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# PESTICIDE RESIDUE AND PHYTOCHEMICAL ANALYSIS IN TOBACCO AND CHILLI


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**Manual on Pesticide Residue and Phytochemical Analysis in Tobacco and Chilli**

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**May, 2024**

# Preface

To surge the commercial agriculture, providing sustenance for millions of farmers, increasing foreign exchange reserves, and farmers' incomes bolstering export potential is a viable option in India.

To increase the export potential, post-harvest management is a critical phase in the tobacco and other commercial crops *viz.*, Chillli, Turmeric, Castor and Ashwagandha, as it is significantly impacts the quality of the product. Several factors and practices come into play during post-harvest handling, crop compliance, and quality standards to meet the global demand. Hence, the institute has standardized various analytical methodologies for estimating pesticide residues and bio-functional components of commercial crops. These methods will ensurethe quality to comply with the international standards i.e. Guidance Residue Levels (GRLs) set by Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA), thereby enhancing the marketability of the crops on a global scale.

Various analytical methods of pesticide residue as well as phytochemical analysis, such as capsanthin, capsaicin, carotenoids, solanesol etc. are presented in a crisp manner in this laboratory manual titled "**Manual on Pesticide Residues and Phytochemical Analysis in Tobacco and Chilli**".

The contributions of the CTRI organic chemistry fraternity in establishing new analytical facilities in institute premises are duly recognized. I extend my heartfelt appreciation to the authors for their dedicated efforts in producing this pioneering publication, encompassing comprehensive information on novel analytical methods. This invaluable resource will serve as a reliable guide for analysts at laboratory or industry level, in this year of foundation day of ICAR-CTRI.

**Date: 26-04-2024**

**(M. SHESHU MADHAV)**
**DIRECTOR**

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# Introduction

Tobacco is a high-value, pesticide-intensive crop, in terms of the amount of pesticide applied per acre, tobacco ranks sixth (potatoes, tomatoes, citrus, grapes, and apples are in the first five ranks). Although pesticides play a significant role in increasing production of tobacco, food and other crops by reducing the pest population, but exposure to these pesticides is harmful. Few pesticides remain in its leaves, even after curing (postharvest processing treatment) might have higher effect on human. According to the different chemical groups, pesticides are classified in wide number of groups such as organochlorines, organophosphates, carbamates, triazines, chloroacetanilides, pyrethroids, pyrethrins, sulfonylureas, neonicotinoids, imidazolinones, isothiazolinones etc, Whereas, pesticides are widely classified into insecticides, herbicides, fungicides, rodenticides, plant growth regulator and botanicals etc.

Tobacco contaminated with pesticides have similar harmful effect as any other crop but it was not shown enough attention, as tobacco products got attention for causing deadly diseases such as mouth and lung cancer. Like many other crops, the monitoring of pesticide residues in tobacco requires appropriate analytical methods for detection, quantification and safety evaluations to comply with the Guidance Residue Levels (GRLs) set by Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA). The estimation of pesticide residues will help in understanding their residue levels for regulatory analysis in tobaccos which will promote the Indian tobacco exports in comply with the trade barriers. Among various spectroscopic, chromatographic techniques and mass analysers, gas chromatography tandem mass spectrometry (GC-MS/MS) liquid chromatography tandem mass spectrometry (LC-MS/MS), Quadrupole-Orbitrap technology provide the highest mass resolution in residue analysis. Using these technologies, screening, identification and quantification of pesticide residues can be done in various matrices.

In addition to that, it is essential to quantify the bioactive compounds found in tobacco and chili as these compounds have significant role in pharmaceutical and nutraceutical industry and have export value. This manual encompasses different analytical methods of pesticide residue of widely used pesticides and phytochemicals of chilli and tobacco which have been used and demonstrated its suitability for sensitivity and quick screening.

## Determination of *Organochlorine pesticide residues* in tobacco- GC-MS method

### Scope

A gas chromatography-single quadrupole mass spectrometry (GC-MS) method is used to determine organochlorine pesticide residues (α-HCH, β-HCH, γ-HCH, δ-HCH, 2,4-DDT, 4,4- DDT, endrin, α-endosulfan, β-endosulfan and endosulfan sulphate) in tobacco leaf.

### Principle

The pesticide residues from a dried and sieved leaf sample is to be extracted with acetonitile and water (1:1) mixture and filtered. The filtrate is further reconstituted with n-hexane and filter through PTFE membrane (0.22 μm). The residues are determined with GC-MS.

### Reagents

All regents are suitable for pesticide residue analysis.

a) **Water,** degas, in accordance with atleast grade 2of ISO 3696.

b) **Acetonitrile,** HPLC and Spectroscopy grade

c) **n-hexane,** HPLC and Spectroscopy grade

d) **Sodium Sulphate,** AR grade

e) **PTFE syringe filter,** 0.2-0.4 μm
Or **Florisil filter** : It is a special, selected variety of magnesium silicate. The nominal aperture size of 150 μm to 250 μm corresponds to a mesh size range designated as 60 mesh to 100 mesh.

f) **Acetone,** HPLC and Spectroscopy grade

g) **Standard pesticide solutions**
Store all pesticide solutions at between 0 $^{0}$C and +4 $^{0}$C in dark.

h) **Individual standard stock solutions**
The stock solutions of the individual pesticide standards are to be prepared by accurately weighing 10 (±0.01) mg of each analyte in to volumetric flasks (certified "A" class) and dissolving the same in 10 ml hexane. These are to be stored in dark vials in a refrigerator at 20 (±2) °C.

i) **Mixed Intermediatestandard solution**
An intermediate stock standard mixture of 10 μg mL$^{-1}$ to be prepared by mixing the appropriate quantities of individual stock solutions followed by requisite volume makeup with hexane and stored at 20 (±2) °C.

j) **Mixed working standard solution**
A working standard mixture of 1 µg $mL^{-1}$ is to be prepared by diluting the intermediate stock solution, from which the calibration standards are to be prepared by serial dilution with hexane. Stability of the working solvent standards is to be checked against freshly prepared working standards (1 µg $mL^{-1}$) from the intermediate stocks as per SANTE guidelines (SANTE guideline 2011).

k) **Calibration standard solution**
A set of six calibration standards at 0.005, 0.01, 0.025, 0.050, 0.10 and 0.25 µg $mL^{-1}$ are to be freshly prepared from the working standard mixture of 1 µg mL-1 concentration by appropriate dilutions. Matrix-matched standards at the same concentration levels are to be prepared by extracting control tobacco and spiking the extract with appropriate volumes of the working standard solutions.

## Apparatus

It is essential to clean all glassware very thoroughly before use and to avoid the use of plastic containers and stopcock grease; otherwise impurities may be introduced in to the solvents. All volumetric flasks and pipettes is to be comply with class A of ISO 1042 and class A of ISO 648 respectively.

a) **Rotary evaporator.**
b) **Sieve:** with 2mm mesh
c) **Oven,** with ventilation
d) **Gas Chromatograph with Mass Spectrometer (GC-MS)**

Operate the QP-2010 Plus GC-MS (single quadrupole, Shimadzu Corporation, Kyoto, Japan) in accordance with manufactures instructions. The injection port, oven and MSD each be equipped with a separate heating unit.

## Preparation of test sample

The leaf samples are to be oven dried at 60 °C for 2 h. The dried leaves (after removing mid rib) are to be powdered, homogenized, sieved (through 1 mm) and used for extraction.

## Procedure

## Extraction

1 g leaf powder is to be taken in a 150-mLErlenmeyer conical flask, 20 mL of acetonitrile/water (1:1) mixture is to be added to the flask. Laboratory observation showed that the resulting coloured solvent extract from the tobacco matrix extracted with acetonitrile/water (1:1) mixture became clear upon florisil clean-up as compared to extraction with pure acetonitrile. This indicated chances of matrix interference from tobacco samples are less for acetonitrile/water (1:1) mixture.

Further, use of acetonitrile/water(1:1) mixture ensured low use of solvent which reduced the cost of solvent. The samples are to be agitated for 45 min over an orbital shaker at 150 rpm and filtered.The filtrate to be partitioned with 40 mL of hexane, and the coloured hexane fraction to be collected for clean-up.A column is to be prepared by fabrication of a column bed which is made of 2 g florisil (60/100 mesh) (activated at 200 °C for 6 h, followed by deactivation with 2% distilled water) sandwiched between two layers of anhydrous sodium sulphate (2 g each layer). Florisil works as an adsorbent for removal of large organic molecules from tobacco matrix extract like pigments,lipids, long-chain organic acids and other matrix compounds. Filter with syringe filter (0.2-0.4 $\mu$m) also removes the traces. Anhydrous sodium sulphate plays dual purposes.First, it acts as a protective layer for underneath adsorbent layer of florisil while loading the sample for column clean-up, and secondly, it removes the traces of water from the solvent extract before injecting to the instrument. Before clean-up, the column is eluted with pure hexane and then coloured hexane extract is passed through the column. Clear and colourless hexane fraction is to be collected from the column and evaporated to dryness under reduced pressure. The residuum isre-dissolved in a volume of 2.5 mL hexane and analyzed by a QP-2010 Plus GC-MS (single quadrupole, Shimadzu Corporation, Kyoto, Japan).

## GC-MS conditions

1) Oven program: The GC separation of pesticides is achieved by formulating an optimized oven temperature program that started from an initial temperature of 100 °C (hold for 0.5 min), ramped at the rate of (@) 30 °C $min^{-1}$ up to 180 °C (hold 1 min), @ 10 °C $min^{-1}$ up to 240 °C (hold for 2 min), @ 10 °C $min^{-1}$ up to 250 °C min (hold for 1 min), @ 10 °C $min^{-1}$ to 260 °C (hold 2 min) and finally @ 40 °C $min^{-1}$ up to 320 °C (hold for 10 min). This program results in a run-time of 18.67 min.
2) Gas flow rates:
   — Carrier gas helium, 3ml/min
   — linear velocity 64.4 cm/sec
3) Injector:
   Injector Automated injector with microsyringe (1$\mu$l-5 $\mu$l)
   Injector temperature 250 °C
   Injection mode Splitless
4) Column: The GC system (GC 2010 Plus) is to be be equipped with ZB-5 (5 % diphenyl, 95 % dimethyl polysiloxane, 30 m (l)×0.25 mm (id), 0.1 µm film thickness) capillary column.
5) MS conditions: A typical GC-MS batch consisted of five matrix-matched multiresidue calibration standards, samples, one matrix

blank and one recovery sample for performance check after a set of every six samples. The detector voltage is set at 1 kV, and the data acquisition is carried out in the selected ion monitoring (SIM) mode with compound-specific m/z ions for selective identification of each pesticide.

## Expression of results

The amounts of respective pesticide, Rp, expressed in milligrams per gram of dried tobacco is given by the equation,

$$R_p = \frac{A_p.E_p.100}{m.(100-w)}$$

where

$A_p$ is the peak area or peak height for the respective pesticide in the sample extract

$E_p$ is the response factor for the respective pesticide in the sample extract

$$E_p = \frac{C_{pst}}{A_{pst}}$$

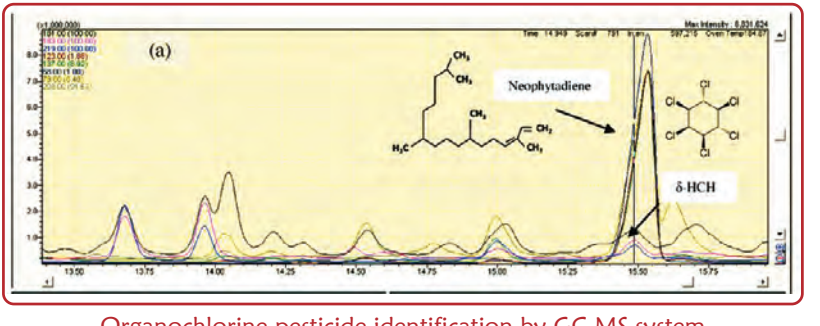
$A_{pst}$ is the peak area or peak height for the respective pesticide in the standard calibration solution

$C_{pst}$ is the concentration, in micrograms per milliliter, of the respective pesticide in the standard calibration solution.

m is the mass of tobacco test portion, in grams

w is the mass fraction of moisture of dried tobacco, as a percentage

- The organochlorine pesticide residue found to be present in the range of 0.01-0.02 mg $kg^{-1}$ in SBS, 0.01-0.08 mg $kg^{-1}$ in NLS, 0.001-0.18mg $kg^{-1}$ in SLS, 0.01-0.12 mg $kg^{-1}$ in CBS, 0.01-0.04 mg $kg^{-1}$ in NBS region which is far below GRL (0.1, 0.05, 0.05 mg $kg^{-1}$ for Chlordane, HCH (α-, β-, δ-), HCH (γ- Lindane) respectively).




Organochlorine pesticide identification by GC-MS system

## Determination of *Imidacloprid residues* in tobacco- HPLC method

### Scope

To determine the imidacloprid (a class of neonicotinoids) residues in the Flue Cured Virginia (FCV) tobacco by high performance liquid chromatography (HPLC) coupled with ultra-violet (UV) detector.

### Principle

The extraction method involves mechanical agitation of tobacco leaf matrix with acetone or ethyl acetate (1:10) for 45 min at 200 rpm, followed by adsorption based clean up (dSPE). The residues are determined with HPLC or UHPLC.

**Reagents :** All regents are to be be suitable for pesticide residue analysis.

a) **Imidacloprid (99.9% purity)**

b) **Water,** degassed, in accordance with at least grade 2of ISO 3696.

c) **Acetonitrile,** HPLC grade

d) **n-hexane,** HPLC grade

e) **Acetone,** HPLC grade

f) **Ethyl acetate,** HPLC grade

g) **Dichloromethane,** HPLC grade

h) **Sodium sulphate,** AR grade

i) **Sodium chloride,** HPLC grade

j) **Imidacloprid standard stock solution**
The stock solution of imidacloprid (200 µg $mL^{-1}$) is to be prepared in a volumetric flask (certified "A" class) by dissolving 5 (± 0.1) mg of reference standard in 25 mL of acteonitrile. This is stored in a dark vial in a refrigerator at 20 (±2) °C.

k) **Working imidacloprid standard solution**
The working solutions of desired imidacloprid concentrations (0.05-7.5 µg $mL^{-1}$) are prepared by serial dilution technique.

## Apparatus

It is essential to clean all glassware very thoroughly before use and to avoid the use of plastics containers and stopcock grease; otherwise impurities may be introduced in to the solvents. All volumetric flasks and pipettes are to be comply with class A of ISO 1042 and class A of ISO 648 respectively.

a) **Rotary evaporator.**

b) **Tobacco mill,** with 2mm mesh

c) **Oven,** with ventilation

d) **Ultra High Performance Liquid Chromatograph (UHPLC) or High Performance Liquid Chromatograph**

## Preparation of test sample

Tobacco leaves are oven dried at 60 $^0$C for 2 h. The dried leaves (after removing mid rib) are to be powdered, homogenized, sieved (through 1 mm) and stored in plastic bags, until analysis.

## Procedure

## Extraction

To extract the imidacloprid residue, 5 g of tobacco matrix with 50 mL of acetone is agitated at 200 rpm for 45 minutes over an orbital shaker at room temperature (32 ± 2 °C) and vacuum filtered. The leachates, thus collected, are reduced to 2 mL under vacuum evaporation at 22 $^0$C. Then 50 mL of 10% NaCl solution is to be added to the reduced acetone extract and liquid-liquid partitioning (L-L-P) is performed by adding 60 mL of n-hexane, thrice (20 mL in each time). The hexane fractions are discarded, and the aqueous layer is again partitioned (L-L-P) with a total 100 mL of dichloromethane, thrice (50 mL + 30 mL + 20 mL). The organic fractions are collected through anhydrous $Na_2SO_4$ layer and evaporated to dryness under vacuum. The residuum is cleaned-up with ready-made dSPE kit. Finally the extract is syringe filtered and analyzed by HPLC.

## HPLC conditions

A Shimadzu LC-8A series HPLC system, coupled with an UV-detector, is used. The stationary phase, a RP-18 column, is maintained at 30 $^0$C, and the isocratic mobile phase, composed of acetonitrile: water (30:70, v/v), is eluted at a flow rate of 0.7 ml min$^{-1}$. The UV

detector is set at 270 nm wavelength of maximum absorbance ($\lambda_{max}$). The HPLC analysis is performed by injecting 20 $\mu$L of sample through a rheodyne injector.

## Expression of results

The amount of imidacloprid, I, expressed in milligrams per gram of dried tobacco is given by the equation,

$$I = \frac{A.R.100}{m.\ (100-w)}$$

where

A is the peak area or peak height for the imidacloprid residue in the sample extract

R is the response factor for the imidacloprid residue in the sample extract

$$R = \frac{C_{ist}}{A_{ist}}$$

$A_{ist}$ is the peak area or peak height for the imidacloprid in the standard calibration solution

$C_{ist}$ is the concentration, in micrograms per milliliter, of the imidacloprid in the standard calibration solution.

m is the mass of tobacco test portion, in grams

w is the mass fraction of moisture of dried tobacco, as a percentage

- Imidaclorprid residue found to be present in the range of 0.005-0.15 mg $kg^{-1}$ across NLS, SLS and SBS regions which is below GRL (5 mg $kg^{-1}$).

HPLC, LC-8A, Shimadzu

## Determination of *Pendimethalin residues* in tobacco- GC-MS method

### Scope

To determine the pendimethalin (belongs to dinitroaniline group) residues in the Flue Cured Virginia (FCV) tobacco by Gas Chromatography mass spectroscopy (GC-MS) .

### Principle

The extraction method involves mechanical agitation of tobacco leaf matrix with acetone or ethyl acetate (1:10) for 45 min at 200 rpm, followed by adsorption based clean up (dSPE). The residues are determined with GC-MS.

**Reagents :** All regents are to be be suitable for pesticide residue analysis.

a) **Pendimethalin (98.9 % purity)**
b) **Acetone, HPLC grade**
c) **Ethyl acetate, GC-MS grade**
d) **Anhydrous sodium sulphate($Na_2SO_4$), AR grade**
e) **Magnesium sulphate ($MgSO_4$)** AR grade
f) **Glacial acetic acid,** AR grade
g) **Primary secondary amine (PSA), graphitized carbon black (GCB) and $C_{18}$**
h) **pendimethalin standard stock solution**

The stock solution of pendimethalin (200 µg $mL^{-1}$) is prepared in a volumetric flask (certified "A" class) by dissolving 5 (± 0.1) mg of reference standard in 25 mL of ethyl acetate. This is stored in a dark vial in a refrigerator at 20 (±2) °C.

i) **Working pendimethalin standard solution**

The working solutions of desired pendimethalin concentrations (0.05-7.5 µg $mL^{-1}$) are to be prepared by serial dilution technique.

### Apparatus

It is essential to clean all glassware very thoroughly before use and to avoid the use of plastics containers and stopcock grease; otherwise impurities may be introduced in to the solvents. All volumetric flasks and pipettes are to be comply with class A of ISO 1042 and class A of ISO 648 respectively.

**a) Rotary evaporator.**
**b) Tobacco mill,** with 2mm mesh
**c) Oven,** with ventilation
**d) Ultra High Performance Liquid Chromatograph or (UHPLC)High Performance Liquid Chromatograph**

## Preparation of test sample

Tobacco leaves are oven dried at 60 $^0$C for 2 h. The dried leaves (after removing mid rib) are powdered, homogenized, sieved (through 1 mm) and stored in plastic bags, until analysis.

## Procedure

### Extraction

The entire sample are mixed thoroughly and selected randomly for further analysis for both tobacco leaf powder. Powdered samples of 20 g homogenate (2 g tobacco + 18 mL water containing 0.5 % acetic acid) are extracted by 10 mL ethyl acetate (vortex for 1 min) followed by the addition of 10 g $Na_2SO_4$ (vortex for 1 min). This is subjected to phase separation by centrifugation at 5000 rpm for 5 min. A supernatant of 2 mL ethyl acetate is cleaned up using two different combinations of dispersive solid-phase extraction (dSPE) sorbents as follows: 25, 50, 75 and 100 mg of PSA per mL of supernatant. The supernatant of the above extract (1 mL) is centrifuged at 10000 rpm for 5 min. It is further filtered through a 0.2 $\mu$m polytetrafluoroethylene (PTFE) membrane filter and finally injected (1 $\mu$L) into the GC-MS system and analyzed in SCAN mode with reference standards (based on retention time) and in SIM mode with quantifier and qualifier ions (m/z).

### GC-MS conditions

The GC-MS QP-2010 Plus (single quadrupole, Shimadzu Corporation, Kyoto, Japan) system is equipped with ZB-5 (5% diphenyl, 95% dimethyl polysiloxane, 30 m (l) x 0.25 mm (id), 0.1 $\mu$m film thickness) capillary column and autosampler. The GC-MS separation of pendimethalin is achieved by an optimized oven temperature program that started from an initial temperature of 90 °C (hold for 0.5 min), ramped at the rate of (@) 20 °C min$^{-1}$ up to 180 °C (hold 1 min), increased to 240 °C @ 12 °C min$^{-1}$ (hold for 1 min), again increased to 260°C @ 15 °C min$^{-1}$ (hold for 1 min) and finally increased up to 280°C @ 12 °C min$^{-1}$ (hold 0.5 min). This program results in total run-time of 16.5 min. The sample solutions are injected in split injection mode (split ratio 10 and pressure 29.1 psi for 1 min) with the injection volume of 1 $\mu$L. The injector temperature is set at 250°C. The ion source temperature is 200°C and the interface is at 280°C. The detector voltage is set at 0.87 kV and the data acquisition is carried out in the selected ion monitoring (SIM) mode with specific *m/z* ions for selective identification of pendimethalin. Ultra-pure (99.999 %) grade helium (INOX Limited, Hyderabad) is used as the carrier gas. The flow rate of Helium is maintained as 3.14 mL/min with a linear velocity of 65.6

cm/sec. The mass spectrometer is operated using electron impact ionization (EI, 70 eV).

## Expression of results

The amount of pendimethalin, I, expressed in milligrams per gram of dried tobacco is given by the equation,

$$I = \frac{A.R.100}{m.\ (100-w)}$$

where

A is the peak area or peak height for the imidacloprid residue in the sample extract

R is the response factor for the imidacloprid residue in the sample extract

$$R = \frac{C_{ist}}{A_{ist}}$$

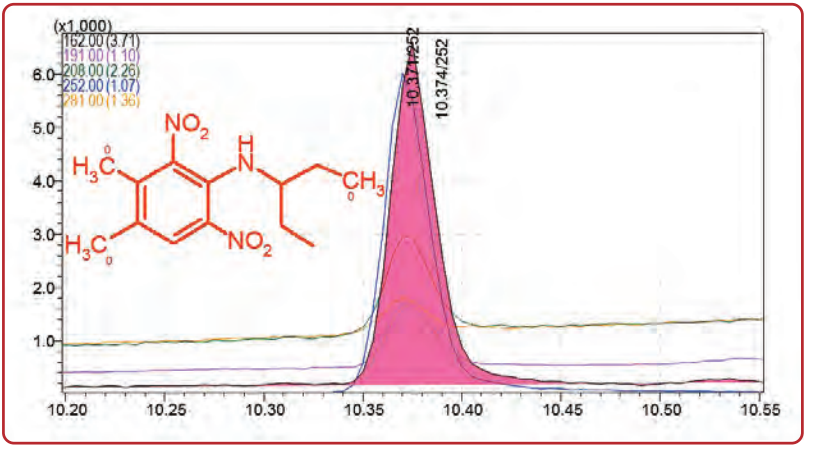
$A_{ist}$ is the peak area or peak height for the pendimethalin in the standard calibration solution

$C_{ist}$ is the concentration, in micrograms per milliliter, of the pendimethalin in the standard calibration solution.

m is the mass of tobacco test portion, in grams

w is the mass fraction of moisture of dried tobacco, as a percentage

- The pendimethalin residue found to be present in the range of 0.06-0.13 mg $kg^{-1}$ in NLS, 0.08-0.13 mg $kg^{-1}$ in KLS, 0.008-0.05 mg $kg^{-1}$ in SLS region which is below GRL (5 mg $kg^{-1}$).




Total Ion chromatogram of Pendimethalin present in tobacco leaf matrix

## Simultaneous screening and quantitative method for *multiresidue analysis of pesticides* in tobacco

### Scope

A total of 181 multiclass pesticides can be analysed including organophosphates, neonicotinoids, triazines, triazoles, urea derivatives, macrocyclic compounds and selective metabolites, among several others using ultra-high performance liquid chromatography-high resolution (Orbitrap) mass spectrometry

### Principle

In quest to develop a rapid and sensitive method for multi-pesticide residue determination, LC high resolution MS/MS (ORBITRAP) technique in combination with triple quadrupole mass spectrometry (MS/MS) are undertaken for large-scale multiresidue analysis of pesticides in tobacco matrix to comply with the reducing trend in GRLs.

**Reagents :** All regents are to be be suitable for pesticide residue analysis.

a) **Restek pesticide mix (99.99% purity)**
b) **Acetone, HPLC grade**
c) **Ethyl acetate, GC-MS grade**
d) **Anhydrous sodium sulphate ($Na_2SO_4$), AR grade**
e) **Magnesium sulphate ($MgSO_4$)** AR grade
f) **Glacial acetic acid,** AR grade
g) **Primary secondary amine (PSA), graphitized carbon black (GCB) and $C_{18}$**
h) **Restek pesticide mix standard stock solution**
The stock solution of pesticide mix (200 µg $mL^{-1}$) is prepared in a volumetric flask (certified "A" class) by dissolving 5 (± 0.1) mg of reference standard in 25 mL of ethyl acetate. This is stored in a dark vial in a refrigerator at 20 (±2) °C.
i) **WorkingRestek pesticide mix standard solution**
The working solutions of desired pesticide CRM concentrations (0.05-7.5 µg $mL^{-1}$) are to be prepared by serial dilution technique.

### Apparatus

It is essential to clean all glassware very thoroughly before use and to avoid the use of plastics containers and stopcock grease; otherwise impurities may be introduced in to the solvents. All volumetric flasks and pipettes are to be comply with class A of ISO 1042 and class A of ISO 648 respectively.

a) **Rotary evaporator.**
b) **Tobacco mill,** with 2mm mesh
c) **Oven,** with ventilation
d) **Ultra High Performance Liquid Chromatograph or (UHPLC)High Performance Liquid Chromatograph**

## Preparation of test sample

Tobacco leaves are oven dried at 60 $^0$C for 2 h. The dried leaves (after removing mid rib) are powdered, homogenized, sieved (through 1 mm) and stored in plastic bags, until analysis.

## Procedure

## Extraction

The entire sample are mixed thoroughly and selected randomly for further analysis for both tobacco leaf powder. Powdered samples of 20 g homogenate (2 g tobacco + 18 mL water containing 0.5 % acetic acid) are extracted by 10 mL ethyl acetate (vortex for 1 min) followed by the addition of 10 g $Na_2SO_4$ (vortex for 1 min). This is subjected to phase separation by centrifugation at 5000 rpm for 5 min. A supernatant of 2 mL ethyl acetate is cleaned up using two different combinations of dispersive solid-phase extraction (dSPE) sorbents as follows: 25, 50, 75 and 100 mg of PSA per mL of supernatant. The supernatant of the above extract (1 mL) is centrifuged at 10000 rpm for 5 min. It is further filtered through a 0.2 $\mu$m polytetrafluoroethylene (PTFE) membrane filter and finally injected (1 $\mu$L) into the GC-MS system and analyzed in SCAN mode with reference standards (based on retention time) and in SIM mode with quantifier and qualifier ions (m/z).

## HPLC-Quadrupole-Orbitrap MS

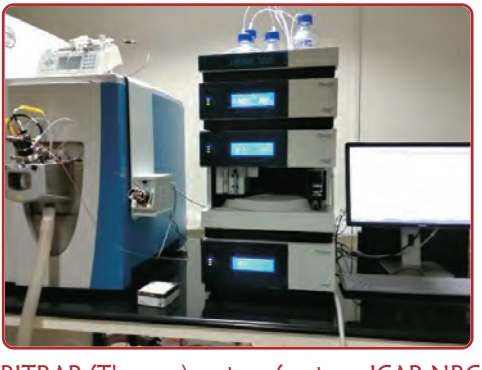
An Ultimate 3000-series HPLC hyphenated to a QExactive MS (ThermoFisher Scientific, Bremen, Germany) is used with an As- centis Express C18 (100 ×2.1 mm, 2.7 µm) column (Supelco, PA, USA), thermostated at 30 °C. The mobile phase comprised the followings: A: methanol:water (10:90) and B: methanol:water (90:10), each containing 5 mmol/L of ammonium formate and 0.2% formic acid. With a flow rate of 0.4 mL/min, the gradient programme is set at 0–1 min/90% A, 1–6 min/90-50% A, 6–9 min/50-2% A, 9–14 min/2% A, 14–15 min/2–90% A and 15–20 min/90% A.

The heated- electrospray ionisation (H-ESI, positive mode) parameters are as follows: sheath gas flow rate, 45; auxiliary gas flow rate, 8; sweep gas flow rate, 1; spray voltage, 3.50 kV; capillary temperature, 320 °C; S-lens RF level, 50.0 and heater temperature, 300 °C. The MS analysis is performed in the FS-vDIA mode. At three

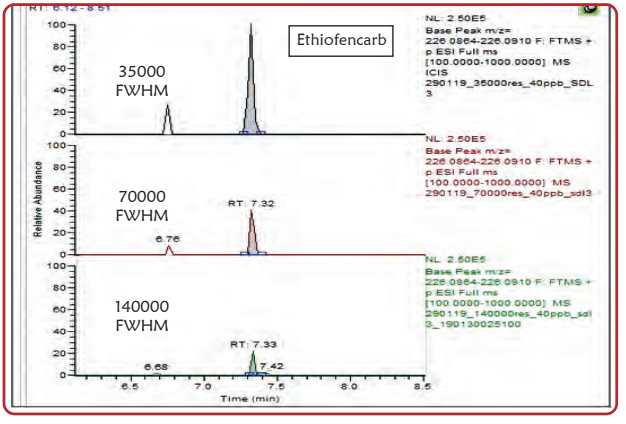
different resolutions of 35000, 70000 and 140000 "Full Width at Half Maxima" (FWHM) (at *m/z* 200), FS is performed in the mass range of 100–1000 Da. This is followed by vDIA at 17500 resolutions (at *m/z* 200) with stepped collision energy, operated at 18, 35 and 70 V. The automatic gain control (AGC)- targets for the FS and vDIA methods are maintained at $3e^6$ and $1e^6$ respectively. In vDIA, eight isolation windows, each of 50 Da width, are chosen from *m/z* 100 until 500, beyond which a single window of 500 Da is maintained upto *m/z* 1000. The compounds are identified by matching them against an in-house updated high resolution accurate mass database.

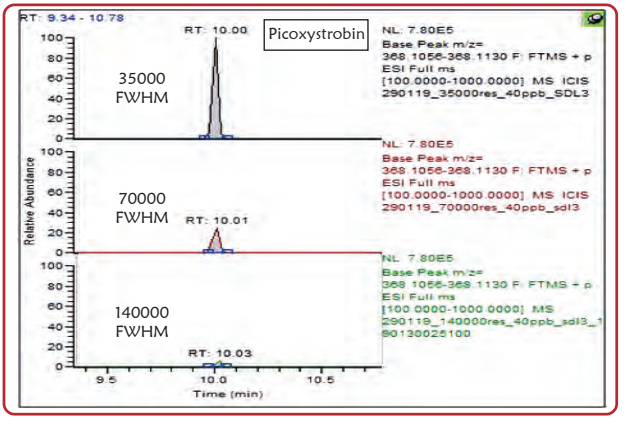
## Expression of results

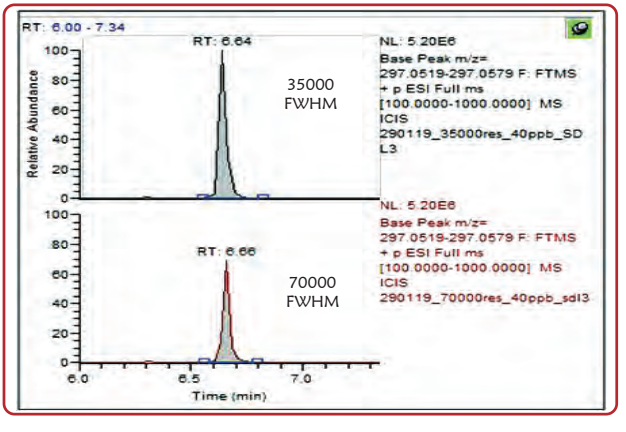
The mass acquisition in the vDIA mode provides a full digital record of all product ions across the mass range of interest. The total number of detected compounds at 40 ng/g is the same, irrespective of mass resolutions (35,000, 70,000 and 140,000 FWHM). So, a resolution of 35,000 is enough to exclude all matrix-related interferences. At all resolutions, the mass error of the test compounds is below 5 ppm. In general, a higher or equivalent signal intensity is recorded at 35,000 FWHM for most of the compounds including ethiofencarb, picoxystrobin and imazalil. Also, the use of 50 Da as the isolation window (in vDIA) ensured better peak shapes and sufficient data- points (>10) across each signal, which in turn facilitated satisfactory measurement accuracies. Among all, 137 pesticides (74.45% of the total number) had their recoveries within 70–120% at 10 ng/g level. Nine compounds-such as acephate, aldicarb-sulfoxide, anilofos, bensulfuron-methyl, carbosulfan, diazinon, dithiocarbamates, difenoxuron, pyrimethanil and pyriproxyfen showed recoveries between 50 and 70%. Whereas, the recoveries of atrazine-desethyl, buprofezin, butachlor, chloridazon, carbofuran, carbofuran-3-OH, carboxin, chlorimuron-ethyl, clethodim, cyanazine, cycloxydim and dinotefuran are noted in the range of 120–140%.



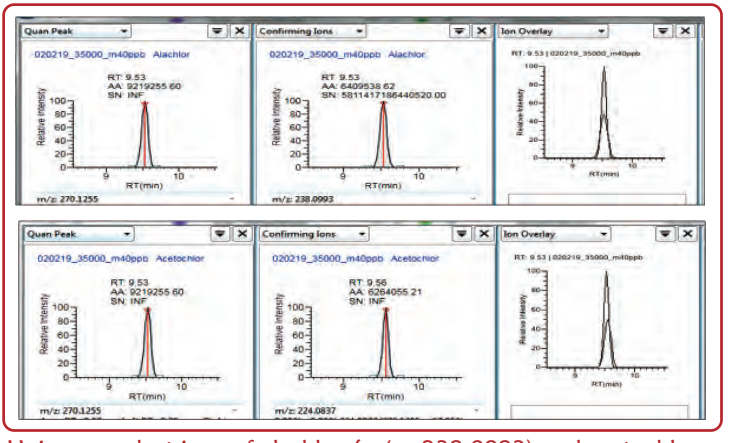
UPLC-ORBITRAP (Thermo) system (curtsey, ICAR-NRCG, Pune)



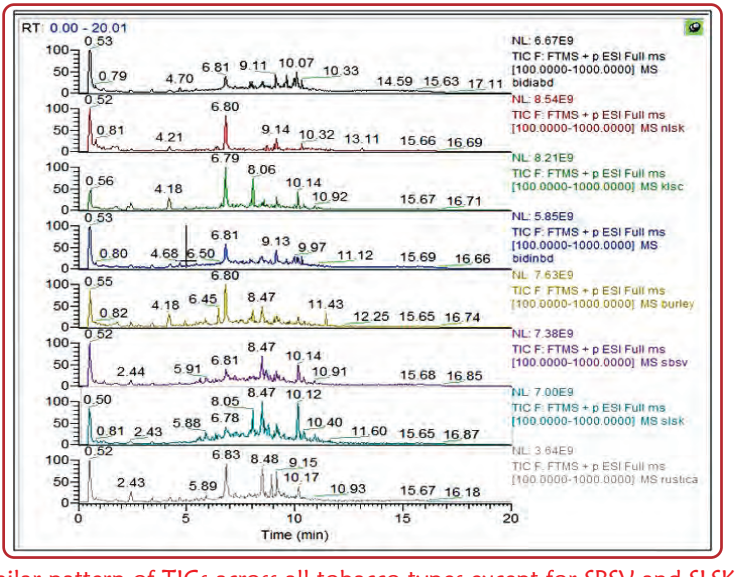




Signal intensity of ethiofencarb (m/z=226.0896), picoxystrobin (m/z=368.1104) and imazalil at 35000, 70000 and 140000 FWHM




Unique product ions of alachlor (m/z=238.0993) and acetochlor (m/z=224.0838) for selective identification




Similar pattern of TICs across all tobacco types except for SBSV and SLSK

## Reference

**Paul A**, Khan Z, Bhattacharyya A, Majumder S, Banerjee K (2021) Multiclass pesticide residue analysis in tobacco (*Nicotiana tabacum*) using high performance liquid chromatography-high resolution (Orbitrap) mass spectrometry: A simultaneous screening and quantitative method. *Journal of Chromatography A*, **1648**: 462208, doi.: https://doi.org/10.1016/j.chroma.2021.462208

## Determination of *Chlorantraniliprole residues* in tobacco- GC-MS method

### Scope

To determine the Chlorantraniliprole (a ryanoid class of insecticide) residues in the Flue Cured Virginia (FCV) tobacco by Gas Chromatography mass spectroscopy (GC-MS) .

### Principle

The extraction method involves mechanical agitation of tobacco leaf matrix with ethyl acetate and other reagents, followed by adsorption based clean up (dSPE). The residues are determined with GC-MS.

**Reagents :** All regents are to be be suitable for pesticide residue analysis.

a) **Chlorantraniliprole(99.9% purity)**

b) **Acetone, HPLC grade**

c) **Ethyl acetate, GC-MS grade**

d) **Anhydrous sodium sulphate ($Na_2SO_4$), AR grade**

e) **Glacial acetic acid,** AR grade

f) **Primary secondary amine (PSA), graphitized carbon black (GCB) and $C_{18}$**

g) **Chlorantraniliprole standard stock solution**
The stock solution of Chlorantraniliprole (100 µg $mL^{-1}$) is prepared in a volumetric flask (certified "A" class) by dissolving 5 (± 0.1) mg of reference standard in 25 mL of ethyl acetate. This is stored in a dark vial in a refrigerator at "20 (±2) °C.

h) **WorkingChlorantraniliprole standard solution**
A working standard solution (10 mg $L^{-1}$) is prepared from which further calibration standards of 0.02, 0.04, 0.08, 0.16, 0.32 and 0.64 mg $L^{-1}$ concentrations are to be prepared freshly by serial dilution for matrix matched calibration

### Apparatus

It is essential to clean all glassware very thoroughly before use and to avoid the use of plastics containers and stopcock grease; otherwise impurities may be introduced in to the solvents. All volumetric flasks and pipettes are to be comply with class A of ISO 1042 and class A of ISO 648 respectively.

**a) Rotary evaporator.**
**b) Tobacco mill,** with 2mm mesh
**c) Oven,** with ventilation
**d) Gas Chromatography Mass spectroscopy**

## Preparation of test sample

Tobacco leaves are oven dried at 60 $^0$C for 2 h. The dried leaves (after removing mid rib) are powdered, homogenized, sieved (through 1 mm) and stored in plastic bags, until analysis.

## Procedure

## Extraction

Samples of 20 g homogenate (2 g tobacco + 18 mL water containing 0.5% acetic acid) extracted with 10 mL ethyl acetate (vortex for 2 min) followed by addition of 10g $Na_2SO_4$ and vortexing for 2 minutes. After that for phase separation centrifugation is done (5000 rpm, 5min). The supernatant of 2 mL is cleaned by d-SPE (100 mg PSA+ 100 mg C18 + 50 mg GCB + 200 mg $MgSO_4$), vortexed for 2 min and centrifuged at 5000 rpm for 5 min at room temperature. The supernatant is filtered through a 0.2 $\mu$m polytetrafluoroethylene (PTFE) membrane filter and finally injected (10 $\mu$L) into the Gas chromatography mas spectrometry (GC/MS) system under standardised environment. The residue analysis of leaf and soil matrix are to be done of each samples using matrix matched calibration.

## GC-MS conditions

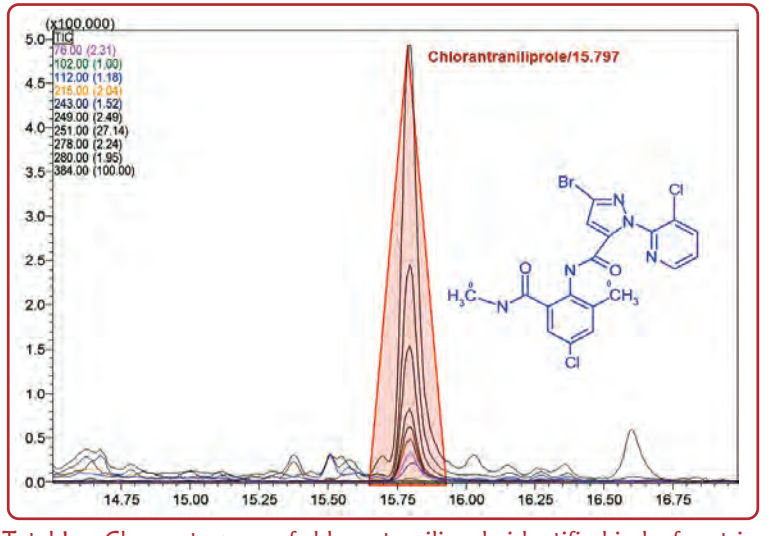
Analytes (1 $\mu$L) injected to a QP-2010 Plus gas chromatography mass spectrometry system (GC-MS; single quadrupole, Shimadzu Corporation, Kyoto, Japan) which is equipped with ZB-5 (5% diphenyl, 95% dimethylpolysiloxane, 30 m (l) x 0.25 mm (id), 0.1 $\mu$m film thickness) capillary column and autosampler. Ultra-pure grade helium (INOX Limited, Hyderabad) is used as the carrier gas. The GC-MS separation of chlorantraniliprole is achieved by formulating an optimized oven temperature program that started initially at temperature of 100 °C (hold for 0.5 min), ramped at the rate of (@) 30 °C min$^{-1}$ up to 180 °C (hold 1 min), increased to 240 °C @ 10 °C min$^{-1}$ (hold for 2 min), @ 10°C min$^{-1}$ up to 250 °C min (hold for 1 min), and finally increased upto 280 °C @ 2 °C min$^{-1}$ (hold 2 min). This program results in a run time of 35.04 min. The ion source temperature is 200°C and the interface is at 280°C. The detector voltage is set at 1 kV. The injector temperature is maintained at 250 °C in a split injection mode (split raion 10 and pressure 29.1 psi for 1 min) with injection volume of 1 $\mu$l and data acquisition is carried out in the selected ion monitoring (SIM) mode with specific m/z ions for selective identification of chlorantrainiliprole The flow rate of helium gas is maintained at 3.14 mL min$^{-1}$ with linear velocity of 64.4 cm s$^{-1}$. The mass spectrometer is operated using electron impact ionization (EI, 70 eV).

## Expression of results

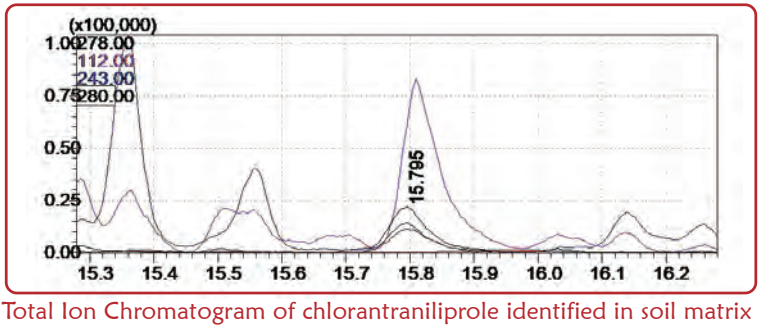
The amount of chlorantraniliprole, I, expressed in milligrams per gram of dried tobacco is given by the equation,

$$I = \frac{A.R.100}{m.\ (100\text{-}w)}$$

Reference ion (m/z 243, 280) and quantifier ion of m/z 278 gave good coefficient of determination ($R^2$) value, 0.994. The LOD and LOQ value obtained through this precise method is 0.02 mg $L^{-1}$ and 0.04 mg $L^{-1}$ respectively. This is an unique method developed for residue analysis of chlorantraniliprole in case of tobacco matrix. It has a very good recovery of 83.4% with acceptable RSD value of 5%, besides, is less laborious, rapid, and rugged.




Total Ion Chromatogram of chlorantraniliprole identified in leaf matrix




Total Ion Chromatogram of chlorantraniliprole identified in soil matrix

## Determination of *Minor alkaloids present* in tobacco-GC-MS method

### Scope

To determine the minor alkaloidspresent in tobacco by Gas Chromatography mass spectroscopy (GC-MS) .

### Principle

The extraction method involves agitation of tobacco leaf matrix with DCM and other reagents. The alkaloids are determined with GC-MS.

**Reagents :** All regents are to be be suitable for minor alkaloids analysis.

a) **Sodium hydroxide (NaOH)**
b) **Dichloromethane**
c) **Dichloromethane, GC-MS grade**
d) **n-heptadecane (99.90% purity)**

### Apparatus

It is essential to clean all glassware very thoroughly before use and to avoid the use of plastics containers and stopcock grease; otherwise impurities may be introduced in to the solvents. All volumetric flasks and pipettes are to be comply with class A of ISO 1042 and class A of ISO 648 respectively.

**a) Rotary evaporator.**
**b) Tobacco mill,** with 2mm mesh
**c) Oven,** with ventilation
**d) Gas Chromatography Mass spectroscopy**

### Preparation of test sample

Tobacco leaves are oven dried at 60 $^0$C for 2 h. The dried leaves (after removing mid rib) are powdered, homogenized, sieved (through 1 mm) and stored in plastic bags, until analysis.

### Procedure

### Extraction

Tobacco leaf powder samples are extracted with 10 mL 2(N) NaOH to allow the sample complete wet followed by DCM. The DCM layer collected over anhydrous $Na_2SO_4$ and transferred to GC vial. The estimation is done using semiquantitative approach in GC-MS.

### GC-MS conditions

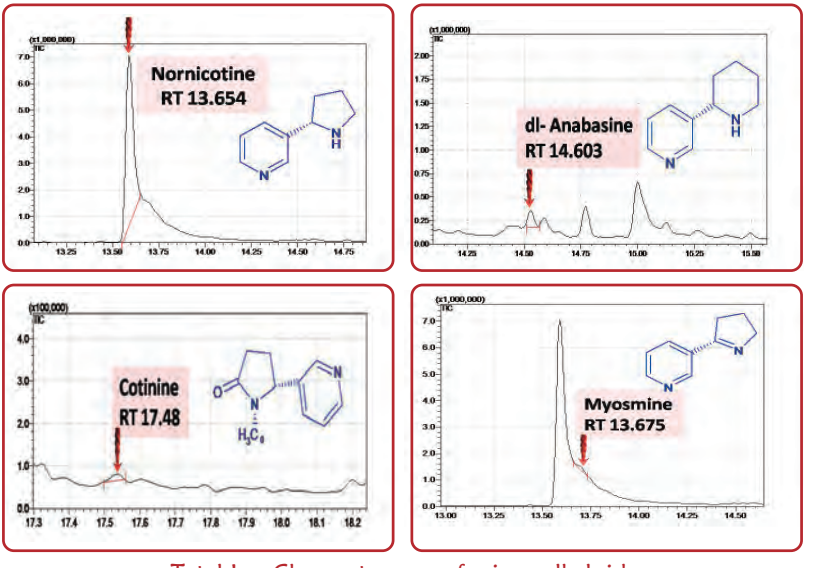
GC-MS QP-2010 Plus (single quadrupole, Shimadzu Corporation, Kyoto, Japan) system is equipped with ZB-5 (5% diphenyl, 95% dimethylpolysiloxane, 30 m (l) ×0.25 mm (id), 0.1 µm film thicknesses) capillary column and auto-sampler (AOC-20i). The GC separation of

minor alkaloids is achieved by an optimized oven temperature program that started initially at a temperature of 60 °C (hold for 1.0 min), ramped @ 10 °C $min^{-1}$ up to 100 °C (hold 1 min), and finally increases upto 280°C @ 10 °C $min^{-1}$ (hold 1 min). N-heptadecane is used as internal standard in SCAN mode for semi quantification of minor alkaloids

## Expression of results

The amount of chlorantraniliprole, I, expressed in milligrams per gram of dried tobacco is given by the equation,

$$I = \frac{A.R.100}{m.(100-w)}$$




Total Ion Chromatogram of minor alkaloids

GC-MS QP , 2010, Shimadzu

## Determination of *Tobacco Specific Nitrosamines (TSNA) present* in tobacco- LC-MS/MS method

### Scope

To determine the TSNAspresent in tobacco by liquid Chromatography mass spectroscopy (LC-MS/MS) .

### Principle

The extraction method involves agitation of tobacco leaf matrix with DCM and other reagents. The alkaloids are determined with LC-MS/MS.

**Reagents :** All regents are to be be suitable for TSNAs analysis.

a) **Sodium hydroxide (NaOH)**
b) **Dichloromethane**
c) **Dichloromethane, GC-MS grade**
d) **N'-nitrosonornicotine (NNN, 99% purity)**
e) **4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone(NNK,99% purity)**
f) **N-nitrosoanabasine (NAB, 99% purity)**

### Apparatus

It is essential to clean all glassware very thoroughly before use and to avoid the use of plastics containers and stopcock grease; otherwise impurities may be introduced in to the solvents. All volumetric flasks and pipettes are to be comply with class A of ISO 1042 and class A of ISO 648 respectively.

**a) Rotary evaporator.**
**b) Tobacco mill,** with 2mm mesh
**c) Oven,** with ventilation
**d) Liquid Chromatography Mass spectroscopy**

### Preparation of test sample

Tobacco leaves are oven dried at 60 $^{0}$C for 2 h. The dried leaves (after removing mid rib) are powdered, homogenized, sieved (through 1 mm) and stored in plastic bags, until analysis.

### Procedure

### Extraction

Tobacco leaf powder samples are to be extracted with 10 mL 2(N) NaOH to allow the sample complete wet followed by DCM. The

DCM layer collected over anhydrous $Na_2SO_4$ and transferred to GC vial. The estimation done by LC-MS/MS.

## LC-MS/MS conditions

### UHPLC conditions (Nexera series)

| | | |
|---|---|---|
| Column | : | RP C18 50 × 2 mm 2.5 µm |
| Mobile phase A | : | 5 mM Ammonium bicarbonate in water |
| B | : | 5 mM Ammonium bicarbonate in methanol |
| Flow rate | : | 0.4 mL/min |
| Time program | : | B conc. 30%(0 min) -90%(0.35 min) - 30%(0.36-1.5 m min) |
| Injection vol. | : | 5 µL (with 1 µL air gaps) |
| Needle ish | : | external ish only with methanol (rinse pump 2 sec) |
| Column temperature | : | 30°C |

### *MS conditions (LCMS/ MS-8045)*

| | | |
|---|---|---|
| Ionization | : | ESI, Positive MRM mode |
| Ion source temperatures | : | Desolvation line : 250°C |
| Heater Block | : | 400°C |
| Gases: Nebulization | : | 2 L/min |
| Drying | : | 15 L/min |

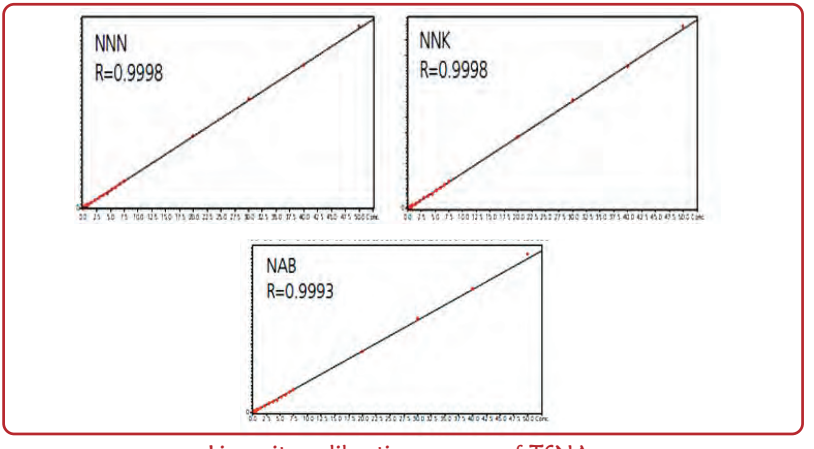
### *MRM Transitions*

| Compound name | MRM | Dwell time (msec) |
|---|---|---|
| NNN | 178.10>148.25 (Qual) | 12 |
| | 178.10>120.25 (Quan) | 12 |
| NNK | 208.10>122.25 (Quan) | 12 |
| | 208.10>79.20 (Qual) | 12 |
| NAB | 192.10>162.30 (Quan) | 12 |
| | 192.10>106.25 (Qual) | 12 |

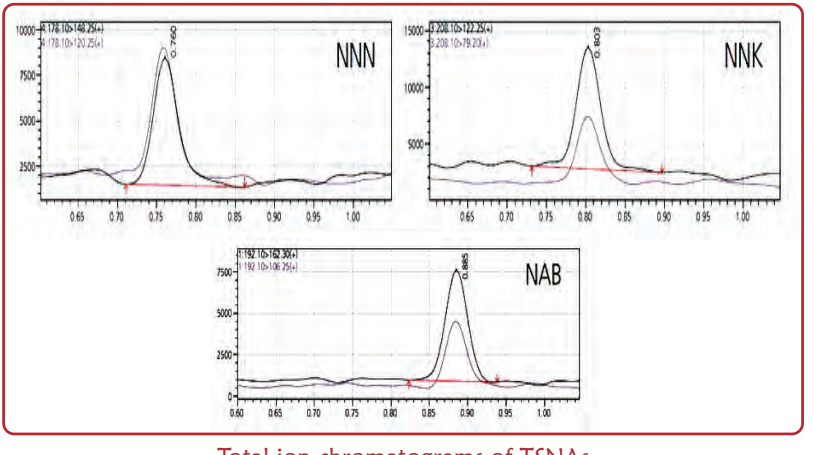
| | |
|---|---|
| Pause time: | 3 msec |
| Loop time: | 0.2 sec |

## Expression of results

Calibration in neat solutions are prepared. For all TSNAs, calibration range is of 0.1 ng/mL to 50 ng/mL. This corresponds to 500 fg to 250 pg of each TSNA injected; and this corresponds to a quantity of 0.004 µg/g to 2 µg/g in tobacco products. All calibration levels are injected in 5 replicates. Intra-level %RSD are inferior to 5% at all levels for all compounds.




Linearity calibration curves of TSNAs




Total ion chromatograms of TSNAs

Very fast and rugged LC-MS/MS analysis lead to high throughput result generation to test many tobacco samples in quality control. The method is sensitive enough to foresee analysis of tobacco smokes or new tobacco products.

## Determination of *Solanesol* in tobacco- HPLC method

### Scope

To determine Solanesol in tobacco by high performance liquid chromatography (HPLC) or Ultra high performance liquid chromatography (UHPLC) coupled with ultra-violet (UV) detector.

### Principle

The extraction method involves mechanical shaking of tobacco leaf matrix with isopropyl alcohol for 30 min at 200 rpm and filtered. The filtrate is analysed by HPLC or UHPLC.

### Reagents

All regents are to be be suitable for solanesol analysis.

a) **Water,** degassed, in accordance with at least grade 2of ISO 3696.

b) **Methanol,** HPLC grade

c) **Isopropyl Alcohol**, HPLC grade

d) **Solanesol standard stock solution**
The stock solution of Solanesol (2000 µg mL-1) is prepared in a volumetric flask (certified "A" class) by dissolving 50 (± 0.1) mg of reference standard Solanesol in 25 mL of isopropyl alcohol. This is stored in a dark vial in a refrigerator at "20 (±2) °C.

e) **Working Solanesol standard solution**
The working solutions of desired Solanesol concentrations (25-200 µg mL-1) are to be prepared by serial dilution technique.

### Apparatus

It is essential to clean all glassware very thoroughly before use and to avoid the use of plastic containers and stopcock grease; otherwise impurities may be introduced in to the solvents. All volumetric flasks and pipettes are to be comply with class A of ISO 1042 and class A of ISO 648 respectively.

**a) Rotary evaporator.**
**b) Tobacco mill,** with 2mm mesh
**c) Oven,** with ventilation
**d) High Performance Liquid Chromatograph (HPLC)**or **Ultra high performance liquid chromatography (UHPLC)**

### Preparation of test sample

Tobacco leaves are oven dried at 60 °C for 2 h. The dried leaves (after removing mid rib) are powdered, homogenized, sieved (through 1 mm) and stored in plastic bags, until analysis.

## Procedure

### Extraction

To extract solanesol, 100 mg of tobacco matrix with 20 mL of isopropyl alcohol is agitated at 200 rpm for 30 minutes over an orbital shaker or sonicator at room temperature (32 ± 2 $^0$C) and filtered through Whatman No.1 filter paper. The filtrate is analysed by HPLC or UHPLC.

### HPLC conditions

A Shimadzu LC-8A series HPLC system, coupled with an UV-detector, is used in the present study. The stationary phase, a RP-18 column, is maintained at 30 °C, and the isocratic mobile phase, composed of isopropyl alcohol and methanol (60:40, v/v), is eluted at a flow rate of 1 ml min$^{-1}$. The UV detector is set at 210 nm wavelength of maximum absorbance ($\lambda_{max}$). The HPLC analysis is performed by injecting 2$\mu$L of sample through a rheodyne injector.

### Expression of results

The amount of Solanesol, S, expressed in milligrams per gram of dried tobacco is given by the equation,

$$S = \frac{A.R.100}{m.(100-w)}$$

where

A is the peak area or peak height for the Solanesol in the sample extract

R is the response factor for the Solanesol in the sample extract

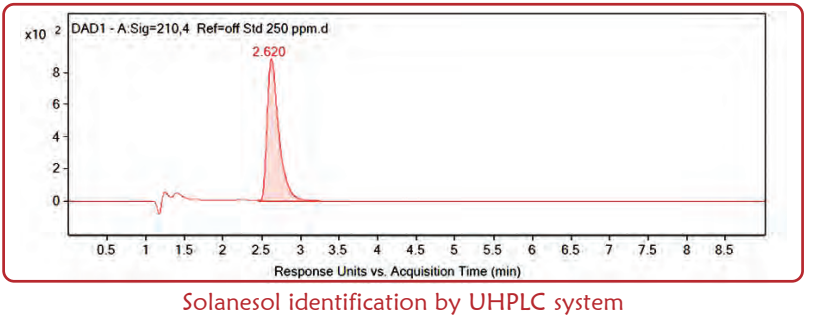
$$R = \frac{C_{sst}}{A_{sst}}$$

$A_{sst}$ is the peak area or peak height for the Solanesol in the standard calibration solution

$C_{sst}$ is the concentration, in micrograms per milliliter, of the Solanesol in the standard calibration solution.

m is the mass of tobacco test portion, in grams

w is the mass fraction of moisture of dried tobacco, as a percentage




Solanesol identification by UHPLC system

## Estimation of *Capsaicin present* in chilli- GC-MS method

### Scope

To determine capsaicinpresent in chillies by Gas Chromatography mass spectroscopy (GC-MS).

### Principle

The extraction method involves sonication of chilies with suitable solvent. The content of capsaicin determined with GC-MS.

### Reagents

a) **Capsaicin (99.9 % purity)**
b) **Acetonitrile, HPLC grade**
c) **Acetonitrile, analytical grade**
d) **Capsaicin standard stock solution**
The stock solution of Capsaicin (100 µg $mL^{-1}$) is prepared in a volumetric flask (certified "A" class) by dissolving 5 (± 0.1) mg of reference standard in 25 mL of acetonitrile. This is stored in a dark vial in a refrigerator at "20 (±2) °C.
e) **WorkingCapsaicin standard solution**
A working standard solution (10 mg $L^{-1}$) is prepared from which further calibration standards of 0.02, 0.04, 0.08, 0.16, 0.32 and 0.64 mg $L^{-1}$ concentrations are to be prepared freshly by serial dilution for matrix matched calibration

### Apparatus

It is essential to clean all glassware very thoroughly before use and to avoid the use of plastics containers and stopcock grease; otherwise impurities may be introduced in to the solvents. All volumetric flasks and pipettes are to be comply with class A of ISO 1042 and class A of ISO 648 respectively.

**a) Rotary evaporator.**
**b) Oven,** with ventilation
**c) Ultrasonicator**
**d) Gas Chromatography Mass spectroscopy**

### Preparation of test sample

Dried chilli (after removing seeds) are powdered, homogenized, sieved (through 1 mm) and stored in plastic bags, until analysis.

## Procedure

### Extraction

Samples of 1 g of chilli powder extracted with 10 mL of acetonitrile. Ultrasonication to be done for 10 mins and repeat for three times. The extract cubed and filtered by whattman filter paper. The supernatant is filtered through a 0.2 $\mu$m polytetrafluoroethylene (PTFE) membrane filter and finally injected into the Gas chromatography mas spectrometry (GC/MS) system under standardised environment.

### GC-MS conditions

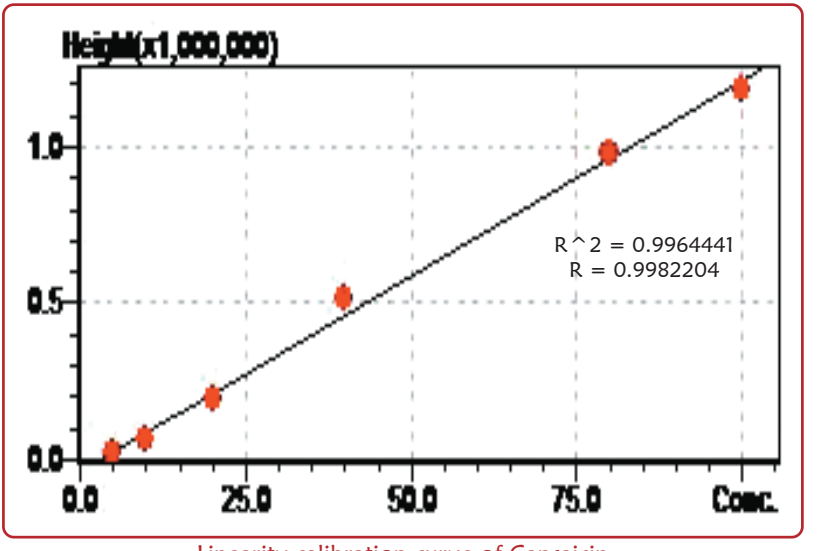
Analytes (1 $\mu$L) injected to a QP-2010 Plus gas chromatography mass spectrometry system (GC-MS; single quadrupole, Shimadzu Corporation, Kyoto, Japan) which is equipped with ZB-5 (5% diphenyl, 95% dimethylpolysiloxane, 30 m (l) x 0.25 mm (id), 0.1 $\mu$m film thickness) capillary column and autosampler. Ultra-pure grade helium (INOX Limited, Hyderabad) is used as the carrier gas. The GC-MS separation of capsaicin is achieved by formulating an optimized oven temperature program that started initially at temperature of 60 °C (hold for 1.0 min), ramped at the rate of (@) 25 °C $min^{-1}$ up to 280 °C (hold 10 min). This program results in a run time of 25.04 min. The ion source temperature is 250°C and the interface is at 250°C.The injector temperature is maintained at 280 °C in a split injection mode with injection volume of 1 $\mu$l and data acquisition is carried out in the selected ion monitoring (SIM) mode with specific m/z ions for selective identification of capsaicin The flow rate of helium gas is maintained at 3.00 mL $min^{-1}$ with linear velocity of 46.3 cm $s^{-1}$. The mass spectrometer is operated using electron impact ionization (EI, 70 eV).

### Expression of results

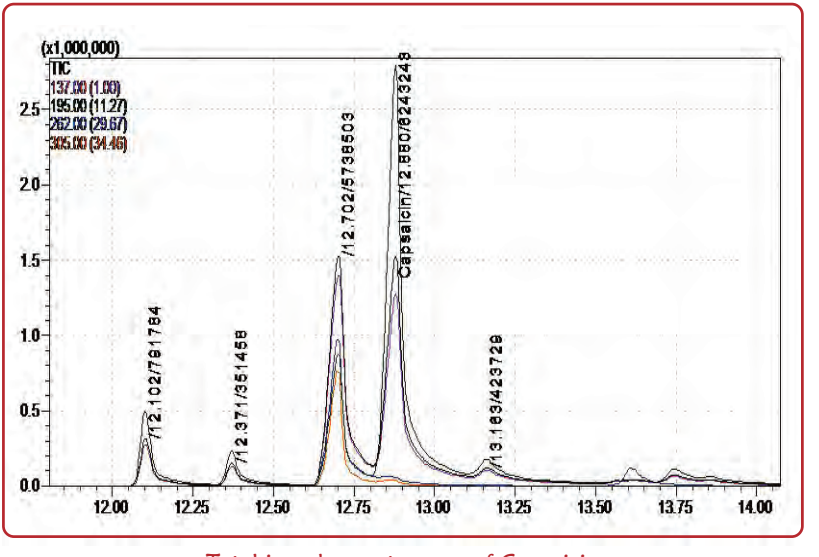
The amount of capsaicin, I, expressed in milligrams per gram of dried chillies is given by the equation,

$$I = \frac{A.R.100}{m.\ (100\text{-}w)}$$

Reference ion (m/z 137, 195, 262) and quantifier ion of m/z 305 gave good coefficient of determination ($R^2$) value, 0.996. The LOD and LOQ value obtained through this precise method is 0.0625 mg $L^{-1}$and 0.125 mg $L^{-1}$ respectively. This is an unique method developed for residue analysis of chlorantraniliprole in case of tobacco matrix. It has a very good recovery of 84.9% with acceptable RSD value of 5%, besides, is less laborious, rapid, and rugged.




Linearity calibration curve of Capsaicin




Total ion chromatogram of Capsaicin

## Estimation of *Capsanthin present* in chilli- LC-MS/MS method

### Scope

To determine the capsanthin present inred chilli by liquid Chromatography mass spectroscopy (LC-MS/MS) .

### Principle

The extraction method involves agitation of dried red chilli matrix with appropriate solvent and determined with LC-MS/MS.

### Reagents

a) **Acetone**
b) **Water, HPLC grade**
c) **Acetone, GC-MS grade**
d) **Capsanthin (NNN, 99% purity)**

### Apparatus

It is essential to clean all glassware very thoroughly before use and to avoid the use of plastics containers and stopcock grease; otherwise impurities may be introduced in to the solvents. All volumetric flasks and pipettes are to be comply with class A of ISO 1042 and class A of ISO 648 respectively.

**a) Rotary evaporator.**
**b) Oven,** with ventilation
**c) Liquid Chromatography Mass spectroscopy**

### Preparation of test sample

Red chillies are oven dried at 40$^0$C. The dried chillies are powdered, homogenized, sieved (through 1 mm) and stored in plastic bags, until analysis.

### Procedure

### Extraction

Red chilli powder samples are to be extracted with acetone/ anhydrous ether (1:1, v/v) mixed solvent provides better extraction of capsanthin by ultrasonication, which is the most important in red chili peppers. The temperature maintained about 35°C.The estimation done using LC-MS/MS.

## LC-MS/MS conditions

### UHPLC conditions (Nexera series)

| | | |
|---|---|---|
| Column | : | RP C18 50 × 2 mm 2.5 µm |
| Mobile phase A | : | water |
| B | : | acetone |
| Flow rate | : | 0.25 mL/min |
| Time program | : | B conc. 80%(0 min) -80%(6.0min) |
| Injection vol. | : | 5 µL (with 1 µL air gaps) |
| Needle ish | : | external ish only with methanol (rinse pump 2 sec) |
| Column temperature | : | 30°C |

### *MS conditions (LCMS/ MS-8045)*

| | | |
|---|---|---|
| Ionization | : | ESI, Positive MRM mode |
| Ion source temperatures | : | Desolvation line : 250°C |
| Heater Block | : | 400°C |
| Gases: Nebulization | : | 2 L/min |
| Drying | : | 15 L/min |

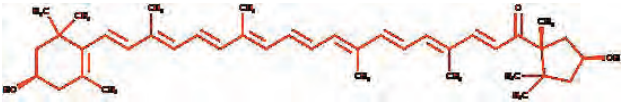
### *MRM Transitions*

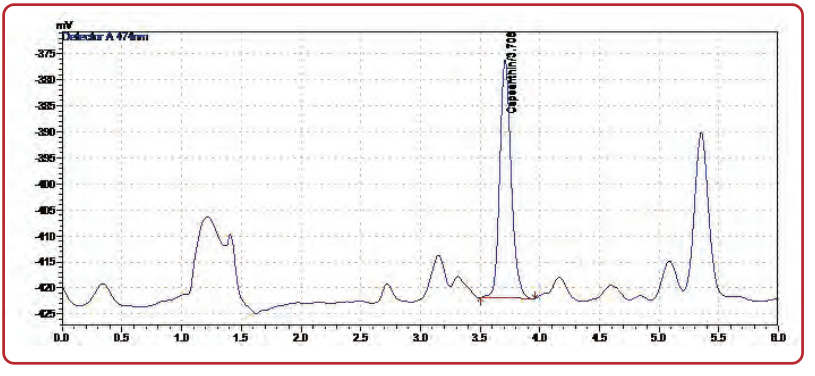
| *Compound name* | *MRM* |
|---|---|
| Capsanthin | 585.40>109.15<br>585.40>567.35<br>585.40>119.10<br>585.40>145.00 |
| Pause time: | 3 msec |
| Loop time: | 0.2 sec |

## Expression of results

Calibration in neat solutions are to be prepared. For capsanthin in MRM mode, calibration range is of 0.05 ng/mL to 0.75 ng/mL. LOD andLOQ level obtained as 0.005 ng/mL and 0.05 ng/mL respectively. In UHPLC, calibration range is of 1 mg/L to 20 mg/L. All calibration

levels are injected in 5 replicates. Intra-level %RSD are inferior to 5% at all levels for all compounds.






Total ion chromatogram of capsanthin present in red chilli sample

This method is very fast and rugged LC-MS/MSmethod to estimate capsanthin as the it is detected very short run time period (3.67 min) lead to high throughput result generation to test many chilli samples in quality control.

LC-MS/MS (LC-MS-8045, TQ, Shimadzu)

## Estimation of *Capsiate present* in chilli- LC-MS/MS method

### Scope

To determine the capsiate present in red dried chilli by liquid Chromatography mass spectroscopy (LC-MS/MS) .

### Principle

The extraction method involves agitation of dried red chilli matrix with appropriate solvent and determined with LC-MS/MS.

### Reagents

a) **Capsiate (99.9 % purity)**
b) **Acetonitrile, HPLC grade**
c) **Acetonitrile, analytical grade**
d) **Capsiatestandard stock solution**
The stock solution of Capsiate (100 µg $mL^{-1}$) is prepared in a volumetric flask (certified "A" class) by dissolving 5 (± 0.1) mg of reference standard in 25 mL of acetonitrile. This is stored in a dark vial in a refrigerator at 20 (±2) °C.
e) **WorkingCapsiate standard solution**
A working standard solution (10 mg $L^{-1}$) is prepared from which further calibration standards of 0.02, 0.04, 0.08, 0.16, 0.32 and 0.64 mg $L^{-1}$ concentrations are to be prepared freshly by serial dilution for matrix matched calibration

### Apparatus

It is essential to clean all glassware very thoroughly before use and to avoid the use of plastics containers and stopcock grease; otherwise impurities may be introduced in to the solvents. All volumetric flasks and pipettes are to be comply with class A of ISO 1042 and class A of ISO 648 respectively.

**a) Rotary evaporator.**
**b) Oven,** with ventilation
**c) Liquid Chromatography Mass spectroscopy**

### Preparation of test sample

Dried chilli (after removing seeds) are powdered, homogenized, sieved (through 1 mm) and stored in plastic bags, until analysis.

### Procedure

### Extraction

Samples of 1 g of chilli powder extracted with 10 mL of acetonitrile. Ultrasonication to be done for 10 mins and repeat for three times. The

extract clubbed and filtered by whattman filter paper. The supernatant is filtered through a 0.2 $\mu$m polytetrafluoroethylene (PTFE) membrane filter and finally injected into the Liquid chromatography mass spectrometry (LC-MS/MS) system under standardised environment.

## LC-MS/MS conditions

### UHPLC conditions (Nexera series)

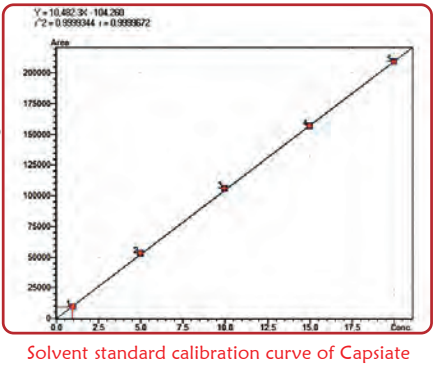
| | | |
|---|---|---|
| Column | : | RP C18 50 × 2 mm 2.5 µm |
| Mobile phase A | : | water |
| B | : | acetonitrile |
| Flow rate | : | 0.4 mL/min |
| Time program | : | B conc. 50%(0 min) -60%(1.0min) – 100% (3-6 min)- 50% (6.1- 8 min) |
| Injection vol. | : | 5 µL |
| Needle ish | : | external ish only with methanol (rinse pump 2 sec) |
| Column temperature | : | 30°C |

### *MS conditions (LCMS/ MS-8045)*

| | | |
|---|---|---|
| Ionization | : | ESI, Positive MRM mode |
| Ion source temperatures | : | Desolvation line : 250°C<br>Heater Block : 400°C |
| Gases: Nebulization | : | 2 L/min |
| Drying | : | 15 L/min |

### *MRM Transitions*

| *Compound name* | *MRM* |
|---|---|
| Capsiate | 307>266 |
| | 307>225 |
| | 307>245 |
| | 307>137 |
| Pause time: | 3 msec |
| Loop time: | 0.2 sec |



Solvent standard calibration curve of Capsiate

## Expression of results

Calibration in neat solutions are prepared. For capsiate in UHPLC, calibration range is of 1 mg/L to 20 mg/L. All calibration levels are injected in 5 replicates. Intra-level %RSD are inferior to 5% at all levels for all compounds. This method is very fast and rugged LC-MS/MSmethod to estimate capsanthin as the it is detected very short run time period (3.67 min) lead to high throughput result generation to test many chilli samples in quality control.

## Estimation of *Total phenols* present in tobacco or dried red chilli

### Scope

To determine the total phenolics present in tobacco or dried chilli by spectroscopic method

### Principle

The extraction method involves agitation of dried red chilli or tobacco matrix with appropriate solvent and determined with UV-VIS spectroscopic method.

### Reagents

a) **Folin-ciocalteu reagent**
b) **Sodium carbonate**
c) **Water**

### Apparatus

It is essential to clean all glassware very thoroughly before use and to avoid the use of plastics containers and stopcock grease; otherwise impurities may be introduced in to the solvents. All volumetric flasks and pipettes are to be comply with class A of ISO 1042 and class A of ISO 648 respectively.

**a) Rotary evaporator.**
**b) Oven,** with ventilation
**c) UV Vis spectroscopy**

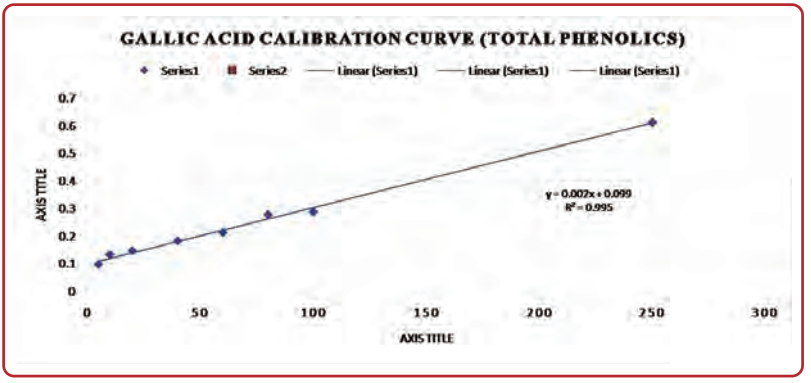
### Preparation of test sample

Dried chilli (after removing seeds) are powdered, homogenized, sieved (through 1 mm) and stored in plastic bags, until analysis. Tobacco leaves are oven dried at 60 $^{0}$C for 2 h. The dried leaves (after removing mid rib) are powdered, homogenized, sieved (through 1 mm) and stored in plastic bags, until analysis.

### Procedure

### Extraction and estimation

Folin-ciocalteu reagent, a mixture of phosphotungstic and phosphomolybdic acids are reduced to blue oxides of tungsten ($W_8O_{23}$) and molybdene ($Mo_8O2_3$) during phenol oxidation. In 100 $\mu$L sample, 3 mL distilled water and 0.5 mL of Folin-ciocalteu reagent (1N) are added. After 3 min; 2 mL of 20 % $Na_2CO_3$ solution is added and mixed thoroughly. The absorbance is measured at 750 nm against blank (Singleton and Rossi., 1996). Then quantity of total polyphenols is expressed as gallic acid equivalents (GAE).




Calibration curve of Gallic acid

## Estimation of *Anthocyanin* presentin chilli

### Scope

To determine anthocyanin, present in dried purple chilli by HPLC or UHPLC method

### Principle

The extraction method involves agitation of dried red chilli with appropriate solvent and determined with UV-VIS spectroscopic method.

### Reagents

a) **Acetone**
b) **Petroleum ether**
c) **Sodium sulphate**

### Apparatus

It is essential to clean all glassware very thoroughly before use and to avoid the use of plastics containers and stopcock grease; otherwise impurities may be introduced in to the solvents. All volumetric flasks and pipettes are to be comply with class A of ISO 1042 and class A of ISO 648 respectively.

**a) Oven,** with ventilation
**b) HPLC or UHPLC**

## Preparation of test sample

Dried chilli (after removing seeds) are powdered, homogenized, sieved (through 1 mm) and stored in plastic bags, until analysis.

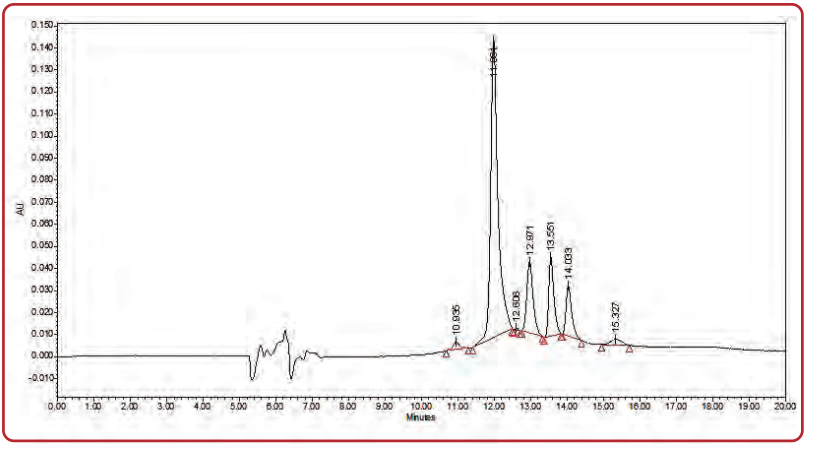
## Procedure

## Extraction

The extraction is done by adding acidified methanol containing 0.1% HCl in the chilli matrices followed by placing into ultrasonic bath sonicator for half an hour (25±2°C) under dim light condition and the process is repeated till the materials became colorless.

## Estimation

Profiling of different anthocyanins compounds analyzed by a HPLC system equipped with an auto injector, quaternary pump, variable wavelength detector (VWD) following the method Singh et al. (2017). A $C_{18}$ column (15 cm × 4.6 mm, 5$\mu$m) column is used for identifying anthocyanins. The binary mobile phase is composed of A- water: ACN: TFA (53:46:1 v/v) and B- water (0.1% TFA) at flow rate of 0.4 mL $min^{-1}$. The gradient elution program is described as follows 0-26 min, 20-60% A; 26-30 min, 60-20% A; and then kept constant for 5min. The column temperature and detection wavelength are 35°C and 520 nm, respectively. Samples of 20 $\mu$L volume are injected, analyzed for a run time of 35 min.




Total ion Chromatogram of Anthocyanins

## Estimation of *Total carotenoids* present in chilli

### Scope

To determine the total carotenoids, present in dried chilli by spectroscopic method

### Principle

The extraction method involves agitation of dried red chilli with appropriate solvent and determined with UV-VIS spectroscopic method.

### Reagents

d) **Acetone**
e) **Petroleum ether**
f) **Sodium sulphate**

### Apparatus

It is essential to clean all glassware very thoroughly before use and to avoid the use of plastics containers and stopcock grease; otherwise impurities may be introduced in to the solvents. All volumetric flasks and pipettes are to be comply with class A of ISO 1042 and class A of ISO 648 respectively.

**c) Oven,** with ventilation
**d) UV Vis spectroscopy**

### Preparation of test sample

Dried chilli (after removing seeds) are powdered, homogenized, sieved (through 1 mm) and stored in plastic bags, until analysis.

### Procedure

### Extraction and estimation

Red chilli powder samples (2g) are extracted with acetone (~30 mL) followed by sonication, The extract (~30 mL) are purified by liquid-liquid partitioning with 20 mL petroleum ether. The petroleum ether fraction collected over sodium sulphate. Dilution done based on color and absorbance. The absorbance is measured at 450 nm against blank.



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