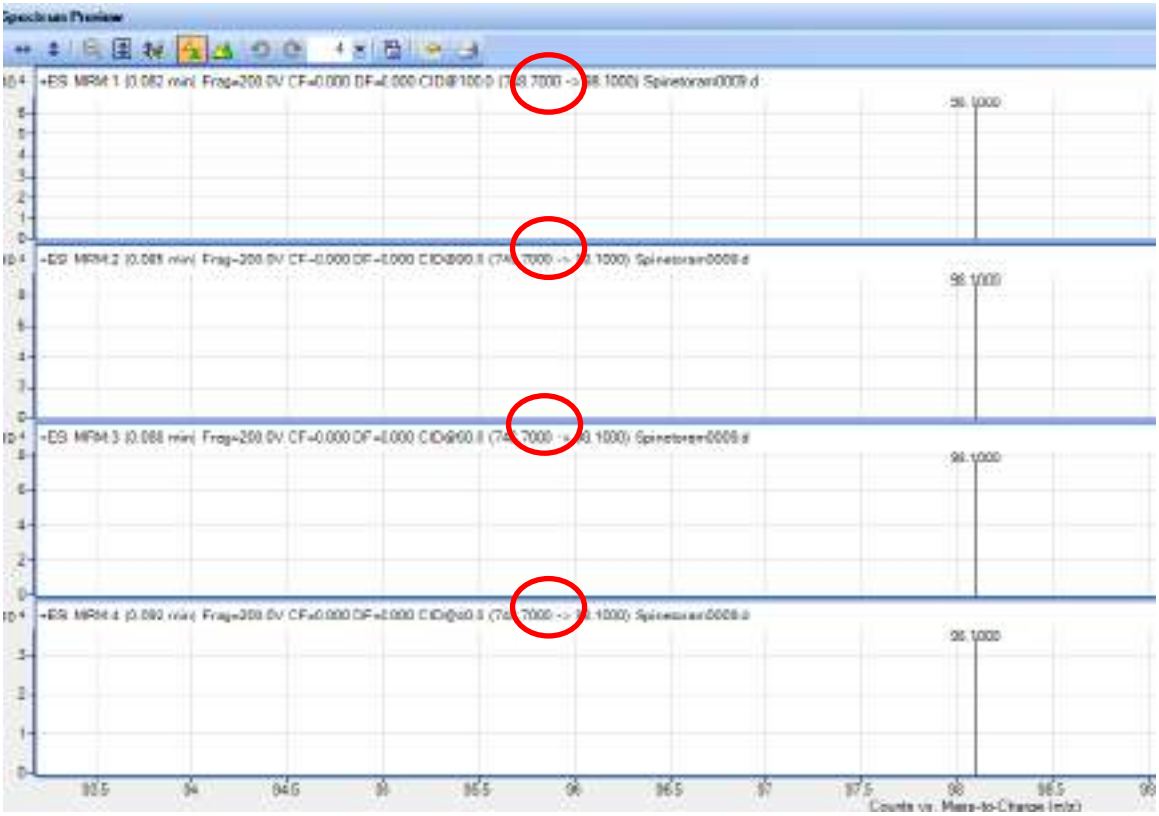
Fragmentation of molecular ion (749) into Product Ions



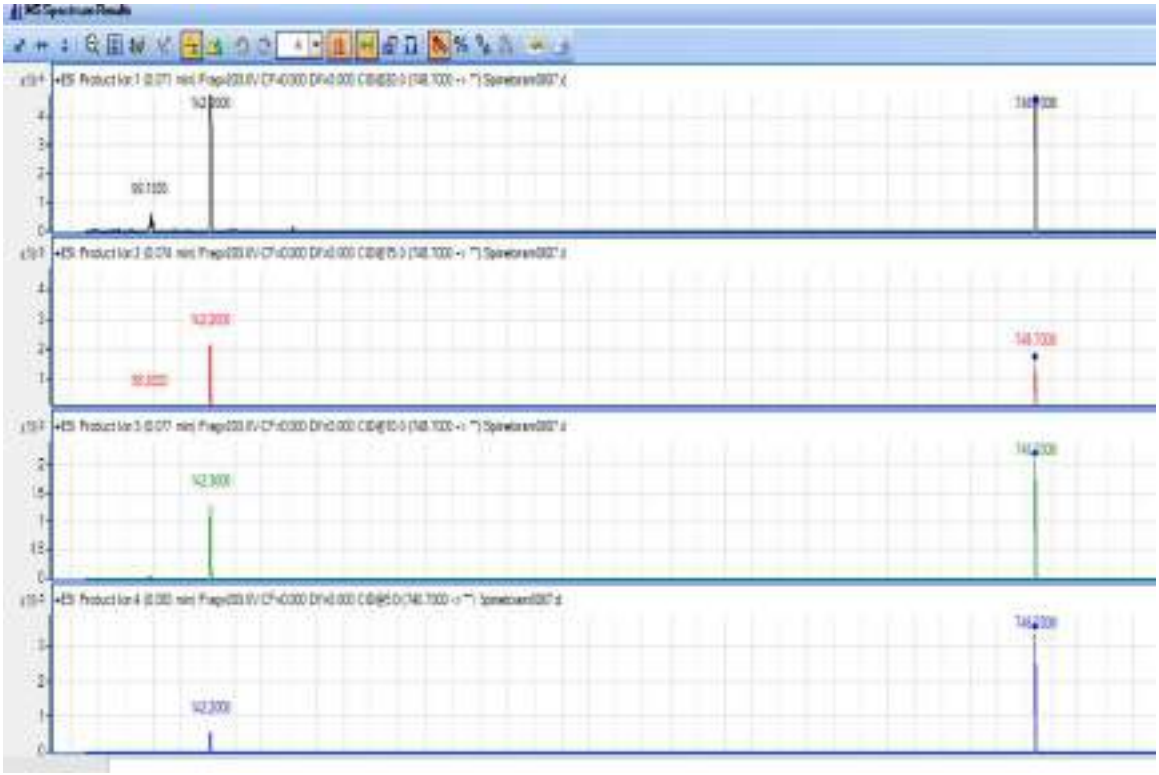
Optimization of Product Ion 749 →142



Optimization of Product Ion 749 →98

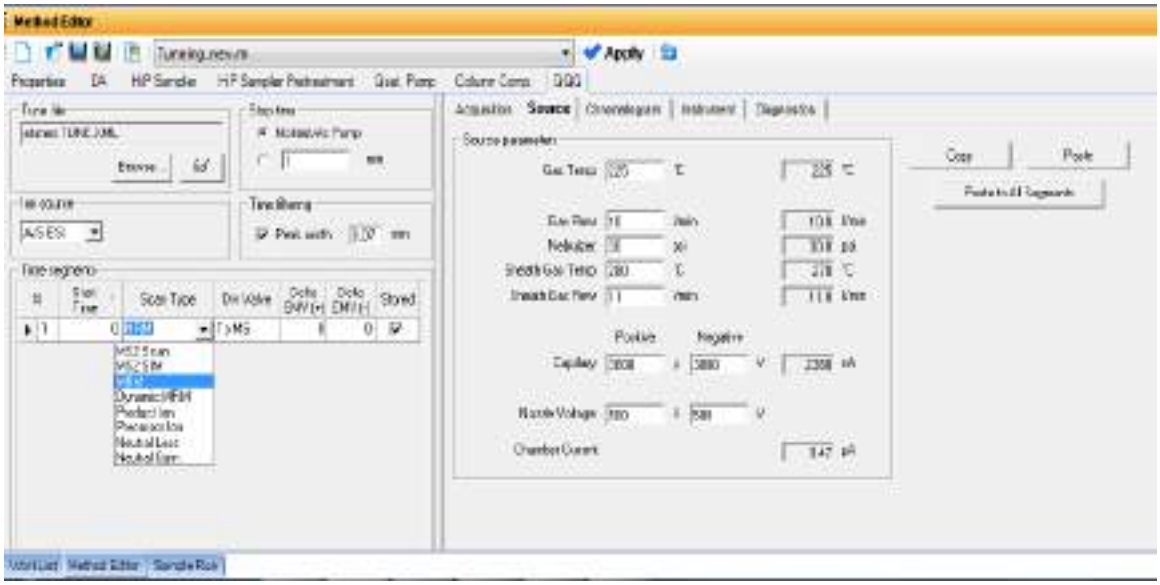


Confirmation of MRM



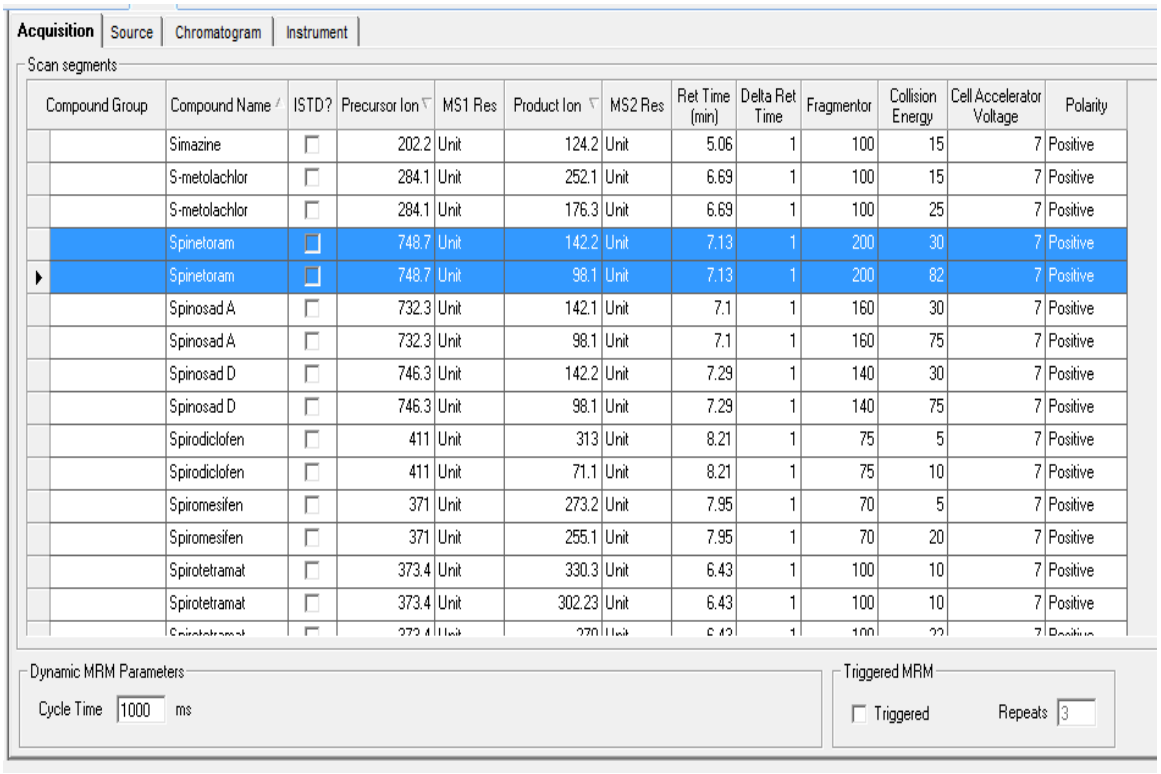
Apart from parent and product ions, Source parameters have to be optimized for highest response of analytes. These parameters include gas flow, nebulizer temperature and sheath gas temperature, sheath gas flow, capillary and nebulizer voltages etc.

Mass Parameters



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

Acquisition method



Acquisition | Source | Chromatogram | Instrument

Scan segments

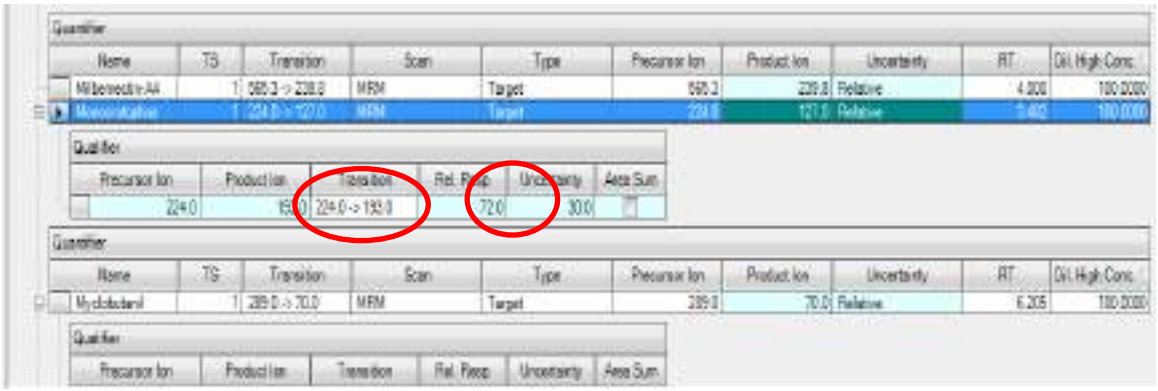
Compound Group	Compound Name	ISTD?	Precursor Ion	MS1 Res	Product Ion	MS2 Res	Ret Time (min)	Delta Ret Time	Fragmentor	Collision Energy	Cell Accelerator Voltage	Polarity
	Simazine	☐	202.2	Unit	124.2	Unit	5.06	1	100	15	7	Positive
	S-metolachlor	☐	284.1	Unit	252.1	Unit	6.69	1	100	15	7	Positive
	S-metolachlor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

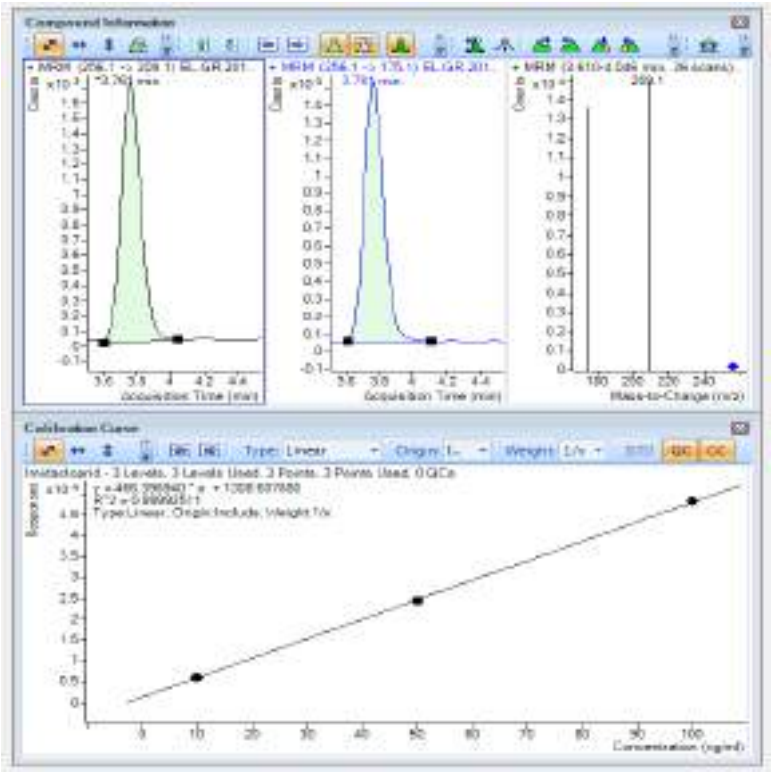
The calibration standards and samples are analysed using this acquisition method. For quantification of samples, a processing method needs to be set up. This processing method addresse following points - Name of the pesticides, their retention times, their MRMs, Calibration levels, quantification ion, 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



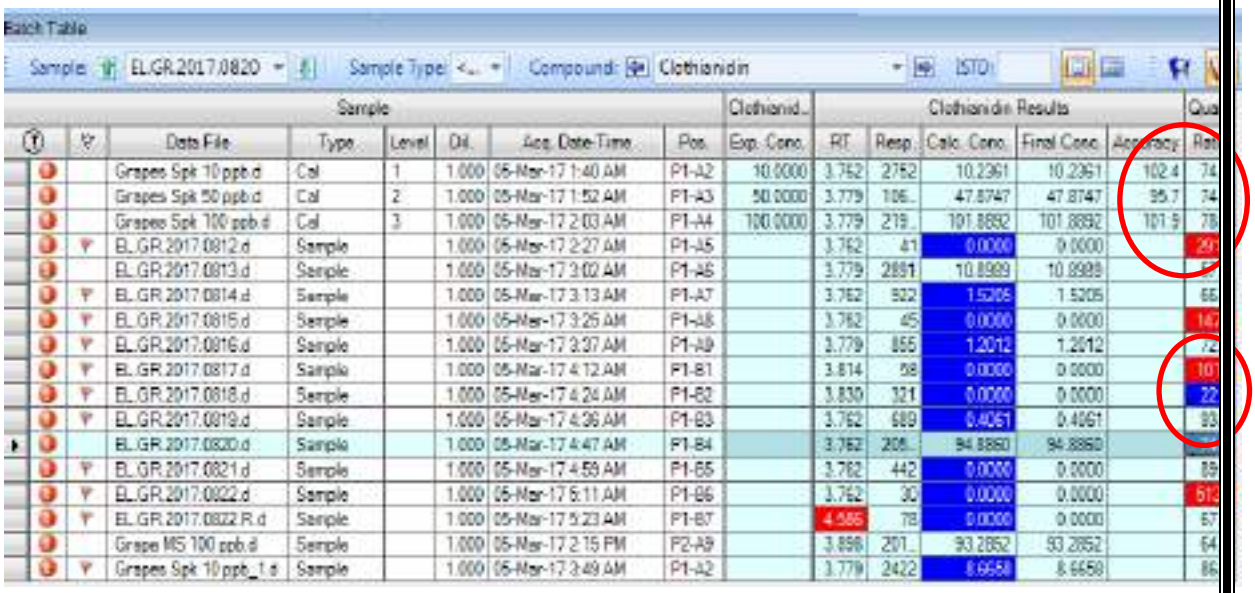
Once the processing method is ready, calibration standards and samples are processed using it and a quantification file is obtained. The quantification file include matrix linearity and results of samples.

Matrix Linearity



Once the results are obtained, confirmation needs to be carried out based on calibration, retention time, area of quantifier ion, qualifier: quantifier ratio and recovery of the pesticide at the LOQ level.

Verification of results based on the ion ratios



Lab Work Notes – Enter the MS parameters

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 beidentified.

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 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 ≥ 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.

Recovery (%) = (Recovered Concentration/Added Concentration) x100

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

Linearity

a) Linearity onStandard

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

b) Matrix Match Standard

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

- 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 followingformula:
- LOD = 3 x (Standard Deviation /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 followingformula:
- LOQ = 10 x (Standard Deviation /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 forrecovery.

Repeatability

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

Reproducibility

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

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 PesticideResidue Analysis

Sr. No.	Parameters	Acceptance Limit
1	**Linearity –** The coefficient of correlation	> 0.99
2	**Specificity –** RSD of Retention time RSD of Area	≤2.0 % ≤5.0 %

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

Laboratory safety:

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

1. Laboratory coat should be worn in thelaboratory
2. Use fume hoods whenever handling volatile or hazardouschemicals
3. Safety goggles should be worn at all times in thelaboratory
4. Appropriate gloves should be worn asneeded
5. Gloves should not be worn outside thelaboratory
6. Label all yoursamples

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.
- European Committee for Standardization/Technical Committee CEN/TC275 (2008), Foods of plant origin: Determination of pesticide residues using GC-MS and/or LC- MS/MS following acetonitrile extraction/ partitioning and cleanup by dispersive SPE QuEChERS-method.
- Validation of a GC–MS method for the estimation of dithiocarbamate fungicide residues and safety evaluation of mancozeb in fruits and vegetables
  SumaiyyaMujawar, Sagar C. Utture, Eddie Fonseca, Jessie Matarrita, Kaushik Banerjee, Food Chemistry, 2014, 150, 175–181.
- Multiresidue Analysis of Plant Growth Regulators in Grapes by Triple Quadrupole (QqQ) and Quadrupole–Time of Flight (Q-ToF) Based Liquid Chromatography/Mass Spectrometry
  Dasharath P. Oulkar and Kaushik Banerjee, Journal of AOAC International Vol. 2011, 94, 6.
- 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 Regulatorsin Grapes by Liquid Chromatography/Tandem MassSpectrometry
  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 andVegetables byBuffered Ethyl Acetate Extraction and LC-MS/MSAnalysis
  Manjusha R. Jadhav, Dasharath P. Oulkar, AhammedShabeer T. P., and Kaushik Banerjee, J. Agric. Food Chem, DOI: 10.1021/jf505221e.