ANALYTICAL TECHNIQUES IN LEAF QUALITY, PESTICIDE RESIDUES AND SMOKE CONSTITUENTS

(A Training Manual)

DIVISION OF CROP CHEMISTRY AND SOIL SCIENCE
ICAR-CENTRAL TOBACCO RESEARCH INSTITUTE
BHASKAR NAGAR, RAJAHMUNDRY-533105

Training Programme

on

"Analytical Techniques for Leaf Quality, Pesticide Residues and Smoke Constituents"

conducted

to

Scientific/Technical Staff, N T T L, Mumbai

during

3-6th April, 2018

By

ICAR-Central Tobacco Research Institute

(An ISO 9001: 2008 Certified Institute)
Rajahmundry, 533 105, Andhra Pradesh, India

PROGRAM SCHEDULE

Date/ Time	Topic	Resource Person
03-04-2018		
9.30 – 10.00 AM	Opening Remarks	
10.00 – 11.30 PM	An overview of Indian Tobacco	Dr D. Damodar Reddy Director, ICAR-CTRI
11.30 -01.00 PM	Visit to CTRI Museum	
1.00 – 2.00 PM	**Lunch Break**	
2.00 - 5.00 PM	Sensitization about the instrumentation facilities, visit to laboratories and BSR Farm, Katheru	Dr CCS Rao Dr J. Poorna Bindu
04-04-2018		
09.30-11.30 AM	Quality parameters in tobacco	Dr J. Poorna Bindu
11.30 – 01.00 PM	**Practical:** Leaf sample preparation, Estimation of moisture content and preparation of reagents for analysis of quality parameters	Smt. K Padmaja & Sri N Johnson
01.00 – 02.10 PM	**Lunch Break**	
02.10 – 05.00 PM	Estimation of leaf chemical quality parameters (Nicotine, reducing sugars) using auto analyser	Smt. K Padmaja & Sri N Johnson
05-04-2018		
09.30-11.30 AM	Tobacco smoke chemistry	Dr C.V. Narasimha Rao
11.30- 01.00 PM	**Practical:** Analysis of smoke constituents – Cigarette selection and pressure drop	Sri N. Johnson
01.00 – 2.10 PM	**Lunch Break**	
2.10-5.00 PM	Analysis of smoke constituents: Estimation of TPM, nicotine and water in smoke condensate	Sri N. Johnson
06-04-2018		
09.30-01.00 AM	Analysis of pesticide residues	Sri N. Johnson
1.00 – 2.00 PM	**Lunch Break**	
2.00-4.00	Good laboratory Practices	Dr L.K. Prasad
4.00 -4.30 PM	Evaluation & Feed back	Dr L.K. Prasad & Dr J.Poorna Bindu
4.30 -5.00 PM	Valedictory programme	

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AN OVERVIEW OF TOBACCO SECTOR

D. Damodar Reddy, Director

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Global Scenario of Tobacco

Tobacco is an international crop grown in more than 120 countries across the world. Among these countries, the top 10 tobacco producers are the China, India, Brazil, United States, Indonesia Malawi, Zimbabwe, Argentina, Pakistan and Turkey. It is grown on less than one percent of the world's agricultural land, and on a wide variety of soils and climates. The global tobacco production was 6,664 million kg during 2016 grown in the area of 3.76 million hectares in the world during 2016. The average tobacco production has increased from 6.69 million tons in 2006-10 to 7.27 million tons in 2011-16 and the average area under tobacco has increased from 3.83 million hectares to 4.05 million hectares during the corresponding period in the world. The global tobacco yield was 1709 kg/ha during 2006 and subsequently increased to 1812 kg/ha during 2012 and again shown a marginal decline to 1774 kg/ha in 2016.

Tobacco Scenario in India

Tobacco is one of the important high value cash crops in India with a production of 761 M kg, India ranks second in the tobacco area and also production, after China during 2016 and stands second in exports after Brazil in the world. During 2016-17, tobacco made a significant contribution of Rs. 28,712 crore to the Indian economy in terms of excise revenue (Rs. 22,737 crore) and export earnings (Rs. 5975 crore) besides providing livelihood security to millions of people.

Indian tobacco has an edge over the leading tobacco producing countries in terms of availability of different styles produced with relatively low production costs. Presently, tobacco is being cultivated in an area of about 4.50 lakh hectares, accounting to 0.24% of the total arable land in the country, covering different varieties of tobacco viz. FCV tobacco, bidi tobacco, chewing tobacco, hookah tobacco, cheroot tobacco, cigar wrapper tobacco, cigar filler tobacco, oriental tobacco, dark fire cured tobacco etc., with an annual production of 761 m.kgs. Out of this, around 190 million kgs is the Flue-cured Virginia [FCV] tobacco which is produced in an area of 1.45 Lakh hectares, mainly in the states of Andhra Pradesh and Karnataka.

The main beneficiaries of tobacco production are the small and marginal farmers, landless agricultural labourers, rural women and tribal youth.

Table1: Major tobacco producing states in India

State	Tobacco types
Andhra Pradesh	FCV, Burley, Oriental, Bidi, Natu
Karnataka	FCV, Bidi
Gujarat	Bidi, Chewing, Rustica
Uttar Pradesh	Bidi, Chewing
Tamil Nadu	Chewing, Cigar
West Bengal	Hookah, Rustica
Bihar	Chewing
Other States	Pikka, Chewing, *Rustica*, *Hookah*

Comparative status of FCV and non - FCV tobacco

FCV	Non-FCV
Area & Production under regulation of Tobacco Board	No regulation
AP, Karnataka	Mostly in Gujarat, UP, WB, Bihar, TN
0.2 m ha ; 300 m kg	0.26 m ha ; 500 m kg
Deforestation	-
Organised and transparent market facility	unorganised market facility
Assured input supply	No
TB extends welfare measures	No

Niche Areas of FCV tobacco cultivation in India

Flue-Cured Virginia tobacco is mainly grown in the states of Andhra Pradesh, Karnataka, and Telangana. The details of main soil domains along with districts covered and area under tobacco cultivation are furnished in Table 2. In Andhra Pradesh, FCV tobacco is grown during *rabi* season by making use of conserved soil moisture in SBS and SLS domains, while 25 percent of tobacco area is under irrigated conditions of NLS domain. In Karnataka, FCV tobacco is predominantly grown as a rain-fed crop during *Kharif* season.

Table.2. Major Production Domains of FCV Tobacco in India

Domains	States covered	Major soil group	Soil order	Rain Fall (mm)	Crop growing conditions
Northern Light Soils (NLS)	AP: East and West Godavari Telangana: Khammam	Red Sandy and sandy loams Soils	Alfisols	1100 - 1200	Irrigated (15th Oct-15th March)
Traditional Black Soils (TBS)	AP: Krishna, Guntur East, and West Godavari	Heavy Black Soils	Vertisols	1000 – 1200	Dry (15th Oct-15th March)
Southern Light Soils (SLS)	AP: Prakasam and Nellore	Red Sandy Loams and Sandy Clay Loams	Alfisols/ Oxisols	750 - 800	Semi - monsoon (15th Oct-15th March)
Southern Black Soils (SBS)	AP: Prakasam and Nellore	Medium Black Soils (silt loams)	Inceptisols/Entisols	750 - 800	Semi - monsoon (15th Oct-15th March)
Karnataka Light Soils (KLS)	Karnataka: Mysore, Hasan	Red Sandy Loams	Alfisols	800-850	Monsoon (May-Sep)

Niche Areas of Non-FCV tobacco cultivation in India

Non-FCV tobacco is mainly grown in the states of Gujarat, Tamil Nadu, West Bengal and Uttar Pradesh. The details of main soil domains along with districts covered and area under tobacco cultivation are furnished in Table.3. In Gujarat, *Bidi* tobacco is mainly cultivated in three districts namely, Anand, Kheda, and Baroda and it is grown during *Kharif* season with60percentof tobacco area is under irrigated conditions and 40 percent under rainfed conditions. In Tamil Nadu, Chewing/ hookah tobacco is mainly cultivated in three districts viz., Dindigul, Erode and Salem and it is grown during *rabi* season predominantly under irrigated conditions. In Uttar Pradesh and West Bengal, Chewing/ hookah tobacco is mainly cultivated in some geographical areas viz., Kanpur, Gursahaiganj, and Ettawa and New Cooch Bihar and Jalpaigudi, respectively and it is mainly grown during *rabi* season predominantly under rainfed conditions.

Table.3. Production domains of Non-FCV tobacco cultivation in India

Tobacco Type	State/Districts Covered	Major Soil Group	Soil Order	Rain Fall (mm)	Crop Growing Conditions
Bidi tobacco	Gujarat: Anand, Kheda, Baroda,	Sandy loams/silt loams	Alfisols/ Vertisols	860	Irrigated (60 percent) and rainfed (40 percent) (July- April)
Chewing/ hookah tobacco	Tamil Nadu: Dindigul, Erode, Salem	Sandy Loam	Inceptisol	800 -900	Irrigated (Oct-March)
	WB: New Cooch Bihar, Jalpaiguri	Red Sandy Loams	Mollisols/ Entisols	2500 - 3000	Rain fed (Oct-March)
	UP: Kanpur, Gursahaiganj,and Ettawa	Alluvial sandy loam	Inceptisol	1100	Rain fed (Oct-March)

Employment Generation

Tobacco provides livelihood security to 45.7 million people in different categories (.Among the different categories maximum livelihood security is provided to Farm labour (43.8 %). Tobacco crop has higher potential to generate the employment during the crop season compared to other crops.

Importance of leaf quality in Tobacco

The tobacco leaf especially FCV tobacco is the most commercial commodity that is marketed in the national and international market. The international market has stringent quality norms especially for FCV tobacco which always influence the Indian exports. The competitive price and sustained market, benefit the Indian buyers at the same time the tobacco farmers.

In order to ensure the better acceptability of our tobacco in the national and international market it is obvious that the quality of tobacco that is produced should be within the limits of acceptability. It is also essential to see that there will be no pesticide residues in the tobacco, even if the traces of residues found must be within the GRLs (Guidance Residue Levels) accepted internationally. Therefore, analyzing important quality parameters of tobacco in the laboratory to find out their contents using standardized procedures and protocols by sophisticated instruments is a paramount activity especially for concerned government agencies and research labs.

Table.5. Important quality parameters and their acceptable limits in flue-cured / Bidi tobacco

Sl.no.	Quality parameters	Acceptable limits
	Leaf quality – FCV tobacco	
1	Total Nitrogen	1.0 - 3.0 %
2	Nicotine	0.70 - 3.0 %
3	Reducing Sugars	8 – 24 %
4	Chlorides	< 1.0 %
	Leaf quality – Bidi tobacco	
1	Total Nitrogen	2.0 - 4.5 %
2	Nicotine	4.5 - 6.0 %
3	Reducing Sugars	2.5 – 4.0
4	Chlorides	1.5 - 2.5

Table 6. Smoke parameters and their range in flue-cured / Bidi tobacco

	Smoke quality – FCV tobacco	Range
1	TAR (mg/cig)	10 - 25
2	Nicotine (mg/cig)	1 - 3
3	Carbon monoxide(mg/cig)	9 - 15
	Smoke quality –Bidi tobacco	Range
1	TAR (mg/bidi)	30- 50
2	Nicotine (mg/bidi)	2 - 5
3	Carbon monoxide (mg/bidi)	20 - 30

Table 7. Pesticide residue GRLs

Pesticide	Gamma BHC	BHC	Chlorpyriphos	Endrin	Total Endosulfon	Total DDT
Guidance Residue Level (ppm)	0.50	0.07	0.50	0.05	1.00	0.20

Concerns of present Indian tobacco

Today tobacco sector is in the whirlpool of diametrically conflicting concerns relating to the livelihood security of those who are associated with tobacco production, processing and marketing on one hand and the serious health risks for those who consume it on the other. Another increasing concern about tobacco is deforestation resulting from the use of huge quantities of wood as source of energy for tobacco curing. Further, the emerging issues relating to climate change impacts, resource degradation, biotic and abiotic stresses, escalating production costs, pesticide residues, consumer preferences and regulatory policies are becoming increasingly complex and represent future challenges for tobacco researchers.

TOBACCO in the whirlpool of conflicting concerns
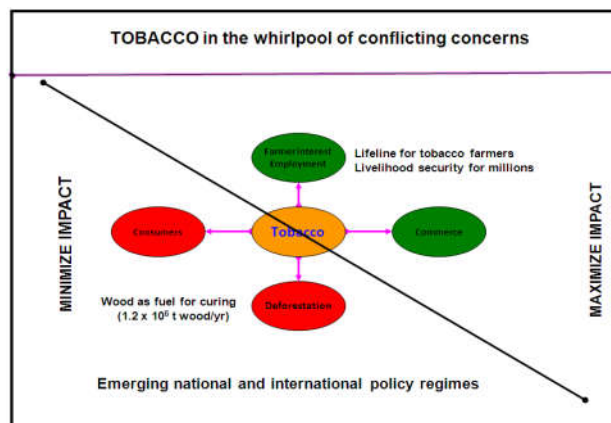
Indian tobacco - Operating Environment

Favourable

1. Diverse climatic conditions favouring production of different tobacco types
2. Price competitiveness and positive features (low TSNA, low CPAs)
3. Growing global market demand for Karnataka tobacco

Unfavourable

1. Changing consumer perceptions and preferences
2. Increased health consciousness
3. Stringent national policies on tobacco control
4. WHO-FCTC: Seeking for demand and supply reduction measures (USA, Argentina, Indonasia – not ratified FCTC and may take advantage of export market)

ICAR-CTRI Research Backup

Vision

Provide vibrant research back-up for Indian tobacco to be less harmful, remunerative and globally competitive in the changing milieu of national and international policy regimes.

Mission

Developing environmentally sustainable agro-technologies for production efficiency, product quality and diversified uses of tobacco.

Mandate

1. Basic and strategic research on domestic and exportable types of tobacco, improvement in quality and value-added products
2. Coordination of tobacco research and developing the alternate usage of tobacco
3. Identification of alternative crops/ cropping systems for tobacco growing regions of the country
4. Dissemination of technologies and capacity building

ICAR-CTRI has a network of six Research Stations situated at Guntur, Kandukur, Jeelugumilli (Andhra Pradesh- FCV and *Natu*), Vedasandur (Tamil Nadu- Chewing, Cheroot and Cigar-filler), Hunsur (Karnataka - FCV) and Dinhata (West Bengal- Chewing, *Hookah* and Cigar-wrapper) and a Burley Tobacco Research Centre at Kalavacharla (Andhra Pradesh).

Research Challenges
- Enhancing farm returns through innovative interventions for sustainable resources use and production efficiency (Related to FCV Tobacco Field Crop Management)
- Production of "tobacco with less harmful constituents"
- Exploring and effective use of alternative energy sources for tobacco curing to reduce dependency on forest wood fuel
- Exploiting tobacco for diversified uses (phytochemicals and value added products)

SENSITIZATION ABOUT THE INSTRUMENTATION FACILITIES

C. Chandrasekhara Rao, Principal Scientist & Head

Division of Crop Chemistry & Soil Science

ICAR-Central Tobacco Research Institute, Rajahmundry - 533105

The Division of Crop Chemistry and Soil Science in ICAR-Central Tobacco Research Institute has four service units *viz,* Soil and Water testing, Leaf quality evaluation, Pesticide residue analysis and Smoke constituents analysis. The division is equipped with the following sophisticated analytical instrument facilities for analysis of different parameters

1.   ATOMIC ABSORPTION SPECTROPHOTOMETER

Make	:	Perkinelmer, USA
Model	:	AA700, 2006

Atomic Absorption Spectrophotometer (AAS) is a spectro analytical instrument for the quantitative determination of chemical elements using the absorption of optical radiation (light) by free atoms in the gaseous state. In analytical chemistry, the instrument is used for determining the concentration of a particular element (the analyte) in a sample to be analyzed. AAS can be used to determine over 70 different elements in solution or directly in solid samples.

Principle

The technique makes use of absorption spectrometry to assess the concentration of an analyte in a sample. It requires standards with known analyte content to establish the relation between the measured absorbance and the analyte concentration and relies therefore on the Beer-Lambert Law. In short, the electrons of the atoms in the atomizer can be promoted to higher orbitals (excited state) for a short period of time (nanoseconds) by absorbing a defined quantity of energy (radiation of a given wavelength). This amount of energy, i.e., wavelength, is specific to a particular electron transition in a particular element. In general, each wavelength corresponds to only one element, and the width of an absorption line is only of the order of a few picometers (pm), which gives the technique its elemental selectivity. The radiation flux without a sample and with a sample in the atomizer is measured using a detector, and the ratio between the two values (the absorbance) is converted to analyte concentration or mass using the Beer-Lambert.

Applications

Determination of following elements in Soil and Tobacco leaf samples

- Total and available forms of Copper
- Total and available forms of Zinc
- Total and available forms of Iron
- Total and available forms of Manganese

2. AUTO ANALYZER

Principle

In continuous flow analysis (CFA) a continuous stream of material is divided by air bubbles into discrete segments in which chemical reactions occur. The continuous stream of liquid samples and reagents are combined and transported in tubing and mixing coils. An essential principle of the system is the introduction of air bubbles. The air bubbles segment each sample into discrete packets and act as a barrier between packets to prevent cross contamination as

they travel down the length of the tubing. Samples and standards are treated in an identical manner as they travel the length of the tubing, eliminating the necessity of a steady state signal. Continuous flow analyzer depends on reactions with the principle of colorimetry using a flow through photometer.

Applications

Estimation of following parameters in Tobacco

- Nicotine
- Reducing Sugars
- Chlorides

3. GAS CHROMATOGRAPH MASS SPECTROMETER

Make: SHIMADZU, JAPAN
Model: GC-MS QP 2010Plus, 2009

The Gas Chromatograph–Mass Spectrometer (GC-MS) is composed of two major building blocks: the gas-chromatograph and the mass-spectrometer. The gas chromatograph utilizes a capillary column, which depends on the column's dimensions (length, diameter, film thickness) as well as the phase properties (e.g. 5% phenyl polysiloxane). The difference in the chemical properties between different molecules in a mixture and their relative affinity for the stationary phase of the column will promote separation of the molecules as the sample travels the length of the column. The molecules are retained by the column and then elute (come off) from the column at different times (called the retention time), and this allows the mass spectrometer downstream to capture, ionize, accelerate, deflect, and detect the ionized molecules separately. The mass spectrometer does this by breaking each molecule into ionized fragments and detecting these fragments using their mass-to-charge ratio.

Principle

The molecules travel the length of the column, pass through the transfer line and enter into the mass spectrometer they are ionized by various methods with typically only one method

being used at any given time. Once the sample is fragmented it will then be detected, usually by an electron multiplier diode, which essentially turns the ionized mass fragment into an electrical signal that is then detected. The ionization technique chosen is independent of using full scan or SIM.

Electron ionization

By far the most common and perhaps standard form of ionization is electron ionization (EI). The molecules enter into the MS (the source is a quadrupole or the ion trap itself in an ion trap MS) where they are bombarded with free electrons emitted from a filament. The electrons bombard the molecules, causing the molecule to fragment in a characteristic and reproducible way. This "hard ionization" technique results in the creation of more fragments of low mass-to-charge ratio (m/z) and few, if any, molecules approaching the molecular mass unit. The molecular fragmentation pattern is dependent upon the electron energy applied to the system, typically 70 eV (electron Volts). The use of 70 eV facilitates comparison of generated spectra with library spectra using manufacturer-supplied software or software developed by the National Institute of Standards (NIST-USA).

Applications
- Organochlorine pesticide residues(11 pesticides)
  (alpha BHC, beta BHC, gamma BHC, delta BHC, chlorpyrifos, endrin, endosulphan-I, endosulphan-II, op' DDT, pp' DDT, endosulphate)
- Pendimethaline residues
- Neutral volatile flavor compounds
- Fatty Acid methyl Esters

4.   HIGH PERFORMANCE LIQUID CHROMATOGRAPH

Make: SHIMADZU, JAPAN
Model: LC-8A, 1995

High-Performance Liquid Chromatograph (HPLC) is an analytical instrument used to separate, identify, and quantify each component in a mixture. It relies on pumps to pass a

pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out the column.

Principle

The sample is being injected through analytical port into the mobile phase stream which carries it into the column. The pumps deliver the desired flow and composition of the mobile phase through the column. Chromatography can be described as a mass transfer process involving adsorption. The active component of the column, the adsorbent, is typically a granular material made of solid particles (*e.g.*silica, polymers, etc.), 2–50 μm in size. The components of the sample mixture are separated from each other due to their different degrees of interaction with the adsorbent particles. The detector generates a signal proportional to the amount of sample component emerging from the column, hence allowing for quantitative analysis of the sample components. A digital microprocessor and user software control the HPLC instrument and provide data analysis.

Applications

Determination of following parameters in Tobacco:

- Solanesol
- Imidacloprid

5. SMOKE MACHINE

Make   : CERULEAN, U.K
Model   : SM 450, 2010

The Smoke Machine, SM450 is a manually operated 20 port linear smoking machine complying with the design requirements of ISO3308. It is capable of smoking ISO and Canadian methods without reduction in smoking throughput or efficiency. A wide variety of cigarettes or bidis can be smoked using the appropriate holders.

Principle:

Carbon monoxide generated during smoking can be collected in bags for analysis via the built-in optional gas analyzer through NDIR technique.

Particulate matter is captured using the supplied Cambridge filter pad holders. The difference of initial and final mass of the filter pad is total particulate matter. Nicotine and water shall be determined by GC-FID and GC-TCD. Eliminating these two parameters from TPM leads to the estimation of Tar or Nicotine Free Dry Particulate matter.

Applications

Cigarette and Bidi analysis for
- Nicotine Free Dry Particulate Matter or Tar
- Nicotine
- Carbonmonoxide

6. TOTAL ORGANIC CARBON ANALYSER

TOC analyzer is for the determination of organically and inorganically bound carbon in liquids, slurries and solids.

Principle

The measuring principle is based on the high temperature digestion of the sample in an air/O_2 stream at 850 ^{0}c to 950 ^{0}c.Totally bound carbon is converted into CO_2 which is quantitatively determined by means of a NDIR detector.

Applications
- Determination of Total organic carbon in solid and liquid samples.

QUALITY PARAMETRES IN TOBACCO

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Tobacco is a quality conscious crop. Especially in FCV tobacco, quality of leaf is of paramount importance as the yield of leaf of high quality only fetches maximum returns to the farmers. The concept of quality in flue-cured tobacco has attained a new dimension in the present days because of higher mobility in international market, ever growing sophistication among smokers taste and increasing automation in cigarette industry. While overall monetary return is a good enough criterion of quality to a farmer.

Tobacco quality is the balance of visible, physical, chemical and biochemical properties of the leaf. Visual characters viz. colour, body, ripeness, size; physical characters viz. filling value, equilibrium moisture content, burning rate, porosity, elasticity, shatterability, strip yield and porosity; Chemical characters viz. total nitrogen, potassium and chlorides, biochemical characters like nicotine, reducing sugars, starch, and their ratios are important in governing the quality of FCV tobacco. Quality management depends on several factors like soils and climate, cultural practices, fertilizers, irrigation, diseases and pests, air pollutants, maturity of leaf, curing and ageing of cured leaf.

VISUAL CHARACTERS

Leaves with undesirable colors particularly green and brown are of poor quality and they are removed by farmers before offering the tobacco for sale. The lemon/orange color is desirable quality and relationship between leaf quality and plant position of leaf is given Table 1.

Table: 1 Relationship between leaf position and leaf quality

S.No.	Visual Characters	Lugs	Cutters/leaf	Tips
1.	Colour	Lemon/ orange	Lemon/ orange	Orange/Mahogany/ green
2.	Finish	Dull	Lustrous	Dull
3.	Ripeness	Over ripe/ ripe	Ripe	Under ripe
4.	Size	Medium	Large	Small/ medium
5.	Texture	Dry	Soft	Dry/woody
6.	Grain	Very grainy	Grainy/ medium	Medium /close
7.	Thickness	Thin	Medium to thin	Medium to thick
8.	Physical damage	High	None	None
9.	Aroma	Low	Medium	High

Physical quality characteristics

Tobacco buyers evaluate tobacco by its visual characteristics. Such a system of subjective quality evaluation varies with personal fancies and hence cannot be considered as precise. The necessity of objective laboratory tests to evaluate physical qualities has become essential particularly in newly evolved varieties and agronomic practices in order to build up the desirable traits in the crop. The important physical properties of leaf are filling value, equilibrium moisture, shatter resistance, porosity and burn rate.

Filling Value

It is an index for the number of cigarettes that can be produced from a given weight of raw material. Good filling value enables the manufacturer to ensure that cigarettes are made as well filled rigid rods. Poor filling results in poorly made soft cigarettes from the ends of which tobacco shreds fall out easily or else cigarette weight has to be increased in order to restore cigarette firmness. Heavy filling on the other hand can result in too much draw resistance and in sharply altered burn characteristics.

Equilibrium Moisture Content

A hygroscopic property of cured leaf as judged by equilibrium moisture content is an important technological criterion for judging quality. It is the moisture absorbing capacity of leaf which depends on the relative humidity of the surrounding environment. Cured leaf low in hygroscopicity is very difficult to get to `order' or `condition' with the result the leaf handling is impeded which gives harsh taste and smoke. High hygroscopicity on the other hand, entails operational difficulty in the cutting and filling machine. Storage of heavily hygroscopic leaf is also a problem. It impairs combustibility. The hygroscopicity of leaf is increased with chloride content of the leaf. Reducing sugars also increase the leaf moisture content. Therefore, it is apparent that cured leaf of acceptable quality should have its equilibrium moisture content within the optimum range i.e. 11 to 15% at 60% relative humidity. Lipophilic colloidal constituents of leaf materials and tissue density (porosity) have direct influence on the water absorption and retention capacities. The water holding capacity of cured leaf increased with potassium content.

Combustibility or burning quality

Combustibility or burning quality of tobacco involves several criteria like fire holding capacity, rate of burn, evenness or completeness of burn and character of residual ash. Leaf burn is very commonly used to determine the burning quality of cured leaf. This test is done by touching a piece of manually stretched pre-conditioned leaf to glowing nichrome wire and noting number of seconds the glow continues. Usually 3-5 seconds burn is considered to be satisfactory. Rates of burn vary with different types of tobacco. Factors affecting combustibility are both physical and chemical in nature. Among the physical characteristics, micro structure of leaf is more important. Thick heavy leaf with fine texture due to a close cell packing would have a poor burn since there would be less air space between the cells and consequently poor aeration during burning. Good burning leaf has a loose open structure having high porosity which was likely to promote burn by better aeration. Good burn is always exhibited by leaf containing high potassium and low chloride. Calcium and magnesium control completion of burning process and production of white ash.

Shatterability

Another important economic factor in tobacco quality is its resistance to breakage during handling. Tobacco is a fragile material that tends to shatter to a great or less degree with each handling.   Breakage becomes accentuated under the stress of mechanical processes in the factory. Tobaccos do differ in their relative brittleness due to various factors. Strength in tobacco leaf is dependent on calcium pectate, the cementing material in the cell wall.

Porosity

Leaf structure or texture is an important physical property of flue-cured tobacco. Texture and grain are synonyms for cigarette tobacco. Graininess in flue cured tobacco is a measure of porosity of leaf which regulates its capacity to absorb and retain additives in the intercellular air chamber. Leaf structure is also defined as degree of cell development of leaf as indicated by its porosity.

Strip yield

Strip yield in flue-cured tobacco is important to manufacturers since it is the lamina portion of leaf that is normally used in cigarette making. Midribs differ substantially from leaf lamina in chemical composition and its inclusion in blends affect smoking flavour unless ground or otherwise reconstituted. Because of this low utility of midrib, a large bulk of exportable flue-cured leaf is despatched only in the form of strips. This makes strip yield an important criterion in developing varieties as the higher the strip yield, the greater is the economic return. The strip constitutes, on an overage, about 75% of leaf by weight, usually ranging from approximately 70-80%. Within this range, the higher the strip yield, the better the usability of tobacco.

Elasticity

Elasticity is considered to be a major quality factor in tobacco.   Elasticity is the ability of the leaf, when moist, to undergo stretching without breaking. Such tobaccos after being compressed, as occur during cutting in the manufacture of cigarettes, will spring back immediately. Springiness in flue-cured tobacco is thus due to its elasticity. Elasticity is dependent upon water soluble constituents, and stands in direct correlation with the moisture content in tobacco.

Chemical and Biochemical Quality Characteristics

Total nitrogen

It is generally considered that flavour and taste of smoke is correlated with the nitrogenous constituents and flue-cured tobacco containing 1.6 to 2.3% total nitrogen gives the most satisfying smoke. Higher nitrogen content of tobacco would result in, apart from curing difficulty, deep brown coloured trashy leaf, which shatters readily, and it has strong – pungent smoke. Generally high level of nitrogen is associated with high level of nicotine. Lower nitrogen content would result in `washed out', pale coloured leaf, lacking in rich colour characteristics of good tobacco, and it has flat insipid smoke.

Phosphorus

Phosphorus deficiency results in dark brown cured leaf, because they do not mature normally and appears in greenish color, Lack of luster of normal leaves.

Potassium

Leaf color, texture, combustibility and hygroscopic properties are believed to be enhanced by potash fertilizer. Increased potassium application leads to thin, more elastic and pliable tobacco. Potassium content in the cured leaf was found to improve the burning quality of tobacco. An adequate level of potassium in cured leaf tends to off-set the deleterious effects of high chlorine on burning quality. Potassium acts as a mineral catalyst and oxygen carrier in promoting burn of tobacco leaf. Cured leaf lacking in potassium content would result in poor coloured trashy leaf and loses its luster, which may not have any commercial value.

Chlorides

Chlorine is one of the essential nutrients in the production of tobacco. It plays an important role in influencing leaf quality and burn. It is absorbed with ease from the soil solution. One of the principal effects of chloride in growing leaf is to increase water content and turgor, which in turn, tends to produce a larger and thinner leaf. When present in small quantities it improves yield and certain quality factors like colour, moisture content and keeping quality. Larger amounts of chlorides produce muddy and uneven colour in the cured leaf with excessive hygroscopicity and poor burn. Regulation of chlorides in the leaf is therefore essential in the production of leaf of good quality. leaf having more chlorides (greater than 2%), which was found to have poor burn and keeping quality. Rate of deterioration in the colour of the leaf is positively correlated with its chloride content. High chloride content of the leaf is known to increase the hygroscopicity of the leaf which in turn induces certain chemical reactions leading to the deterioration of colour during storage. Rate of deterioration increases with increase in relative humidity and both oxygen and moisture were necessary to bring about the degradation in the colour of the leaf. Leaching technique is best suitable for reclaiming soils containing marginally more chlorides.

Biochemical compounds play an important role in the quality of tobacco. Many of the constituents originally present in the green leaf will undergo enzymatic and oxidative reactions during curing. During the curing process loss of volatile constituents, changes in the structure of compounds by oxidation, hydrolysis, degradation and polymerization of chemical components leading to the formation of aromatic and flavorful compounds. Each compound produced in smoke is a potential contributor to organoleptic properties.

Nicotine

Nicotine content of tobacco by virtue of its stimulatory effect on the smoker is next important constituent. Nicotine is synthesized in the root. It is considered that a nicotine level of 1.75 to 2.0% in FCV tobacco is most satisfactory. The nornicotine in acceptable tobacco should not exceed 5% of total alkaloids. High proportion of nornicotine in cigarette leaf leads to abnormal and objectionable smoke flavour due to pyrolysis of nornicotine into myosmine.

Carbohydrates

Carbohydrates account for 40-50 % of tobacco weight and contribute significantly to the smoking quality.

Starch and sugars

During early stage of curing enzymatic hydrolysis of starch results in reducing sugars.

Reducing sugars

Higher content of reducing sugars in flue cured tobacco is undesirable as it imparts to the smoke an acidic character. Lower content imparts alkalinity to smoke due to high nitrogenous constituents. During smoking, sugars are burnt out as CO_2 and water, thus helping to neutralise free base and increase moisture content in smoke and so act as an emollient, if present in excessive quality.

Lignin

High mol. wt. polymer with 100 or more aromatic units with more methyl groups. Lignin is source for flavour compounds like vanillin, benzyl alcohol which are distilled into main stream smoke. Benzyl alcohol contribute to fruity and smoothness to smoke.

Cellulose

High cellulose content in tobacco blend is a negative to smoking quality. It tends to impart sharp a stinging harshness and a burnt paper odor to the smoke.

Pectin

Pectins contribute to the structural stability of the leaf and pyrolysis products that contributes to the smoke chemistry.

Proteins and Aminoacids

During curing, storage and ageing many changes occur in proteins and aa. some proteins will give quality to smoke.

Phenols

Chlorogenic acid, rutin and scopoletin are major phenols in tobacco. FCV has more phenols than burley tobacco. Pyrolisis of chlorogenic acid, rutin and scopoletin produces simple phenols and compounds which produce smoky like aroma. Some phenols found in smoke are derived from other than these phenols have been implicated in bitter, smokey and medicinal taste of smoke.

Organic acids

Malic, citric, oxalic and malonic acids are 90% of organic acids produced in leaf. They found in the form of salts. Quality of tobacco smoke is inversely proportional to citric acid. Aromatic acid phenyl acetic acid gives honey like taste to smoke

Nitrogen/Nicotine ratio

The ratio of nitrogen to nicotine is assumed to give some chemical balance within the leaf. The higher the ratio, the less desirable the tobacco because it tends to be light bodied. A ratio in the neighborhood of 1.35 gives way to paleness of colour, slickness of texture and a general lack of desirable physical characters and deficiency in aroma. In fact, a value exceeding 1.0 has been ascribed as imbalanced. Too low a value (below 0.5), on the other hand, frequently may be considered undesirable because the tobacco is heavy bodied and associated with high nicotine content and low level of reducing sugars. A range of 0.6 - 0.7 ratio has been adjudged as most desirable in medium to light bodied matured tobacco.

Reducing sugars/nicotine ratio

The ratio of sugar to nicotine would give balance of opposing effects and thus serve as a good smoking quality indicator. A high ratio may tend to indicate mildness and smoothness while a very low ratio may be indicative of harsh irritating smoke. If the ratio is too high, it may indicate that the tobacco is too mild to be acceptable to smoker. If cured leaf contains both low level of nicotine and sugars as generally is the case with Indian flue-cured tobaccos, the ratio appear to be comfortable. High sugar content consistent with nicotine level is the most desirable feature for smoking quality in flue-cured tobacco. The desirable ratio is 7-13.

Table 2: Biochemical constituents of Tobacco leaf

Constituent	Range
Wax, wax esters	0.66-1.20
Solanesol and esters	0.8-2.0
Organic acids	3.0-7.76
Polyphenols	0.75-5.70
Reducing sugars	8.0-25.0
Non Reducing sugars	1.00-5.00
Starch and pectin	0.8-8.00
Nicotine	0.25-3.00
Volatile acids and oils	0.25-1.00
Cellulose and lignin	25-28.5
Proteins	1.00-3.00
Water	11-24.00

Table 3: Acceptable limits for the important chemical constituents and quality indices in flue-cured tobacco

Constituent/Quality index	Acceptable limits
Total nitrogen %	1.0 - 3.0
Nicotine %	0.7 - 3.0
Total Sugars %	10.0 - 26.0
Reducing sugars %	8.0 - 24.0
pH	4.6 - 5.5
Reducing sugars/ Total N	7 - 13
Reducing sugars/Nicotine	7 - 13
Total N/ Nicotine	< 1.2
Filling value at 60% R.H. & 20^0C	3.3 - 3.8 cc/g shreds
Equilibrium moisture content at 60% R.H. & 20^0C	11 - 15%
Pore volume	0.13 - 0.18 ml/g
Combustibility	2.5 - 3.5 mm/min
Leaf burn	3 - 6 sec.
Shatterbility index	> 3

Source: Krishnamurthy *et al.,* 2007
Note: The individual chemical constituents alone should not be taken into consideration for quality evaluation. The ratios of the constituents are also very important and should be taken into consideration for quality appraisal of tobacco.

TOBACCO SMOKE CHEMISTRY AND ANALYSIS OF SMOKE CONSTITUENTS

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Introduction

The WHO Framework Convention on Tobacco Control (FCTC) provides a comprehensive framework for global tobacco control efforts. The FCTC covers all aspects of tobacco control, including tobacco product regulation, advertising, health warnings, price and tax issues, illicit trade (smuggling) and programs for smoking cessation. Article 9 of FCTC addresses the regulation of the contents of tobacco products, including their emissions. The implementation of article 9 requires product regulation measures based on the empirical testing of tobacco products using standardized methods. According to The Cigarettes and Other Tobacco Products (Prohibition of Advertisement and Regulation of Trade and Commerce, Production, Supply and Distribution) Act (COTPA), 2003 enacted by the Parliament, "For purposes of testing nicotine and tar contents in cigarettes and any other tobacco products the Central Government shall by notification in the Official Gazette grant recognition to such testing laboratory as the Government may deem necessary". In this background, an overview on major chemical constituents in tobacco leaf, formation of smoke, chemical constituents in tobacco smoke, analysis of smoke constituents particularly tar, nicotine and carbon monoxide in cigarette smokeis presented in this lecture notes.

Major Constituents in Tobacco Leaf

Tobacco contains a wide spectrum of chemical compounds broadly classified as alkaloids, nitrogenous constituents, carboxylic acids, phenolics, lipids and inorganic substances (Stedman, 1968 and Narasimha Rao and Krishnamurthy, 2007). Important groups and major constituents are listed below. (Table.1)

Table.1. Important groups and major constituents of tobacco leaf

Alkaloids	Nicotine, Nornicotine, Anabasine
Carbohydrates	Sucrose, Glucose, Fructose
Nitrogenous substances	Proteins, Nitrate nitrogen, Ammoniacal nitrogen
Non-volatile aliphatic acids	Malic acid, Citric acid, Oxalic acid
Polyphenols	Chlorogenic acid, Rutin
Inorganic constituants	Chloride, Potassium, Calcium
Structural constituants	Cellulose, Lignin, Pectin
Hydrocarbon	Neophytadiene
Fatty acids:	Palmitic acid, Linoleic acid, Linolinic acid
Sterols:	Stigmasterol, Campesterol, β-Sitosterol, Cholesterol
Terpenes:	Solanesol

Formation of Smoke

The process of cigarette smoking consists of alternate puffs and puff intervals. Smoke generated during puffing which issues through the end of the cigarette is called the main-stream (MS) smoke. Smoke that emanates from the fire-cone, primarily during puffing intervals, directly into the surrounding atmosphere is called the side-stream (SS) smoke. During puffing, air enters the cigarette mainly around the periphery of the fire-cone base i.e. at the burn-line. The tobacco burns back on the cigarette surface and the fire-cone becomes elongated. The highest temperatures attained will be 925 – 950 °C in front of the burn-line. During puff interval or static burn, the burning process is sustained by the diffusion of atmospheric oxygen to the fire-cone surface resulting in the consumption of tobacco near the cone apex and the cone becomes shorter. The peak temperature in a statically burning cigarette is about 850 °C. The main-stream smoke mainly originates from the peripheral portion of tobacco, while the side-stream smoke is derived predominantly from the concentric core of the cigarette. As the two streams originate from different regions of the cigarette and under varying conditions, they differ physically and chemically in some respects.

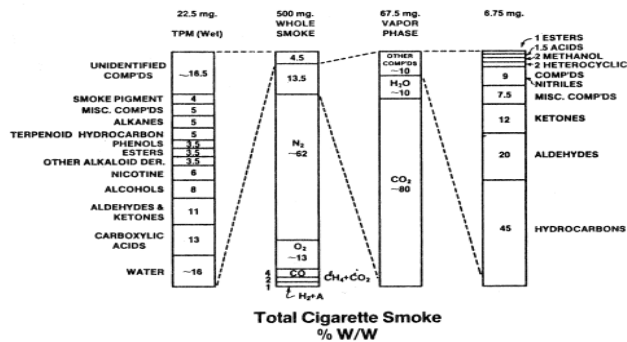## Chemical Constituents in Tobacco Smoke

FIGURE 13.—Composition of cigarette mainstream smoke
SOURCE: Dube and Green (1982).

Fig.1: Mainstream smoke composition *(U.S. Surgeon General's Report, 1989, Fig. 13, page 80).*

The composition of cigarette mainstream smoke, inhaled by the smoker is depicted in the figure showing four vertical bars. The second vertical bar represents the main chemical constituents of MS smoke, labeled "whole smoke", dominated by N_2 (nitrogen) ~62% by weight, and O_2 (oxygen) ~13% by weight. TPM (Wet) accounts for 4.5% of this symbolic cigarette and the main components are shown in the first vertical bar. Of the 4.5% only about 16% is water (H_2O), i.e. only about 0.7% of the total. The main constituents in the "vapor phase," which constitute 13.5% of the total, are shown in the third vertical bar. Only 10% of that is H_2O by weight, i.e. about 1.35% of the total is water. Thus, the amount of H_2O in the mainstream smoke inhaled by a smoker is only about 2% of total (0.7% Wet + 1.35% Vapor Phase).

Osdene (1976) reported that at least three types of reactions occur simultaneously during smoking: pyrolysis, pyrosynthesis and distillation. The process of tobacco burning leads to thermal degradation, in which organic matter is broken down into smaller molecules (pyrolysis). The newly formed fragments, or radicals, are often unstable and may recombine to form components that were not originally present in tobacco. This process is called pyrosynthesis. Distillation of certain compounds from the tobacco into the smoke is the third process occurring during the smoking. Compounds such as nicotine and some low molecular weight terpenes participate in this third process. They decompose only to a small extent. The smoke components are distributed between the gas phase and particles which constitute the aerosol. The smoke itself is produced from the smoking article in two distinct streams: main-stream and side-stream as indicated above. Main-stream smoke is drawn from the mouth or butt end of the cigarette when a smoker puffs on the cigarette. The smoke that rises from lit end of the cigarette, especially during the smolder period between puffs, is known as side stream-smoke. According to Thornton (1978), the yield of components in cigarette main-stream smoke depends upon the volume of puff and the puff 'profile', the number and frequency of puffs and butt length left at the end of smoking.

Cigarette smoke comprises a highly complex chemical mixture of non-specific products of organic material combustion and chemicals that are specific to the combustion of tobacco and other components of the cigarette. For most of the compounds and substances added to tobacco, little is known of their combustion chemistry. This creates difficulties in determining relationship between chemicals in tobacco and chemicals actually inhaled in the smoke.

The particulate fraction of cigarette smoke contains many harmful carcinogenic constituents, including metals, PAHs, dioxins and some non-volatile nitrosamines. The nature of the chemical components in tar and their toxicity vary widely across tobacco from various sources. Tar is defined as the nicotine-free, dry, particulate matter of tobacco smoke (U.S. Surgeon General Report, 1989). Therefore, measurement of tar, per se, is only a crude measure of the relative toxic potential of tobacco combustion products.

In addition to the particulate fraction of tobacco smoke, many chemicals are found in the gaseous phase. Boyd *et al.* (1972) reported that the major gas phase constituents (percent value) in the cigar smoke are Nitrogen: 51.8-54.6%, Oxygen: 4.1-4.2%, CO_2: 15.25-16.7% and CO: 9.7-12.7%. The most widely reported of the gaseous chemicals is carbon monoxide (CO). Carbon monoxide is emitted in high concentrations in cigarette smoke. The toxicity of carbon monoxide is a function of its ability to form carboxy-hemoglobin, a stable chemical complex with hemoglobin. This effectively serves to remove oxygen-carrying hemoglobin from the circulating blood and to vital tissues. Carboxy-hemoglobin concentrations in the blood of about 2% or more of hemoglobin have been associated with angina pain in the people with cardiovascular disease and can result in cardiac ischemia and diminished blood flow to the heart. Some other important chemicals in tobacco smoke, such as benzene, are also found in the gaseous phase of the smoke, but are correlated with the amount of tar (Smith *et al.,* 1997).

Tar is defined as the weight of total particulate matter less the weight of nicotine and water (nicotine free dry TPM). Tar includes the majority of mutagenic and carcinogenic agents in tobacco smoke. Kim *et al*. (2000) concluded that dry TPM delivery is greatly dependent on the level and position of the vent as well as on the usual tipping parameters, filter length, pressure drop and wrapping paper porosity.

Nicotine is a probably the most thoroughly investigated tobacco component in terms of its transfer to smoke. This is because nicotine has such an important role in the smoker's sensory assessment of main-stream smoke, in particular, its impact and irritation. The most comprehensive study on nicotine transfer to smoke was published by Houseman (1973). He found that 70% of the nicotine originally present in the tobacco burnt was recovered intact after smoking and 27% of the original nicotine was converted into other substances which ended up in the mainstream (0.5% in particulate phase, 4% in gas phase), side-stream (4% in particulate phase, 16% in the vapor phase) and on the butt (1.7%). He found that 70% of the nicotine originally present in the tobacco burnt was recovered intact after smoking and 27% of the original nicotine was converted into other substances which ended up in the

mainstream (0.5% in particulate phase, 4% in gas phase), side-stream (4% in particulate phase, 16% in the vapor phase) and on the butt (1.7%).

Carbon monoxide and carbon dioxide are the major constituents in cigarette smoke and are formed by both thermal decomposition and combustion of many of the components of the tobacco viz., starch, cellulose, sugars, carboxylic acids, esters, amino acids etc. (Baker, 1981). Baker (1987) investigated that within the burning cigarette, the carbon monoxide is formed by about 30% of thermal decomposition of tobacco components, about 36% by combustion of tobacco and at least 23% by carbonaceous reduction of carbon dioxide.

$$C + CO_2 \rightarrow 2CO$$
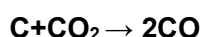
Analysis of Smoke Constituents

It has been estimated that there are over 4000 chemical constituents in tobacco smoke. Cigarette smoke contains numerous known or suspected human carcinogens. The International Agency for Research on Cancer (IARC) has listed 36 chemicals that are known to cause cancer in humans. Cigarette smoke contains at least 10 of these 36 compounds, plus many more mutagenic chemicals that are in the "probably carcinogenic" or "possibly carcinogenic" categories. It is not feasible to measure all 5000 cigarette smoke components for product monitoring and subsequent regulation purposes. Therefore, a list of smoke components needs to be selected with a sufficiently broad chemical, toxicological, and pharmacological profile (Table.2).

Table.2. Analytical methods for estimation of smoke components

Analytes	Matrix	Analytical Method
Tar, Nicotine and Carbon monoxide	Smoke	GC-FID (Nicotine), GC-TCD (Water in TPM), Non-dispersive infrared (NDIR) analyzer (CO)
Tobacco Specific Nitrosamines (TSNAs) [N-Nitrosonornicotine (NNN), 4-Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-Nitrosaminoanabasine (NAB) and N-Nitrosaminoanatabine (NAT)].	Smoke	HPLC-MS-MS
Poly nuclear Aromatic Hydrocarbons (PAHs) – Benzo(a) Pyrene (BaP)	Smoke	GC-MS
Volatile Organic Compounds (VOCs) – Benzene and 1,3-Butadiene	Smoke	GC-MS
Carbonyls (Formaldehyde, Acetaldehyde and Acrylaldehyde)	Smoke	HPLC-Diode Array Detector (DAD)

Analysis of Tar, Nicotine and Carbon monoxide in Cigarette Smoke

The sample cigarettes are conditioned in a humidity cabinet at 25 °C and 60% RH as per the ISO method 3402 (ISO, 1999a). The selection criteria for the cigarettes are adopted as prescribed in the ISO method 4387 (ISO, 2000a). The cigarettes falling within ±20 mg of the average weight are selected using a sorter balance (Model HB1867, Heinr-Borgwaldt, Germany). Then the pressure drop of the cigarettes is measured on a Pressure Drop apparatus (Model BVM 102, Filtrona, UK) and cigarettes of pressure drop within ±5 mm WG of the mean value are selected. The circumference of the cigarettes is measured employing a tape gauge (Model MTG 102, Filtrona, UK). The cigarettes so selected on weight, circumference and pressure drop basis are smoked on a 20– port harmonized cigarette smoking machine (Model SM 400, Cerulean, UK) following the standard parameters as per the ISO method 4387 (ISO, 2000a). After completion of smoking, the Cambridge filter pads are transferred to 150 ml glass stoppered conical flasks, 20 ml isopropyl alcohol is added and shaken for 15 min on a rotary shaking machine for estimation of water as per the ISO method 10362-1 (ISO, 1999b), nicotine as per the ISO method 10315 (ISO, 2000b) using GC (Model 5890 Series II, Agilent, USA). Carbon monoxide is estimated by the NDIR method of ISO 8454 (ISO, 2007). Puff count values are recorded.
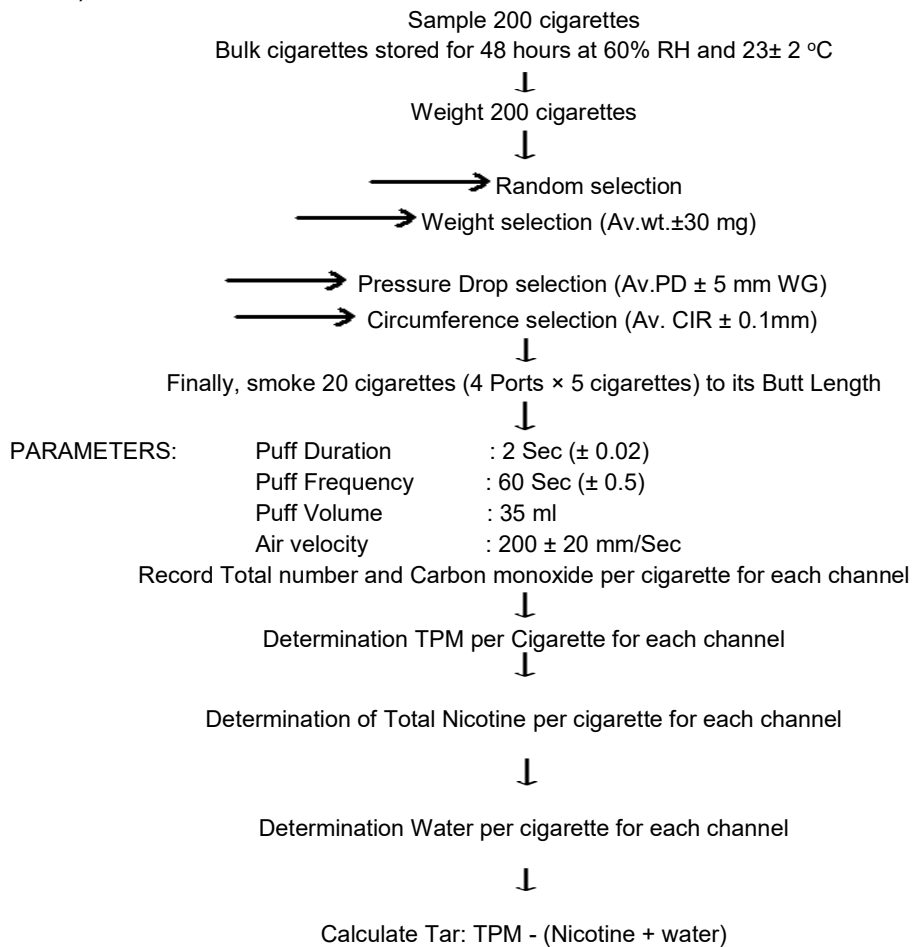
Sample 200 cigarettes
Bulk cigarettes stored for 48 hours at 60% RH and 23± 2 °C
↧
Weight 200 cigarettes
↧
⟶ Random selection
⟶ Weight selection (Av.wt.±30 mg)

⟶ Pressure Drop selection (Av.PD ± 5 mm WG)
⟶ Circumference selection (Av. CIR ± 0.1mm)
↧
Finally, smoke 20 cigarettes (4 Ports × 5 cigarettes) to its Butt Length
↧

PARAMETERS: Puff Duration   : 2 Sec (± 0.02)
                  Puff Frequency   : 60 Sec (± 0.5)
                  Puff Volume   : 35 ml
                  Air velocity   : 200 ± 20 mm/Sec
Record Total number and Carbon monoxide per cigarette for each channel
↧
Determination TPM per Cigarette for each channel
↧

Determination of Total Nicotine per cigarette for each channel

↧

Determination Water per cigarette for each channel

↧

Calculate Tar: TPM - (Nicotine + water)

Fig.2. Flow chart for the determination of TPM, Tar, CO and Nicotine

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GOOD LABORATORY PRACTICES

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The Good Laboratory Practice or GLP is a set of principles intended to assure the quality and integrity of laboratory studies that are intended to support research regulated by government agencies.

Ten Golden Rules of the Laboratory are

- *Establish and Follow Procedures*
- *Maintain Your Proficiency*
- *Validate Methods*
- *Use Traceable Standard Reference Materials/ Certified Reference Materials*
- *Run in Duplicate*
- *Keep Original Data*
- *Assign Instruments and Equipment to Analysts*
- *Calibrate Instruments*
- *Use Control Charts*
- *Document Everything and Maintain Good Records*
-

Dos and Don'ts in a laboratory

- ❖ Keep the first aid kit handy at a conspicuous working place in the laboratory.
- ❖ Personal safety aids such as laboratory coat, hand protection gloves, safety glasses, face shield and proper footwear should be used while working in the laboratory.
- ❖ Ensure rinsing of pipette before use with the next solution.
- ❖ Do not return the liquid reagents back into the bottle after they are taken out for use.
- ❖ Do not put readily soluble substances directly into volumetric flax but first transfer into a beaker, dissolve and then put in the flask.
- ❖ Store oxidizing chemicals like iodine and silver nitrate only in amber colour bottles.
- ❖ Keep the working tables/space clean. Clean up spillage immediately.
- ❖ Wash hands after handling toxic / hazardous chemical.
- ❖ Never suck the chemicals with mouth but use automatic pipetting device.
- ❖ Use fume hood while handling concentrated acids, bases and hazardous chemicals.
- ❖ Never open a centrifuge cover until the machine has stopped.
- ❖ Add acid to water and not water to acid while diluting the acid.
- ❖ Always put labels on bottles, vessels and wash bottles containing reagents, solutions, samples and water.
- ❖ Do not heat glass wares and inflammable chemicals directly on the flame.
- ❖ Read the labels of the bottles before opening them

Laboratory Quality Assurance/Control

For the uniformity of expression and understanding, the definitions of the terms quality, quality-assurance and quality control as defined by the International Standardization Organization (ISO).

Quality

The Quality has been defined as "the total features and characteristics of a product or service that bear on its ability to satisfy stated and implied need." A product can be stated to possess good quality, if it meets the predetermined parameters. In case of an analytical laboratory, the quality of the laboratory may be considered adequate and acceptable if it has the capacity to deliver the analytical results on a product within the specified limits of errors and as per other agreed conditions of cost and time of analysis so as to enable an acceptable judgement on the product quality.

Quality Assurance

As per ISO, it means "the assembly of all planned and systematic action necessary to provide adequate confidence that a product, a process or service will satisfy given quality requirements". The results of these actions are checked by another independent laboratory/person to conform the pronouncement on the quality of a product by a given laboratory. This could be referred as inter-laboratory check.

Quality Control

Quality control is an important part of quality assurance which is defined by ISO as "the operational techniques and activities that are used to satisfy quality requirements". Quality assessment or evaluation is necessary to see if the activities performed to verify the quality are effective. **Thus, an effective check on all the activities and processes in a laboratory can only ensure that the results pronounced on a product quality are within the acceptable parameters of accuracy.**

In quality control system, the following steps are involved, which when implemented properly, ensure that the results delivered are acceptable and verifiable by another laboratory.

- ✓ Check on the performance of the instruments.
- ✓ Calibration or standardization of instruments and chemicals.
- ✓ Adoption of sample check system as a batch control within the laboratory.
- ✓ External check: inter-laboratory exchange programme.

To ensure obtaining accurate and acceptable results of analysis on a sample, the laboratory has to run in a well-regulated manner where the equipment are properly calibrated and the methods and techniques employed are scientifically sound which will give reproducible results. For ensuring the high standards of quality, Good Laboratory Practice (GLP) has to be followed. The GLP can be defined as "the organizational process and the conditions under which laboratory studies are planned, performed, monitored, recorded and reported". Thus, the GLP expects a laboratory to work according to a system of procedures

and protocols whereas the procedures are also specified as the Standard Operating Procedure (SOP).

Standard Operating Procedure (SOP)

As per Reeuwijk and Houba (1998), a Standard Operating Procedure (SOP) is a document which describes the regularly recurring operations relevant to the quality of the investigation. The purpose of a SOP is to carry out the operation correctly and always in the same manner. A SOP should be available at the place where the work is done. If, for justifiable reasons, any deviation is allowed from SOP, the deviated procedure may be fully documented.

In a laboratory, SOP may be prepared for:

- Safety precaution.
- Procedure for operating instruments.
- Analytical methods and preparation of reagents.
- Registration of samples.

To sum up, all the operations have to be properly documented so as no chance is left for adhocism in any manner.

Error, Precision, Accuracy and Detection Limit

Error

Error is an important component of the analysis. In any analysis, when the quantity is measured with the greatest exactness that the instrument, method and observer are capable of, it is found that the results of successive determination differ among themselves to a greater or lesser extent. The average value is accepted as most probable. This may not always be true value. In some cases, the difference in the successive values may be small, in some cases it may be large, the reliability of the result depends upon the magnitude of this difference. There could be a number of factors responsible for this difference which is also referred as 'error'. The error in absolute term is the difference between the observed or measured value and the true or most probable value of the quantity measured. The absolute-error is a measure of the accuracy of the measurement. The accuracy of a determination may, therefore, be defined as the concordance between it and the true or most probable value. The relative error is the absolute error divided by the true or most probable value. The error may be caused due to any deviation from the prescribed steps required to be taken in analysis. The purity of chemicals, their concentration/strength and the accuracy of the instruments and the skill of the technician are important factors.

Precision and accuracy

In analysis, other important terms to be understood are precision and accuracy. Precision is defined as the concordance of a series of measurements of the same quantity. The mean deviation or the relative mean deviation is a measure of precision. In quantitative analysis, the precision of a measurement rarely exceeds 1 to 2 parts per thousand.

Accuracy expresses the correctness of a measurement, while precision expresses the reproducibility of a measurement. Precision always accompanies accuracy, but a high degree of precision does not imply accuracy. In ensuring high accuracy in analysis, accurate

preparation of reagents including their perfect standardization is critical. Not only this, even the purity of chemicals is important.

For all estimation, where actual measurement of a constituent of the sample in terms of the "precipitate formation" or formation of "colored compound" or "concentration in the solvent" is a part of steps in estimation, chemical reagents involved in such aspects must always be of high purity which is referred as AR-grade (Analytical Reagent).

Detection limit

In the analysis for trace elements in soils, plants and fertilizers and for environmental monitoring, need arises to measure very low contents of analytes. Modern equipments are capable of such estimation. However, while selecting equipment and the testing method for such purpose, it is important to have information about the lowest limits up to which analytes can be detected or determined with sufficient confidence. Such limits are called as detection limits or lower limits of detection. The capacity of the equipment and the method may be such that it can detect the traces of analyte in the sample. In quantitative terms, the lowest contents of such analyte may be decided through appropriate research as the values of interpretable significance. The service laboratories are generally provided with such limits.

Quality Control of Analytical Procedures

Independent Standards

The ultimate aim of the quality control measures is to ensure the production of analytical data with a minimum of error and with consistency. Once, an appropriate method is selected, its execution has to be done with utmost care. To check and verify the accuracy of analysis, independent standards are used in the system. The extent of deviation of analytical value on a standard sample indicates the accuracy of the analysis. Independent standard can be prepared in the laboratory from pure chemicals.

When new standard is prepared, the remainders of the old ones always have to be measured as a mutual check. If the results are not within the acceptable levels of accuracy, the process of calibration, preparation of standard curve and the preparation of reagents may be repeated till acceptable results are obtained on the standard sample. After assuring this, analysis on unknown sample has to be started.

Apart from independent standard, certified reference samples can also be used as 'standard'. Such samples are obtained from other selected laboratories where the analysis on a prepared standard is carried out by more than one laboratory and such samples along with the accompanied analytical values are used as a check to ensure the accuracy of analysis.

Use of blank

A blank determination is an analysis without the analyte or attribute or in other words, an analysis without a sample by going through all steps of the procedure with the reagents only. **Use of blank accounts for any contamination in the chemicals used in actual analysis**. The 'estimate' of the blank is subtracted from the estimates of the samples. The use of 'sequence control' samples is made in long batches in automated analysis. Generally two samples, one with a low content of analyte and another with very high content of known analyte (but the contents falling within the working range of the method) are used as standards to monitor the accuracy of analysis.

Blind sample

A sample with known content of analyte. This sample is inserted by the head of the laboratory in batches and times unknown to the analyst. Various types of sample material may serve as blind samples such as control samples or sufficiently large leftover of test samples (analysed several times). It is essential that analyst is aware of the possible presence of a blind sample but is not able to recognize the material as such.

Validation of procedures of analysis

Validation is the process of determining the performance characteristics of a method /procedure. It is a pre-requisite for judgement of the suitability of produced analytical data for the intended use. This implies that a method may be valid in one situation and invalid in another. If a method is very precise and accurate but expensive for adoption, it may be used only when the data with that order of precision are needed.

The data may be inadequate, if the method is less accurate than required. Two types of validation are followed.

Validation of own procedure

In-house validation of method or procedure by individual user laboratory is a common practice. Many laboratories use their own version of even well-established method for reasons of efficiency, cost and convenience. A change in liquid solid ratio in extraction procedures for available soil nutrients and shaking time etc. result in changed value, hence need validation. Such changes are often introduced to consider local conditions, cost of analysis, required accuracy and efficiency.

Validation of such changes is the part of quality control in the laboratory. It is also a kind of research project, hence all types of the laboratories may not be in a position to modify the standard method. They should follow the given method as accepted and practiced by most other laboratories.

Apart from validation of methods, a system of internal quality control is required to be followed by the laboratories to ensure that they are capable of producing reliable analytical data with minimum of error. This requires continuous monitoring of the operation and systematic day to day checking of the produced data to decide whether these are reliable enough to be released.

Following steps need to be taken for internal quality control:

❖ Use a blank and a control (standard) sample of known composition along with the samples under analysis.
❖ Round off the analytical values to the 2nd decimal place. The value of 3rd decimal place may be omitted if less than 5. If it is more than 5, the value of second decimal may be raised by 1.

Since the quality control systems rely heavily on control samples, the sample preparation may be done with great care to ensure that the:

- ➢ Sample is homogenous.
- ➢ Sample material is stable.
- ➢ Sample has uniform and correct particle size as sieved through a standard sieve.
- ➢ Relevant information such as properties of the sample and the concentration of the analyte are available.

The samples under analysis may also be processed / prepared in such a way that it has similar particle size and homogeneity as that of the standard (control) sample. As and when an error is noticed in the analysis through internal check, corrective measures should be taken. The error can be due to calculation or typing. If not, it requires thorough check on sample identification, standards, chemicals, pipettes, dispensers, glassware, calibration procedure and equipment. Standard may be old or wrongly prepared. Pipette may indicate wrong volume, glassware may not be properly cleaned and the equipment may be defective or the sample intake tube may be clogged in case of flame photometer or Atomic Absorption Spectrophotometer. Source of error may be detected and samples be analyzed again.

Validation of the Standard Procedure

This refers to the validation of new or existing method and procedures intended to be used in many laboratories including procedures accepted by national system or ISO. This involves an inter-laboratory programme of testing the method by a member of selected renowned laboratories according to a protocol issued to all participants. Validation is not only relevant when non-standard procedures are used but just as well when validated standard procedures are used and even more so when variants of standard procedures are introduced. The results of validation tests should be recorded in a validation report from which the suitability of a method for a certain purpose can be deduced.

Inter-laboratory sample and data exchange programme

If an error is suspected in the procedure and uncertainty cannot readily be solved, it is not uncommon to have the sample analysed in another laboratory of the same system/organisation. The results of the other laboratory may or may not be biased, hence doubt may persist. The sample check by another accredited laboratory may be necessary and useful to resolve the problem.

An accredited laboratory should participate at least in one inter-laboratory exchange programme. Such programmes do exist locally, regionally, nationally and internationally. The laboratory exchange programme exists for method performance studies and laboratory performance studies. In such exchange programme, some laboratories or the organizations have devised the system where periodically samples of known composition are sent to the participating laboratory without disclosing the results. The participating laboratory will analyse the sample by a given method and find out the results. It provides a possibility for assessing

the accuracy of the method being used by a laboratory, and also about the adoption of the method suggested by the lead laboratory.

For quality check, each laboratory will benefit if it becomes part of some sample/method check and evaluation programme. The system of self-check within the laboratory also has to be regularly followed.

Laboratory safety Precautions/measures

Special Care is required while operating equipment, handling the chemicals and in waste disposal.

Equipment: Electrical cables, plugs and tubing need proper check to avoid accident. Various types of gas cylinders needed in the laboratory like acetylene, nitrous oxide and LPG may be kept under watch and properly sealed/capped and may be stored in ventilated cupboards.

Chemical reagents: Hazardous chemicals may be stored in plastic bottles. While working with chemicals such as perchloric acid, fume hood may be used. Chemicals may be properly labeled indicating their hazardous nature.

Bottles with inflammable substances need to be stored in stainless steel containers.

Waste disposal: Cyanides, chromates, arsenic, selenium, cobalt and molybdate are very commonly used but hazardous chemicals and should never be disposed off in the laboratory sink but collected in a metal container for proper disposal at the specified places.

References:

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ESTIMATION OF LEAF QUALITY PARAMETERS BY AUTO ANALYZER

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I.   Leaf sample preparation, estimation of moisture content and preparation of reagents

a.   Leaf sample preparation

Midrib is removed from the leaf samples and the stripped lamina portions are divided into two sub samples by keeping right and left halves alternately in each of these sub samples. Thus each sub sample is made up of equal number of right and left halves. As physical quality, characteristics of the leaf are related more to manufacturing factors these determinations are to be carried out under simulated conditions of manufacture.

Sample powders are prepared by drying the lamina samples in an air oven at 60°C and powdered to pass through a 60-mesh sieve. The leaf samples should never be dried at temperatures higher than 60°C as certain organic constituents are lost at higher temperatures.

b.   Estimation of moisture

Weigh accurately duplicate samples of 2-3 gm of tobacco powder each in aluminum or stainless steel dishes, which are provided with covers. Distribute the samples evenly over the bottom of the dish, dry uncovered at 100-105 ° C in a desiccator over anhydrous calcium chloride, and weigh again soon after the samples reach room temperature. Calculate the loss in weight as percent of moisture.

$$\text{Per cent moisture} = \frac{\text{Moisture retained by oven dried shreds}}{\text{Weight of oven dried shreds}} \times 100$$

Preparation of Reagents:

1. Extracting solution: Dissolve 50 ml Acetic Acid and 200 ml Methanol in 500 ml distilled water and make up to 1000 ml with distilled water.
2. Carbon Suspension: Dissolve 135 g Darco-G in 900 ml solution of 1:1 Glycerol and distilled water. Mix the components thoroughly.

Nicotine estimation

3. Cyanogen Bromide (CNBr) Solution: Dissolve 100 g of CNBr solid in 1000 ml Alcohol and make up the volume up to 5000 ml with filtered distilled water.

4. Buffer Solution: Dissolve 8.2g of Citric acid and 11.24g of Sodium phosphate di basic in 200 ml distilled water. Add 3 ml of Aniline drop wise, make up to 1000 ml with distilled water, and add 30 drops of Brij.

Reducing Sugars estimation

5. K_3 [Fe (CN) $_6$] Solution (0.015%): Dissolve 0.015g of K_3 [Fe (CN)$_6$] in 100ml of 1N NaOH solution.
6. NaCl solution: Dissolve 9 g of NaCl in 1000ml distilled water.

Chlorides estimation

7. Mercuric Thio Cyanate (Hg (SCN) $_2$) Solution: Dissolve 2.085g of Mercuric Thio Cyanate in 500 ml of methanol.
8. Ferric Nitrate (Fe (NO) $_3$) $9H_2O$ Solution: Dissolve 500g Ferric Nitrate of in 90 ml of Con HNO_3 and make the volume up to 2.5 litres with distilled water.
9. Colour Reagent: 6.5 ml of Mercuric Thio Cyanate solution and 6.5 ml of Ferric Nitrate solution are mixed and made up to the volume of 100ml with distilled water.

Calibration solutions

10. Stock Standard Solution: 0.5120g of Nicotine Hydrogen Tartarate, 1.25g of Dextrose and 0.2749g of NaCl is taken in 250 ml volumetric flask and made up to 250 ml with Extracting solution.
11. Working Standards: 15ml, 7.5ml and 3.75ml of stock standard solution is pipetted out in three individual 50ml volumetric flasks and made up to the volume with extracting solution to get 200 ppm, 100 ppm and 50 ppm of Nicotine, 1500 ppm, 750 ppm and 375 ppm of Reducing Sugars, 200 ppm, 100 ppm and 50 ppm of Chlorides, respectively.

Procedure:

a. Extraction:
0.25 g of tobacco leaf lamina powder is taken in 150 ml conical flask, 2 ml of carbon suspension and 48 ml extracting solution are added and shaken well for 15 minutes at 170 rpm. The solution is filtered and taken into cuvettes as sample.

b. Estimation:
Pass all the reagents to the instrument till it acquires a stable baseline and after getting the stable baseline switch on the sampler. Reagents when mixed with samples produce different colour complexes of respective compounds.

Nicotine: Cyanogen bromide is used as reagent, as it reacts with nicotine in the presence of aniline buffer forms a yellow complex. The intensity of the yellow colour is proportionating to

the nicotine concentration in the sample and is measured at 460nm. Cyanogen bromide releases pyridine ring ($C_5H_5N_2$) from nicotine, which reacts with aniline forming yellow colour complex. (Direct calorimetry)

Reducing sugars: Reducing sugars in the sample reduces yellow coloured $K_3Fe(CN)_6$ (ferri cyanide) to colourless $K_4Fe(CN)_6$ (ferro cyanide) in an alkaline medium. The degree of decolourization will be measured at 420nm. (Inverse calorimetry).

Chlorides: A mixture of mercuric thio cyanate and ferric nitrate will be used as a colour reagent for chlorides estimation. Chloride ions release thio cyanate ions which react with ferric nitrate forming blood red coloured ferric thio cyanate complex. The intensity of colour is proportional to the original chloride concentration and measured at 480nm. The number of thio cyanate ions released are proportional to the chloride ions present in the sample. (Direct calorimetry)

The peak value readings represents the sample concentration and is compared with the standard calibration curve. The percentage of Nicotine, Reducing Sugars and Chlorides present in the leaf can be calculated by the formula given below:

Nicotine (%) or Reducing Sugars (%) or Chlorides (%) = (0.005 x ppm) / weight of the tobacco powder.

References:

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ANALYSIS OF SMOKE CONSTITUENTS

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I. Cigarette Selection

Cigarette samples for smoking are to be selected based on weight, circumference, pressure drop and ventilation of the cigarettes.

a. <u>Selection by weight:</u>

Weigh individually 200 cigarettes taken randomly from the conditioned laboratory sample to the nearest mg and calculate the weight of the cigarette. Cigarettes are then selected from the conditioned laboratory using QTM-8 on the basis of mean weight± 30mg.

b. <u>Selection by Circumference:</u>

The selected cigarettes on basis of weight are measured for circumference using QTM-3 and average is calculated. Then the cigarettes are selected on basis of circumference±0.1mm.

c. <u>Selection by Pressure Drop and Ventilation</u>

The cigarettes selected on the basis of weight and circumferences are measured for Pressure Drop and Ventilation using QTM-5. The average values are calculated. Then the cigarettes are selected on the basis of P.D±5 mm WG and Ventilation±2%.

II. Estimation of TPM, CO, Nicotine and Water in Smoke Condensate

The selected cigarettes shall be sorted out for approximately same values of weight, circumference, PD and ventilation and following operations shall be made before entering in to the smoke run.

a. Marking the butt length

<u>Standard butt length</u>
The standard butt length to which cigarettes shall be marked shall be the greatest of the following three lengths;
  ---- 23 mm,
  ---- length of filter + 8 mm, or
  ---- length of overwrap +3 mm,

where the overwrap is defined as any wrapper applied to the mouth end of the cigarette, and the length of the filter is defined as the total length of the cigarette minus the length of the tobacco portion.

Draw a line, using a fine soft-tipped marker, at the standard butt length, to an accuracy of 0.5 mm, from the mouth end for the particular cigarette type.

b. Preparation of Smoke traps and cigarette holders

For all operations, the operator shall prevent contamination from fingers by wearing gloves of a suitable material. Insert filter discs which have been conditioned in the test atmosphere for at least 12 hrs in to their holders, and assemble placing the rough side of the filter disk so that it will face the oncoming smoke. After assembly examine the filter holders to ensure that the discs have been properly fitted. If the smoke trap is designed to contain the perforated disc (washer), insert it and fit the ceiling devices (end caps). If the cigarette holder is designed to contain a perforated disc, insert it in to the cigarette holder before attaching the labyrinth seals (see ISO 3308:2000), 4.8). Weigh the assembled smoke traps to the nearest 0.1 mg. Because of absorption of water by smoke traps and solvent, it is necessary to determine a value for the sample blank. Prepare sample blanks by treating additional smoke traps (at least 2 per 100 cigarettes) in the same manner as that used for smoke collection.

c. Setting up the Smoking machine

If necessary, replace any protective filters on the machine. Switch on the machine and allow it to warm up on automatic cycling for at least 20 min. With the machine warmed up, check that the puff duration and puff frequency on each channel are in accordance with the standard conditions. The puff volume should be checked if it is suspected that the smoking machine is subject to a large change in temperature during use.

d. Measurement of puff volume

The displacement of a bubble in soap bubble flow meter gives a direct measurement of puff volume and also provides a check for leaks in the system. A suitable indicator graduated at 35 ml shall have a resolution of 0.1 ml. It shall be connected through a standard pressure drop device of 1kPa±5% to the cigarette holder of the smoking machine channel under test. Before use for a series of measurements, wet the instrument twice with detergent solution and then allow it to drain a period of between 30 s and 45 s.

The bubble flow meter shall contain a mass fraction of 15% aqueous solution of a surface active agent. Teepol L ® has been found to be satisfactory. The concentration of Teepol as purchased must be known before carrying out further dilution.

Fit the prepared smoking trap or traps and cigarette holders in to the machine. Attach a plastic insert of an appropriate size for the labyrinth seals in the cigarette holder to the resistance in the tube from the soap bubble flow meter indicator. Prepare the soap bubble flow meter by wetting the inside of the tube with the detergent solution to above the top graduation mark. Connect the indicator to the cigarette holder in port 1 and determine the puff volume; adjust if necessary to (35.0±0.3) ml. Repeat for all remaining ports in turn.

Repeat the determinations until the necessary precision of measurement is obtained. If the number of replicates exceeds three, continue until the correct precision is obtained but replace the pad before smoking, reweigh the smoke trap and recheck the puff volume with the new pad in place. Measure the temperature and relative humidity of the air surrounding the smoking machine and note the atmospheric pressure.

e. **Measurement of Air velocity**

1) <u>Air velocity measurement locations</u>
The reference points at which the measurement of air velocity shall be made shall be given. The required measurements shall be made such that the centre of the air velocity meter probe is within 2mm in each plane of the specified position.

2) <u>Specification of air velocity meter</u>
An air velocity meter capable of accuracy of not less than 20 mm/s at 200 mm/s shall be used. The air velocity measuring equipment shall be capable of integrating air velocity data over a min of 10 s. The value of measurement of air velocity shall consist of the average of not less than 10 replications of 10 s integrations.

3) <u>Standard value of the air velocity</u>
The standard value of the air velocity shall be 200 mm/s. Laboratory procedures should aim to ensure that the air velocity average during a smoking run lies in the range of 170mm/s to 230mm/s.

4) <u>Setting and checking air velocity</u>
Air velocity should be checked, and adjusted if necessary, when the machine is used. Extreme atmospheric conditions, external to the test atmosphere, may affect air flow in smoking machine enclosures. In such circumstances, more frequent checks of air velocity should be made.

Standard Conditions

- <u>Machine pressure drop</u>
  The whole of the flow path between the butt end of the cigarette and the suction mechanism shall offer the least possible resistance, and its pressure drop shall not exceed 300 Pa.
- <u>Puff duration</u>
  The standard puff duration shall be (2.00±0.02) s.
- <u>Puff volume</u>
  The standard puff volume measured in series with a pressure drop device of 1kPa±5% shall be (35.0±0.3) ml. In one puff duration not less than 95% of the puff volume shall leave the butt end of the cigarette.
- <u>Puff frequency</u>
  The standard puff frequency shall be one puff every (60±0.5) s measured over 10 consecutive puffs.
- <u>Air velocity</u>
  The standard air velocity shall be (200±20) m/s.

f. Procedure for Smoking run

The cigarettes are marked for Butt length and inserted in a cigarette holder, weighed initially with an empty Cambridge filter pad kept inside. Ensure that the cigarettes are positioned correctly so that the axes of the cigarettes coincide with the axes of the ports. Adjust the position of each cigarette so that when burning coal reaches the butt mark, the puff termination device is activated. 100% cotton thread of (48±4) is used to terminate smoking at the butt mark, without modifying the cigarette position. Zero the puff counters and light each cigarette at the beginning of its puff. Should it be necessary to relight a cigarette, a hand-held electrical lighter may be used. When each butt mark has been reached, remove the burning coal from the cigarette and note the final reading of the puff counters. After smoking process is complete, leave the cigarette butt in place for at least 30 s to enable deposition of any residual smoke in the trap. Avoid disturbance of the smoking by artificial removal of ash. Allow the ash to fall naturally in to the ash tray. If required, new cigarettes shall be inserted immediately and the smoking process repeated until the predetermined number of cigarettes, in accordance with the smoking plan, has been smoked in to the smoke trap. Immediately begin the determination of total particulate matter as described below.

g. Determination of Total Particulate Matter

Remove the smoke traps from the smoking machine (gloves shall be worn). Where necessary remove the cigarette holder from the smoke trap. Cover the front and back apertures of the trap with the sealing devices. It is recommended, particularly when plain cigarettes have been smoked, that the removal of the holder be conducted with the smoke trap held with its cigarette- facing side downwards to avoid any possible contaminants from the cigarette holder reaching the filter disc. Immediately after smoking, weigh the smoke traps to the nearest 0.1 mg. Check the back of each filter disc to ensure that there are no brown stains indicating overloading or pad damage. Discard any disc showing such stains or damage. Glass fibre filter pads of 44 mm diameter are capable of retaining up to 150 mg of total particulate matter and pads of 92 mm diameter are capable of retaining 600 mg of TPM. If, during smoking, this mass is exceeded, the number of cigarettes shall be reduced and a calculation made to allow for the reduced number of cigarettes smoked.

Calculations

The TPM content, m_{TPM}, for each channel , expressed in milligrams per cigarette, is given by the equation:

$$m_{TPM} = \frac{(m_1 - m_0)}{q}$$

where,

m_0 is the mass of the smoke trap before smoking , in milligrams;

m_1 is the mass of the smoke trap after smoking , in milligrams

q is the number of cigarettes smoked in to the trap.

h. Treatment of total particulate matter

Extraction procedure

Remove the sealing devices from the smoke trap (gloves shall be worn). Open it and remove the filter disc with forceps. Fold it twice, total particulate matter inwards, being careful to handle only the edge with forceps and gloved fingers. Place the folded disc in an appropriately shaped dry flask (maximum 150 ml for 44 mm discs, maximum 250 ml for 92 mm discs). Wipe the inner surface of the filter holder front with two separate quarters of an unused conditioned filter disc and add these to the flask. Pipette solvent (Propan-2-ol containing the internal standards for both nicotine and water determinations) in to the flask (20 ml for 44 mm discs or 50 ml for 92 mm discs) (see ISO 10315 and ISO 10362-1).

Stopper the flask immediately and shake gently on an electric shaker for at least 20 min. ensuring that the disc does not disintegrate. The shaking time should be adjusted to ensure full extraction of the nicotine and water in the particulate matter. Follow the same procedure with each of the blank smoke traps used for the determination of water.

i. Determination of water

The water content of the aliquot of the extracted solution is determined by gas chromatography, and water content of the whole of the smoke condensate is calculated.

1. Reagents : Use only reagents of recognized analytical reagent grade.
   i. Carrier gas: helium or nitrogen
   ii. Propan-2-ol with maximum water content of 1.0 mg/ml.
   iii. Internal standard: ethanol, or methanol (of purity at least 99%).
   iv. Extraction solvent: propan-2-ol containing an appropriate concentration of internal standard, normally 5 ml/lit.
   v. Reference substance: Distilled water or deionized water.

2. Calibration solutions

   Prepare a series of at least four calibration solutions whose concentrations of added water cover the range expected to be found in the test portion (usually up to 4mg/ml) by adding weighed amounts of water to the solvent. One of these calibration solutions shall be the solvent with no added water (solvent blank). To prevent water being absorbed, the bulk solvent container shall be fitted with a water trap and all solutions shall be kept sealed. The solvent shall be stirred continuously to ensure the homogeneity of the water concentration in the solvent. The calibration solutions shall be made up using an extraction solvent from the same batch. It is recommended that the calibration solutions be made up at least each week.

3. Apparatus
   Usual laboratory apparatus and, in particular, the following items
   i. **Gas-chromatograph,** equipped with a thermal conductivity detector, recorder and integrator or other suitable data-handling equipment. Glassware and septa for vials should be stored in a desiccator until use.

ii. **Column,** of internal diameter between 2 mm and 4 mm and preferably of length. 5 m to 2 m. Stationery phase is Porapak Q 150 μm(100 mesh) to 190 μm (80 mesh). The column is preferably made of deactivated stainless steel but other materials such as glass or nickel may be used. Alternative stationery phases, Porapak QS or Chromosorb 102, may be used.

iii. **Dispensing system**, preferably automated, capable of delivering the required volume of solvent. The dispensing system should be flushed prior to use by dispensing a volume of solvent of at least 50 ml which will then be rejected.

4. Procedure

<u>Setting up the apparatus</u>

Set up the apparatus and operate the gas chromatograph in accordance with the manufacturer's instructions. Ensure that the peaks of water, the internal standard and solvent are well resolved, the analysis time being about 4 min. Condition the system just prior to use by injecting 2μl aliquot of the extraction solvent as a primer.

Suitable operating conditions are as follows:
- ---- column temperature : 170 °C (isothermal);
- ---- injection temperature : 250 °C
- ---- detector temperature : 250 °C
- ---- carrier gas : helium at a flow rate of about 30ml/min;
- ---- injection volume : 2μl

Note: Nitrogen may also be used as an alternative carrier gas if the detector sensitivity is sufficiently high.

<u>Blank test</u>

Due to the absorption of water, by smoke traps and solvent, it is necessary to determine a value for the sample blank. Prepare sample blanks by treating additional smoke traps including filters (at least 2 per 100 cigarettes smoked) in the same manner as that used for smoke collection. Place them near the smoking machine during smoking and extract and analyse them together with the smoke samples.

<u>Determination</u>

Inject aliquots (2μl) of the test portion from the smoke traps and blank traps. Calculate the ratio of the water peak/internal standard peak from the peak area (or height) data.

Carry out the determination at least twice under identical conditions. Calculate the mean value of the ratio from the replicate determinations.

<u>Expression of results</u>

Calculate the water concentration of the smoke trap and blank trap extracts using the graph or linear regression equation prepared.

The water content of the smoke, m_w, in milligrams per cigarette, is given by the equation:

$$m_W = \frac{(\rho_{WS} - \rho_{WB}).V_{ES}}{q}$$

where

ρ_{WS} is the concentration of water in the sample smoke trap, in milligrams per milliliter;

ρ_{WB} is the concentration of water in the blank smoke trap, in milligrams per milliliter;

q is the number of cigarettes smoked through each smoke trap;

V_{ES} is the volume of extraction solvent in which the contents of the smoke trap were dissolved.

Express the test results in milligrams per cigarette for each channel to the nearest 0.01 mg, and the average per cigarette to the nearest 0.1 mg.

j. Determination of Nicotine:

The nicotine content of the aliquot of the extracted solution is determined by gas chromatography, and nicotine content of the whole of the smoke condensate is calculated.

1. <u>Reagents:</u>
Use only reagents of recognized analytical reagent grade.
   I. Carrier gas, helium or nitrogen of high purity
   II. Auxiliary gases: air and hydrogen of high purity for the flame ionization detector.
   III. Propan-2-ol, with maximum water content of 1.0 mg/ml.
   IV. Internal standard: n-heptadecane or quinaldine (of purity at least 99%).
   V. Extraction solvent: propan-2-ol containing an appropriate concentration of internal standard, this is normally in the range of 0.2 mg/ml to 0.5 mg/ml. Solvent not stored in a temperature – controlled laboratory shall be allowed to equilibrate to (22±2) ^{0}C before use.
   VI. Reference substance: nicotine of known purity and verified in accordance with ISO13276. Store this at between 0 ^{0}C and 4 ^{0}C and exclude light. Nicotine salicylate of known purity and verified in accordance with ISO13276 may also be used.

2. <u>Calibration solutions:</u> Pure Nicotine standards shall be prepared ranging from 1 mg to 20 mg dissolving in 25 ml IPA solution.

3. <u>Apparatus</u>
   Usual laboratory apparatus and, in particular, the following items

   a. **Gas-chromatograph,** equipped with a flame ionisation detector, recorder and integrator or other suitable data-handling equipment.
   b. **Column,** of internal diameter between 2 mm and 4 mm and preferably of length 1.5 m to 2 m. Stationery phase: 10% PEG 20000 plus 2% potassium hydroxide on an acid washed silanized support material, 150 μm (100 mesh) to 190 μm (80 mesh). The column is preferably made of glass but other materials such as deactivated stainless steel or nickel may be used.

4. **Procedure**

   <u>Setting up the apparatus</u>
   Set up the apparatus and operate the gas chromatograph in accordance with the manufacturer's instructions. Ensure that the peaks of nicotine, the internal

standard and solvent and other smoke component peaks especially neophytadiene (which can appear on the tail of the nicotine peak under certain circumstances) are well resolved, the analysis time being about 6-8 min. Condition the system just prior to use by injecting 2µl aliquot of the extraction solvent as a primer.

Suitable operating conditions are as follows:
- ---- column temperature : 170 °C (isothermal);
- ---- injection temperature : 250 °C
- ---- detector temperature : 250 °C
- ---- carrier gas : helium at a flow rate of about 30ml/min;
- ---- injection volume : 2µl

Determination

Inject aliquots (2µl) of the test portion from the smoke traps and blank traps. Calculate the ratio of the nicotine peak/internal standard peak from the peak area (or height) data.
Carry out the determination at least twice under identical conditions. Calculate the mean value of the ratio from the replicate determinations.

Expression of results
Calculate the nicotine concentration of the test portion using the graph or linear regression equation prepared.
The nicotine content of the smoke, m_N, in milligrams per cigarette, is given by the equation:

$$m_N = \frac{\rho_{NS} . V_{ES}}{q}$$

Where

ρ_{NS} is the concentration of nicotine in the sample test portion, in milligrams per milliliter;
q is the number of cigarettes smoked through each smoke trap;
V_{ES} is the volume of extraction solvent in which the contents of the smoke trap were dissolved.
Express the test results in milligrams per cigarette for each channel to the nearest 0.01 mg, and the average per cigarette to the nearest 0.1 mg.

k. **Determination of Nicotine Free Dry Particulate Matter (NFDPM, also termed as Tar)**

If water is eliminated from total particulate matter, the resulting material is known as Dry Particulate Matter, DPM in mg/cig=TPM in mg/cig- m_w
If nicotine is eliminated from DPM, the resulting material is known as Nicotine Free Dry Particulate Matter, NFDPM in mg/cig=DPM in mg/cig- m_N

l. **Determination of Carbon monoxide (CO)**

Definitions
a. Vapourphase: The portion of the smoke which passes particulate phase trap during smoking in accordance with ISO 4387 using a machine confirming to ISO 3308.

b. Clearing puff: Any puff taken after cigarette has been extinguished or removed from the cigarette holder.

Principle: After completion of smoking, the vapour phase of the cigarette smoke shall be collected for the measurement of the carbon monoxide using a non-dispersive infrared (NDIR) analyser calibrated for carbon monoxide.

Non-dispersive infra-red (NDIR) analyser, selective and calibrated for the measurement of carbon monoxide in vapours and gases. Analysers are available from several manufacturers and should have a preferred working range of 0%-10% (V/V) CO and a sampling rate of between 0.5 l/min and 2 l/min. The analyser shall have a precision of 1% of full scale, a linearity of 1% of full scale and a repeatability of 0.2% of full scale, under conditions of constant temperature and pressure. Its response to 10% of (V/V) of CO_2 shall not exceed 0.05% (V/V) of CO. Its response to 2% (V/V) of water vapour shall not exceed 0.05% (V/V) of CO.

Measurement of Carbon monoxide:

After completion of smoking, the average mass of carbon monoxide per cigarette is measured through COA- Analyser automatically, in accordance with the equation:

$$m_{cig} = \frac{C \times V \times N \times p \times T_o \times M_{CO}}{S \times 100 \times p_o \times (t+273) \times V_m}$$

m_{cig} is the average mass of carbon monoxide per cigarette, in milligrams.

M_{CO} is the molar mass of carbon monoxide, in grams per mol.

V_m is the molar volume of an ideal gas, in litres per mole.

V_{as} is the average volume of carbon monoxide per cigarette, in millilitres ;

C is the percentage by volume of carbon monoxide observed;

V is the puff volume, in millilitres;

N is the number of puffs in the measured sample (including clearing puffs);

p is the ambient pressure, in kilopascals ;

p_o is the standard atmospheric pressure, in pascals ;

S is the number of cigarettes smoked;

T_o is the temperature for the triple point of water, in kelvin ;

t is the ambient temperature, in degrees

For V = 35 ml and using rounded values of (101,3 kPa) and Ta (273 K), equation above yields :

$$V_{as} = \frac{0.9432 \times C \times N \times P}{S \times (t+273)}$$

These three NFDPM, Carbon monoxide (CO) and Nicotine gives smoke quality parameters for the analysed cigarettes.

j. Terms and definitions used in smoke analysis

1. Test atmosphere: Atmosphere to which a sample or test piece is exposed throughout the test.
2. Butt length: Length of un burnt cigarette remaining at the moment when the smoking is stopped
3. Restricted Smoking: Condition that exists when the butt end of a cigarette is closed to the atmosphere between successive puffs
4. Pressure Drop:
   Static pressure difference between the two ends of test piece completely encapsulated in a measuring device such that no air can pass through the outer

membrane(or wrapping) or Static pressure difference between the two ends of a pneumatic circuit when it is traversed by an air flow under study conditions in which the measured volumetric flow, under standard conditions, at the output end is 17.5 ml/s.

5. <u>Puff duration:</u> Interval of time during which the port is connected to the suction mechanism.
6. <u>Puff volume</u>: Volume leaving the butt end of a cigarette and passing through the smoke trap
7. <u>Puff number</u>**:** Number of puffs necessary to smoke a cigarette to a specified butt length
8. <u>Puff frequency:</u> Number of puffs in a given time
9. <u>Cigarette holder:</u> Device for holding the mouth end of a cigarette during smoking
10. <u>Smoke trap:</u> Device for collecting such part of the smoke from a sample of cigarettes as is necessary for the determination of specified smoke components
11. <u>Port:</u> Aperture of the suction mechanism through which a puff is drawn and to which is attached a smoke trap.
12. <u>Channel:</u> Element of a smoking machine consisting of one or more cigarette holders, one trap and a means of drawing a puff through the trap.
13. <u>Mainstream smoke:</u> All smoke which leaves the butt end of a cigarette during the smoking process
14. <u>Sidestream smoke:</u> All smoke which leaves a cigarette during the smoking process other than from the butt end
15. <u>Ashtray</u>**:** Device positioned under the cigarettes in their holders to collect ash falling from the cigarettes during smoking
16. <u>Clearing puff:</u> Any puff taken after the cigarette has been extinguished or removed from the cigarette holder
17. <u>Ambient air-flow:</u> Air flow around the cigarettes during the smoking process

References:
1. IS 16023:2012, ISO 4387:2000 -Determination of total and nicotine-free dry particulate matter using a routine analytical smoking machine.
2. IS 16025:2012, ISO 10315:2000 -Determination of Nicotine in smoke condensates - Gas-Chromatographic method.
3. IS 16042 (Part 1):2012, ISO 10362-1:1999 -Determination of water in smoke condensates -Gas-Chromatographic method.

ANALYSIS OF PESTICIDE RESIDUES

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Estimation of Pesticide Residues

All regents shall be of required grade for pesticide residue analysis.

1. Reagents
   a) Water, degassed, in accordance with at least grade 2 of ISO 3696.
   b) Acetonitrile, HPLC and Spectroscopy grade
   c) n-hexane, HPLC and Spectroscopy grade
   d) Sodium Sulphate, AR grade
   e) Florisil: Florisil 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 ^{O}C and +4 ^{O}C and exclude light.
   h) Individual standard stock solutions:
      The stock solutions of the individual pesticide standards shall 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 stored in dark vials in a refrigerator at   −20 (±2) °C.
   i) Mixed Intermediate standard solution:
      An intermediate stock standard mixture of 10 μg mL^{-1} shall 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} shall be prepared by diluting the intermediate stock solution, from which the calibration standards were prepared by serial dilution with hexane. Stability of the working solvent standards shall be checked against freshly prepared working standards (1 μg mL^{-1}) from the intermediate stocks as per SANCO guidelines (SANCO/12495 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} was freshly prepared from the working standard mixture of 1 μg mL−1 concentration by appropriate dilutions. Matrix-matched standards at the same concentration levels were prepared by extracting control tobacco and spiking the extract with appropriate volumes of the working standard solutions.
2. 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 shall 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 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 shall each be equipped with a separate heating unit.

3. Procedure

Preparation of test sample

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

Extraction

1 g powder was taken in a 150-mL Erlenmeyer conical flask, and 20 mL of acetonitrile/water (1:1) mixture was added to the flask. Our 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 were less for acetonitrile/ water (1:1) mixture. Further, use of acetonitrile/water (1:1) mixture ensured low use of solvent and reduced the cost of solvent. The samples were agitated for 45 min over an orbital shaker at 150 rpm and filtered. The filtrate was partitioned with 40 mL of hexane, and the coloured hexane fraction was collected for clean-up. A column was prepared by fabrication of a column bed which was 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 worked as an adsorbent for removal of large organic molecules from tobacco matrix extract like pigments, lipids, long-chain organic acids and other matrix compounds. Anhydrous sodium sulphate played dual purposes. First, it acted as a protective layer for underneath adsorbent layer of florisil while loading the sample for column clean-up, and secondly, it removed the traces of water from the solvent extract before injecting to the instrument. Before clean-up, the column was eluted with pure hexane and then coloured hexane extract was passed through the column. Clear and colourless hexane fraction was collected from the column and evaporated to dryness under reduced pressure. The residuum was re-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 was 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 resulted 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 (1µl-5 µl)
Injector temperature : 250 °C
Injection mode: Splitless

4) Column   : The GC system(GC 2010 Plus) shall be equipped with ZB-5 (5 % diphenyl, 95 % dimethylpolysiloxane, 30 m (l)×0.25 mm (id), 0.1 µm film thickness) capillary column.

5) M S 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 was set at 1 kV, and the data acquisition was 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

References:

1. Rakesh Kumar Ghosh, Zareen S. Khan, C. V. N. Rao, Kaushik Banerjee , D. Damodar Reddy, T. G. K. Murthy, Nalli Johnson, Deb Prasad Ray (2014).Assessment of organochlorine pesticide residues in Indian flue-cured tobacco with gas chromatography-single quadrupole mass spectrometer. Environmental Monitoring and Assessment 186:5069–507

2. Zareen S. Khan, Rakesh Kumar Ghosh, Rushali Girame, Sagar C. Utture, Manasi Gadgil, Kaushik Banerjee, D. Damodar Reddy, Nalli Johnson (2014). Optimization of a sample preparation method for multiresidue analysis of pesticides in tobacco by single and multi-dimensional gas chromatography-mass spectrometry. Journal of Chromatography A 1343: 200–206.

ICAR-CTRI QUALITY POLICY

- *Ensuring* production of "quality tobacco" with reduced levels of harmful constituents

- *Enhancing* farm returns through innovative interventions for sustainable resource use and production efficiency

- *Exploring* and effective use of green energy sources for FCV tobacco curing

- *Exploiting* tobacco for diversified uses (phytochemicals and value added products

- *Effective* technology transfer/ consultancy services to address the stakeholders needs

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