Sections in This Document

1. Introduction

The Tobacco analysis training program provides a preliminary training in basic technical, mass spectrometry instrumentation and specific instrumentation for the testing of tobacco products. The ORA U New Hire Laboratory Analyst Training curriculum (Bingo card), specifies sections of the ORA Lab Manual as part of the New Hire Curriculum. The laboratory manual is a basic training document for analysts and smoke technicians in the FDA tobacco laboratory area.

The Center for Tobacco Product (CTP) is responsible for establishing the assignments and Compliance Program for tobacco product analysis. These assignments and Compliance Program documents have analytical guidelines and instructions for sample handling and analysis.

This chapter does not include the entire range of methodologies and technologies performed in the tobacco laboratory. Most of the technology used in the tobacco laboratory will be discussed with the analyst. The trainee will be trained in only one of the analytical methods of the tobacco laboratory. During the course of training, the trainer will demonstrate the method to the trainee, the trainee will run the method with the assistance of the trainer, and finally the trainee will be required to perform the method independently as a final test. After the training is completed, the employee will be trained in other methodologies. A proficiency test will be required after training.

Tobacco Laboratory receives different types of assignments from CTP therefore the training should include an evaluation of the sample assignment. As part of training, the trainer should consider and discuss topics with the trainee that will cultivate a thoughtful and responsible approach to each assignment. Topics may include the following:

- Reason for the sample collection:
  - New Tobacco Product Application: This type of sample is related to either a Modified-Risk Tobacco Product Application (MRTP) or Pre-Market Tobacco Application (PMTA).
  - Research: Project requested by CTP/ORS for information purposes.
  - For-cause Sample: CTP/OCE assignment for a specific sample due to an ongoing investigation.
  - Survey Sample: This samples are collected as part of a survey program generated by CTP and ORS.
- Method to be use for analysis: Check assignment or compliance program.
- Methods need validation or verification
- Correct sample handling:
  1. Is there a microbiological analysis? The microbiologists handle the sample first.
  2. Are there enough samples for all the tests requested?

After the trainee has completed each exercise, the analysis, and worksheet preparation, any trainee questions and answers should be discussed thoroughly. At the end of training, the analyst should be proficient in the analytical technique, instrumentation, and documentation requirements for the method trained.

2. Historical Background and Law

The FDA Center for Tobacco Products (CTP) is responsible for carrying out the Family Smoking Prevention and Tobacco Control Act, which Congress passed in 2009. This law, commonly called the Tobacco Control Act, gives FDA authority to regulate the manufacturing, distribution, and marketing of tobacco products. CTP mission is to protect Americans from tobacco-related death and disease by regulating the manufacture, distribution, and marketing of tobacco products and by educating the public, especially young people, about tobacco products and the dangers their use poses to themselves and others.

In 2010, the Southeast Tobacco Laboratory (STL), in Atlanta, GA, was established as the first FDA tobacco regulatory laboratory, responsible for providing the analytical support for testing Harmful and Potentially Harmful Constituents (HPHC's) in both tobacco and tobacco smoke. STL develops, validates, and/or verifies methods for testing in a wide variety of tobacco products to support CTP application review, product standard development, and compliance & enforcement.

E.g. STL developed and validated the analytical method to detect and quantitate flavor compounds in different tobacco products to support of the ban on flavored cigarettes.

3. Methodology

3.1. Introduction

The source for the methodology to regulate the tobacco industry can be STL, literature, CDC, CORESTA, ISO and/or Tobacco industry. All methods used in STL must be either validated or verified as per STL SOP.. STL uses the following technologies: GC-MS, LC-MS, LC, GC, ICP-MS, IC (Ion Chromatography), and GCxGC-TOF. Assignments and Compliance Programs from CTP will establish methodology source.

3.2. Methodology Sources

A. STL (Southeast Tobacco Laboratory)

STL develops, validates, and verifies methods for the analysis of HPHCs in tobacco products. STL is validating standard analytical methods (SAM) to be used by the tobacco industry. Analytical method validation should be performed as per CTP requirements. The method validation reports are reviewed and approved by CTP. However, only methodology approved by STL QA can be used to analyze regulatory samples.

B. CORESTA (Cooperation Centre for Scientific Research Relative to Tobacco)

CORESTA is an association founded to promote international cooperation in scientific research relative to tobacco and its derived products. They have many methodologies for the analysis of constituents in tobacco products. All CORESTA methods need either method validation or verification before use. CTP will advise which type of method evaluation is needed. However, only methodology approved by STL QA can be used to analyze regulatory samples.

C. ISO (International Organization for Standards)

ISO is considered a primary source of official methods. All ISO methods must be either validated or verified in STL before use. The method validation or verification must be reviewed and approved by QA. CTP will advise which type of method evaluation is needed.

Examples of ISO methodologies:

1. ISO 2881:1992 Determination of Alkaloid content
2. ISO 4387:2000 Determination of total and nicotine-free dry particulate matter using a routine analytical smoking machine
3. ISO 6488:2004 Determination of water content by Karl Fischer method
4. ISO 16632:2013 Determination of water content by Gas Chromatography
5. ISO 192920:2016 Determination of tobacco specific nitrosamines in mainstream cigarette smoke
6. ISO21045:2018 Determination of ammonia by ion Chromatography

D. MRTPA (Modified Risk Tobacco Product Application) or PMTA (Pre-Market Tobacco Application)

The MRTPA/PMTA's submitted by manufacturers may include methods used by the manufacturer or a third-party contract laboratory to provide application data for the applicant's tobacco product(s). If the method provided is determined to be suitable by CTP, STL may be asked to perform a method verification and use the applicant's method to verify select application data. All method verifications and testing must be done in accordance with ORA laboratory QA requirements.

All the material provided in the MRTPA or PMTA submissions are confidential and subject to protection. The analyst may not cite MRTPA or PMTA methods in a publication or discuss them with persons outside STL. The documents require secure storage; the analysts are to be careful in how they handle the documents.

E. Other sources (CDC, Literature and Journals)

When methods are not located in the conventional sources mentioned above, there may be applicable procedures found in the literature. The *Laboratory Information Bulletin* (LIB) is a popular source of unofficial methodology in FDA published by the Office of Regulatory Science. The LIBs are an excellent source for methods. Other good sources for articles are PubMed, NCBI, NIH and instrument manufacturer technical applications. Any peer-review journal is a good source for analytical methods. When using methods from external or alternative sources, the methods must be fully validated before use in an ORA laboratory and the method validation package must be reviewed and approved by QA and CTP.

F. Questions

1. What do all methodology sources have in common?
2. What special attention is needed for MRTP and PMTA methodologies?
3. Please list all methodology sources?
4. Look for the HPHC table in the FDA website and mention 10 HPHCs on the list.

4. Testing Procedures

4.1. Tobacco Sample Analysis

The trainee must perform successfully one of the mentioned methods below (15.4.1-6). In case the method requires smoke collection, the smoke technician is only responsible for performing the smoking portion

of the sample collection. To certify the smoking technician, a qualified analyst will perform the sample analysis in the adequate instrument. If the trainee is an analyst, the smoking should be performed by a qualified smoke technician. The results must be within acceptable limits. All trainees must pass a proficiency test before starting sample analysis in the laboratory.

The major types of tobacco products are combusted and non-combusted. Non-combusted tobacco products consist of smokeless tobacco, primarily chewing tobacco and snuff (including snus) in the U.S.; Electronic Nicotine Delivery Systems (ENDS); heat-not-burn; and some dissolvable or novel tobacco products. Most smokeless tobacco use involves placing the product between the gum and the cheek or lip. Electronic nicotine delivery system (ENDS) products use an “e-liquid” that may contain nicotine, as well as varying compositions of flavorings, propylene glycol, vegetable glycerin, and other ingredients.

The liquid is heated to create an aerosol that the user inhales. ENDS may be manufactured to look like conventional cigarettes, cigars, or pipes. Some resemble pens or USB flash drives. Larger devices, such as tank systems or mods, bear little or no resemblance to cigarettes. Heat-not-burn tobacco products are products in which the tobacco is heated to release nicotine without combustion. Combustible tobacco products, such as cigarettes, cigars, roll your own (RYO), pipe and waterpipe tobacco are burned and inhaled. The tobacco industry is constantly changing and creating new tobacco products which may require the adaption of existing tobacco methods or the development of new methods (ex. nicotine gel that contains nicotine and can be absorbed through the skin).

STL testing includes, but is not limited to, tobacco product filler, smokeless, aerosol, e-liquids, and smoke. When analyzing smoke and aerosol products, a specialized smoking or aerosol machine is used to generate and collect the sample. The operation of these machines requires significant training and practice. Smoke technicians must be trained in a method that includes smoking. There are different types of smoking machines: linear smoking machine, rotary smoking machine, e-vaping machines, cigar smoking machine, etc. Smoke technicians and analysts must be trained and be proficient in one machine type but must continue training to operate all machine types.

Linear and rotary smoking machines are mainly used for collecting smoke constituents of cigarette and cigarette-like products. The sample can be collected in either an impinger containing an extraction solution,

an electrostatic trap, or on a filter pad depending on the type of analyte to be analyzed. The linear smoking machines allows the collection of multiple individual replicates of mainstream smoke sample at the same time. The rotary smoking machine collects smoke from multiple cigarettes on one pad. By collecting smoke from multiple cigarettes on one pad, the analyte of interest can be concentrated for low level detection. The e-vaping machines is designed to be used with different types of e-cigarettes and vapor devices. A cigar smoking machine is used for cigar and cigar-like products with higher diameter and length than cigarettes.

A. Questions:

1. What types of smoking machines can be used to collect smoke from cigarettes?
2. You need to analyze an analyte that is found at low level in alittle cigar. What would be the appropriate smoking machine to use and why?
3. What are few of the major tobacco product types?
4. What collection technique would be appropriate to analyze nicotine in smoke?
5. What does ENDS stand for?

4.2. Nicotine in Tobacco Filler (GC-MS)

A. Objective

The analyst will become familiar with the quantitation of nicotine in cigarette filler, cigar, little cigars, cigarillo, and smokeless products. The analysts must follow the current SOP for quantitation of nicotine by GC-MS.

B. Reference

1. STL SOP Quantitation of Total Nicotine in Tobacco Filler using GC-MS (SRL-CHEM I.027) or current method. (Pre-requisite – Read STL SOP Quantitation of Total Nicotine in Tobacco Filler using GCMS (SRL-CHEM I .027).

C. Pre-analysis Questions

1. What is nicotine's molecular weight?
2. Why do you need to be careful handling nicotine standards, solutions and samples?
3. Define CCV, ICV and % RSD.

4. What GC-MS type will be used for this analysis? What other types of GC-MS are available?
5. What reference materials can be used as control samples?
6. Why is ISTD added to the sample and standard solutions?
7. Should the samples be analyzed frozen, refrigerated or at room temperature?
8. Sometimes grinding the sample is a requirement for sample preparation, why the grinder should be set on pulse instead of constant?
9. What ions will be used for quantitation?
10. What quality controls are needed for this test?

D. Exercise

Analyst will perform the analysis of a proficiency sample or SRM (Standard Reference Material) following the current method for quantitation of nicotine in cigarette filler and smokeless. The sample will be reported on the FDA form 431.The analyst's results must be within established limits.

E. Post Analysis Questions

1. Which GC-MS mode is used, SIM or full scan?
2. What do SIM and MRM stand for?
3. What was the purity of the nicotine standard? How is this purity used within the method?
4. How can you verify that the instrument used was qualified and ready for use?
5. Are the standards and sample solutions labeled correctly?

4.3. NNN and NNK in Tobacco Filler/smokeless (LC-MS/MS)

A. Objective

N'-Nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), are Tobacco Specific Nitrosamine (TSNA) found in many tobacco products. These compounds are classified as group 1 carcinogens and they are included in the HPHC list. These products are formed during the curing, fermentation, and burning of tobacco. The analysts must quantitate these compounds in tobacco filler or smokeless product using an LC- MS/MS method. **Reference**

1. STL SOP Analytical Method for the Quantitation of TSNA in Cigarette Filler and Smokeless Products (SRL-CHEM I.029) or current methodology for the quantitation of NNN and NNK in tobacco products by LC-MS/MS.

B. Pre-analysis Questions

1. When are NNN and NNK formed in the tobacco manufacturing process?
2. Where are TSNAs found in higher amounts?
3. What LC-MS/MS stands for and what is used for?
4. Explain the most commonly used ionization techniques available for LC-MS? How do the ions get ionized in each type of source?
5. What type of ionization source is used in the TSNA method?
6. What ions are used for quantitation? What ions are used for confirmation? What criteria are used to confirm the molecule?
7. What is a product ion?
8. What is the collision energy of the method and what is the collision energy for?
9. What gas is needed in the collision cell and why?
10. Why is the column heated?
11. What are the QC requirements of the method?

C. Exercise

Analyst will perform the analysis of a proficiency sample or SRM following the current method for quantitation of NNN and NNK in cigarette filler and smokeless products. The sample will be reported on the FDA form 431. The analyst's results must be within established limits.

D. Post-analysis Questions

1. What is critical during sample preparation?
2. Should the standards be used frozen, refrigerated or at room temperature?
3. Why is system suitability needed?
4. What precautions are needed during the sample and standard preparation?

4.4. Tar, Nicotine and Carbon monoxide (TNCO) by GC-FID/TCD

Note: This method must be used with participation of both training analyst and smoke technician.

A. Objective

Cigarette composition and design determine the amount of tar, "Nicotine Free Dry Particulate Matter (NFDPM), nicotine, and carbon monoxide delivered within the smoke. In this method, "tar", nicotine, Total Particulate Matter (TPM), water, and carbon monoxide (CO) are measured in mainstream cigarette smoke. Water content and TPM are used to calculate the amount of "tar" in the smoke residue. Cigarettes are smoked using a linear smoking machine. CO is measured directly by the smoking machine. All remaining analytes are collected on a filter pad for subsequent extraction and analysis using a gas chromatograph (GC). Smoke technicians and analysts must follow a specified smoking regime. STL typically performs smoking runs using either the Canadian Intense (CI) and ISO smoking regimes. The smoke technician does not perform the instrumental analysis for nicotine and water content.

However, the technician must work with an analyst during the technician's proficiency test to obtain results which will be used to evaluate the technician's performance by comparison to TNCO values from a standard reference cigarette. Analysts must also demonstrate proficiency for both cigarette smoking, instrumental analysis, and subsequent calculation of TNCO parameters. Results must be within specified limits of a standard reference cigarette.

B. References

1. Quantitation of "Tar", Nicotine and Carbon Monoxide in Cigarette Smoke Using a Linear Smoking Machine and GC/FID and GC/TCD (SRL-CHEM-I.028) or current method
2. STL SOP for Operation of Linear Smoking Machines (SRL-CHEM-I.040) or current method.

C. Pre-analysis Questions

1. What is "Tar"?
2. Why is it important to measure carbon monoxide (CO)?
3. How is water quantitated?
4. What preparations are necessary for cigarettes prior to smoking?
5. What are the Canadian intense (CI) smoking regime parameters?

6. What parameters must be verified in the smoking machine settings prior to smoking?
7. In what environmental conditions, should the sample be smoked? Where are the required environmental conditions for a smoking machine run found?
8. Why are the Cambridge filter pads weighed prior to and immediately after performing a smoking machine run?
9. What safety precautions are needed during a smoking machine run?

D. Exercise

A smoking machine run is performed by an analyst or smoke technician using a proficiency sample or reference cigarette. An analyst will perform the analysis of these samples following the current method for quantitation of TNCO in cigarette smoke. The sample will be reported on the FDA form 431. Sample results must be within established limits.

E. Post-analysis Questions

1. What is butt length and how is it determined?
2. Describe the appearance of the Cambridge filter pad (CFP) after smoking?
3. How many cigarettes are smoked per pad in ISO regime?
4. What is the reference cigarette?
5. Is it a requirement to smoke a reference cigarette with each smoking run?
6. What are the special preparations necessary for cigarettes to be smoked using the CI regime? Why?
7. What is the GC carrier gas?
8. What does FID mean and how does it work?
9. What does TCD mean and how does it work?
10. What are the QC of the method and what they measure?

4.5. Metals Analysis (ICP-MS)

A. Objective

Toxic metals are a concern to the public health. Arsenic and cadmium are two of the toxic metals monitored in tobacco products filler. Other metals of concern in ENDS aerosol are lead, nickel, iron and copper.

Analysts will be trained in the current ICP-MS/MS method and must pass a proficiency test.

B. Reference

1. STL SOP for Quantitation of Metals in Tobacco Product filler or current method.

C. Pre-analysis Questions

1. What gas is used to generate the plasma?
2. Why are metals monitored in food, drugs, and tobacco?
3. How is the sample prepared before ICP-MS testing?
4. What is digestion?
5. Before starting any sample testing, what needs to be verified in the ICP-MS?
6. What are all the types of cones?
7. What is a critical parameter of the nebulizer?

D. Exercise

The analyst will perform the analysis of a proficiency sample or SRM following the current method for quantitation of metals in tobacco filler. The sample will be reported on the FDA form 431. The analyst's results must be within established limits.

E. Post-analysis Questions

1. What is ICP and how is it used with MS?
2. Describe the change in appearance of the sample after digestion?
3. Why is hydrofluoric acid used in the digestion?
4. Why is pressure used in the digestion system?
5. Is an internal standard used for metal analysis? If so, why?
6. How is the correct internal standard selected for the analysis?
7. What are the QC requirements of the method?

4.6. Carbonyl in Tobacco Filler (LC-UV)

Carbonyl group is a functional group composed of a carbon atom double-bonded to an oxygen atom (C=O). Some carbonyl compounds are potentially toxic (carcinogenic) and they can be found in tobacco product filler, ENDS aerosol and smoke. Some of the most common carbonyl compounds found in tobacco products are: acetaldehyde,

acetone, acrolein, crotonaldehyde and formaldehyde. This method requires derivatization of the carbonyl compounds before LC-UV detection. The analyst will become familiar with the quantitation of carbonyl compounds in tobacco products.

A. Reference

1. STL SOP for Quantitation of Carbonyl Compounds in Tobacco Product or current method.

B. Pre-analysis Questions

1. What type of chemical compounds are carbonyls?
2. Are carbonyls byproducts of tobacco product combustion?
3. Are carbonyl compounds part of the abbreviated HPHC list?
4. What is an UV/VIS detector?
5. What is the HPLC's column stationary phase?
6. Why are carbonyls derivatized before analysis?
7. What is used as reference standard?
8. Are there other technologies used to quantitate carbonyl compounds?

C. Exercise

Analyst will perform the analysis of a proficiency sample or SRM following the current method for quantitation of carbonyl in tobacco products. The sample will be reported on the FDA form 431. The analyst's results must be within established limits.

D. Post-analysis Questions

1. Which two carbonyls elute close to each other?
2. Why is the composition of the mobile phase important?
3. What is the purpose of heating the column?
4. Why do the samples need to be neutralized after derivatization?
5. Why is the gradient increased to a high amount of organic phase at the end of the run?

4.7. Polymerase Chain Reaction (PCR)

A. Objective

Real-time PCR is a technique in molecular genetics that permits the analysis of short sequences of DNA or RNA. This technique is so

sensitive that can detect minute quantities of genetic material. Currently, the real-time PCR is used for the detection of two DNA fragments found in tobacco plants. The presence of tobacco plant DNA in any product will assure CTP's jurisdiction over the product. The analyst will become familiar with the analysis and the detection of tobacco plant genes in tobacco product by real-time PCR.

B. Reference

1. Current SOP, SRL-CHEM I.031, "Detection of Nicotiana genes Nt1 and NtNir in Tobacco and Tobacco Related Products Using Real Time Polymerase Chain Reaction" or any current method.

C. Pre-analysis Questions

1. How does real-time PCR work? Explain PCR reaction and detection.
2. Why is sample handling so important?
3. Can the real-time PCR detect the presence of one DNA fragment in the sample?
4. Why does the sample need to be grinded before analysis?
5. Why does the sample need to be handled first by the analyst performing this test?
6. What are inhibitors and why is it important to be aware of them?

D. Exercise

Analyst will perform the analysis of a proficiency sample or SRM following the current method for the detection of tobacco plant DNA in tobacco product. The sample will be reported on the FDA form 431. The analyst's results must contain satisfactory control and indicate positives and/or negatives based on cycle threshold (Ct) values.

E. Post-analysis Questions

1. How do you know there is tobacco plant DNA in the sample?
2. Which probe dyes are used to target the tobacco genes of interest?
3. What quality controls were used to assure the analysis performed as intended?
4. How many sample replicates are needed? What reagent control is used to determine if inhibition has occurred during an analysis and what steps are taken to rule out false negatives due to inhibition? References

1. Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) website: https://www.coresta.org/
2. International Organization for Standardization (ISO): ISO/TC 126 Tobacco and Tobacco products.
3. Center of Tobacco Product intranet. http://inside.fda.gov:9003/CTP/default.htm

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5. Answer Key

5.1. Methodology Sources

A. What do all methodology sources have in common? All need either validation or verification.

B. What special attention is needed for MRTPA and PMTA methodologies? Confidential, do not distribute.

C. Please list all methodology sources? STL, literature, LIB, CDC, CORESTA, ISO and/or Tobacco industry

D. Look for the HPHC table in the FDA website and mention 10 HPHCs on the list. Example: ammonia, acrolein, BaP, benzene, cobalt, furan, isoprene, 2-nitropropene, NNN, NDMA, phenol, etc.

5.2. Tobacco Sample Analysis

A. What types of smoking machines can be used to collect smoke from cigarettes? Linear and rotary smoking machines

B. You need to analyze an analyte that is found at low levels in a little cigar . What is the appropriate smoking machine to use and why? Rotary smoking machine. Because it can concentrate the analyte of interest.

C. What are the major tobacco product types? Smokeless, ENDS, Smoked Tobacco Products, etc.

D. What collection technique is used to analyze nicotine in smoke? Mainstream smoke collection on a CFP

E. What does ENDS stand for? Electronic nicotine delivery system

5.3. Nicotine in Tobacco Filler (GC-MS)

A. Pre-analysis Questions

1. **What is nicotine's molecular weight?** 162.2 g/mol

2. **Why do you need to be careful handling nicotine standards, solutions and sample?** Additives are highly toxic.
3. **Define CCV, ICV and RSD.** CCV= Continuing Calibration Verification, ICV= Initial Calibration Verification, %RSD = % Relative Standard Deviation
4. **What GC-MS type will be used for this analysis?** Single quadrupole **What other types of GC-MS are available?** Example: Triple Quadrupole, Time of Flight, Ion Trap, Magnetic Sector, Orbitrap.
5. **What reference materials can be used as control samples?** 1R6F, CRMs, current SRM
6. **Why is ISTD added to the sample and standard solutions?** To improve the system reproducibility.
7. **Can the sample be analyzed frozen or refrigerated?** No, the sample must be at room temperature before weighing.
8. **When sample grinding is used, why is the grinder set on pulse instead of constant? To** prevent sample heating. Nicotine can be degraded with heat.
9. **What ions will be used for quantitation?** 102 and 133
10. **What quality controls are needed for this test?** Examples: Linearity 0.995, SRM accuracy ± 10%, CCV accuracy ± 10% and %RSD of sample triplicate (± 10%).

B. Post Analysis Questions

1. **Which GC-MS mode is used, SIM or full scan?** SIM
2. **What do SIM and MRM stand for?** Single Ion Monitoring and Multiple Reaction Monitoring
3. **What was the purity of the nicotine standard used? How is this purity used within the method?** Example 99.9. To correct standard concentration.
4. **How can you verify that the pipette and instrument used are qualified and ready for use?** Pipette and GC will be labeled with qualification due date. Instrument qualification paperwork should be in the instrument's logbook.
5. **How are the standards and sample solutions labeled?** Minimum: content, date prepared, expiration date, and analyst's initials.

5.4. NNN and NNK in Tobacco Filler/smokeless (LC-MS/MS)

A. Pre-analysis Questions

1. **When are NNN and NNK formed in the tobacco manufacturing process?** These products are formed during the curing, fermentation and burning of tobacco leaves.
2. **Where are TSNAs are found in higher amounts?** Cigarette Smoke
3. **What LC-MS/MS stands for and what is used for?** Liquid chromatography with triple quadrupole mass spectrometer detector. Great for quantitation in MRM mode.
4. **Explain the most commonly used ionization techniques available for LC-MS? How do the ions get ionized in each type of source?** APCI (Atmospheric Pressure Chemical Ionization)- the liquid from the LC system is pumped through a capillary and there is also nebulization at the tip, where a corona discharge takes place. First, the ionizing gas surrounding the interface and the mobile phase solvent are subject to chemical ionization at the ion source. Later, these ions react with the analyte and transfer their charge, APPI (Atmospheric Pressure Photo-Ionization)- this interface is like the APCI ion source, but instead of a corona discharge, the ionization occurs by using photons coming from a discharge lamp, and ESI (Electrospray Ionization)- the liquid is nebulized at the tip of the capillary and a fine spray of charged droplets is formed.
5. **What type of ionization source is used in the TSNA method?** ESI (Electrospray Ionization)
6. **What ions are used for quantitation? What ions are used for confirmation? What criteria are used to confirm the molecule?** See current method.
7. **What is a transition ion?** Fragment ion from a parent/precursor molecule.
8. **What is the collision energy of the method and what is the collision energy for?** Collision energy is used to accelerate the molecule and cause collision with the gas. This will increase the molecule fragmentation.
9. **What gas is needed in the collision cell and why?** Nitrogen/argon because they are inert gases or have triple bond stability and low reactivity.

10. **Why is the column heated?** Peak shape and better resolution.
11. **What are the QC requirements of the method?** Linearity, CCV, ICV, SRM, method blanks etc.

B. Post Analysis Questions

1. **What is critical during sample preparation?** Prevent contamination, not overheat the sample during grinding and sample must be at room temperature before handling.
2. **Can the standards be used frozen or refrigerated?** No, must be at room temperature.
3. **Why is system suitability needed?** To assure that the system is working properly before and during sample analysis.
4. **What precautions are needed during the sample and standard preparation?** TSNAs are highly toxic compounds so gloves, lab coat, safety glasses are required. Work must be performed under a fume hood. See also response to question 1.

5.5. Tar, Nicotine and Carbon monoxide (TNCO) by GC-FID/TCD

A. Pre-analysis Questions

1. **What is "Tar"?** Partially combusted particulate matter produced by the burning of tobacco after subtracting nicotine and water content, also known as nicotine free dry particulate matter (NFDPM).
2. **Why it is important to measure CO?** Smoking increases the CO content in the blood. CO replaces oxygen in the blood. It is harmful to the body in any concentration and can kill you.
3. **How is water quantitated in smoke extracts?** By GC-TCD (gas chromatograph-thermal conductivity detector).
4. **What preparations are necessary for cigarettes prior to smoking?** Cigarette samples must be conditioned for a minimum of 48 hours and no more than 10 days at 22°C and 60% RH before smoking.
5. **What are the CI smoking regime parameters?** Puff volume=55mL, puff once every 30 seconds, and all filter ventilation holes must be blocked.
6. **What parameters must be verified in the smoking machine settings prior to smoking?** Puff volume and air velocity.
7. **In what environmental conditions, should the sample be smoked?** 22°C and 60% Relative Humidity. Where are the required

environmental requirements for a smoking machine run found? ISO3308:2000(E).

8. **Why are CFPs weighed prior to and immediately after performing a smoking machine run?** Pre and post smoking run weights are used to calculate TPM and "Tar" (NFDPM,).
9. **What safety precautions are needed during a smoking machine run?** The precautions are needed to ensure that cigarette smoke is pulled into the smoking machine (to minimize personnel contact with cigarette smoke and HPHCs). Avoid contact with the high temperature (ignited ends) of the cigarettes lighters contained within smoking machine lighter bar.

B. Post Analysis Questions

1. **What is butt length and how is it determined?** It is the greatest of either 23 mm, length of filter + 8 mm, or length of overwrap + 3mm. This value is calculated by determining the mean filter and overwrap lengths of 20 cigarettes.
2. **Describe the appearance of the CFP after smoking?** Covered with a brown residue.
3. **How many cigarettes are smoked per pad in the ISO regime?** 3
4. **What is the reference cigarette?** 1R6F
5. **Is there a requirement to smoke a reference cigarette with each smoking run?** Yes, a minimum of one port should be used for a reference cigarette.
6. **What special preparations are necessary for cigarettes to be smoked using the CI smoking regime? Why?** All filter ventilation holes must be covered by cellophane tape or by special filter holders which guarantee 100% blocking of all filter ventilation holes. Blocking all filter ventilation holes is done to maximize the smoke delivery to the pad.
7. **What is the GC carrier gas?** Helium because it is an inert gas.
8. **What does "FID" mean and how does it work?** Flame Ionization Detector. This detector measures organic species ions formed by combustion in a hydrogen-air flame.
9. **What "TCD" mean and how does it work?** Thermal Conductivity Detector. This detector senses changes in the thermal conductivity when compared to a reference flow of carrier gas. Most compounds have a thermal conductivity lower than common carrier gases. This

detector is considered a universal detector because all compounds have a thermal conductivity different from helium (a common carrier gas).

10. **What are the QC requirements of the method for both smoking and sample analysis? What do the analytical QC requirements measure?** Smoking QC requirements include measurements of air flow and puff volume. Analytical QC requirements include the analysis of multiple injections of an ICV standard and CCV standards to ensure they fall within the specified ranges, as well as, calculation of SRM recoveries, and water determination of blanks.

5.6. Metals Analysis (ICP-MS)

A. Pre-analysis Questions

1. **What gas is used to generate the plasma?** Argon
2. **Why metals are monitored in food, drug and tobacco?** Toxic metals like lead, arsenic, nickle and cadmium can cause disease in the body and can accumulate in tissues to dangerous levels.
3. **How is the sample prepared before ICP-MS testing?** The sample is digested in acid, heat and high pressure before analysis.
4. **What is digestion?** Digestion is the process of using acid, heat and pressure to decompose the organic molecules to CO and release the metals from the matrix.
5. **What type of verification is done to the ICP-MS before starting any sample testing?** The ICP plasma is tested against known parameters for operation. This is called tuning.
6. **What is a nebulizer and why is it important to ICP?** The nebulizer is a device that converts a liquid into an aerosol. ICP instruments need a fine aerosol mist to maintain a plasma.

B. Post Analysis Questions

1. **What is ICP and how is it used with MS?** ICP stands for Inductively Coupled Plasma. It uses a high intensity radio frequency field to convert argon gas into a plasma. The hot plasma creates ions which are needed for MS analysis.
2. **Why hydrofluoric acid used in the digestion?** To dissolve silicate, and therefore to release trapped metals.
3. **Why pressure is used in the Ultra wave digestion system?** To prevent the solution from boiling over.

4. **Why are internal standards used for metal analysis?** To correct for the high variability during sample introduction in the nebulizer.
5. **How do you select the correct internal standard for your analysis?** Try to match the ionization potential of the internal standard to that of the element of interest, then select one close to the mass of the element.
6. **What are the QC of the method?** See current method

5.7. Carbonyl in Tobacco Filler (LC-UV)

A. Pre-analysis Questions

1. **What types of chemical compounds are carbonyls?** Aldehydes and ketones
2. **Are carbonyls byproducts of tobacco product combustion?** Yes
3. **Are carbonyl compounds in the abbreviated HPHC list?** Yes, acetaldehyde, acrolein, crotonaldehyde, formaldehyde, others
4. **What is an UV/VIS detector?** A detector that measures the absorption in the ultraviolet-visible spectral region.
5. **What is the HPLC's column stationary phase?** C18 stationary phase
6. **Why are Carbonyls derivatized before analysis?** Unstable due to volatility, thermal instability and sensitive to acid environment.
7. **What is used as reference standard?** 1R6F, NIST3222, or any current SRM
8. **Are there other technologies used to quantitate carbonyl compounds?** Yes, GC-LC-MS, LC-MS/M.

B. Post Analysis Questions

1. **Which two carbonyls elute close to each other?** Acetone and acrolein
2. **Why is the composition of the mobile phase important?** Composition of mobile phase determines the separation and retention of time of the compounds in the chromatogram.
3. **What is the purpose of heating the column?** To improve peak shape and sometime resolution.

4. **Why the samples need to be neutralized after derivatization?** To stop the reaction and prevent the formation of impurities like dimers.
5. **Why is the gradient increased to a high amount of organic phase at the end of the run?** To clean the column from impurities and tobacco residues.

5.8. Polymerase Chain Reaction (PCR)

A. Pre-analysis Questions

1. **How does the PCR work?** Explain PCR reaction and detection. PCR works by repeating cycles of heating and cooling to denature, anneal, extend or elongate specific DNA fragments according to the primers used. This thermal cycling, in the presence of a heat-stable DNA polymerase (Taq polymerase), enables temperature dependent reactions to occur, specifically, DNA melting and replication.
2. **Why sample handling is important?** To prevent contamination from DNA from the analyst, DNA from other samples in the lab and DNA from the DNA ladder used to determine the size of amplified sample, reagents, pipette tips etc.
3. **Can the PCR detect the presence of 1 DNA fragment in the sample?** Yes
4. **Why does the sample need to be grinded before analysis?** To release the DNA from the cell.
5. **Why the sample need to be handle first by the analyst performing this test?** To prevent sample contamination
6. **What are inhibitors and why is it important to be aware of them?** Inhibitors are various materials or compounds that may be found within a sample that interfere with the DNA amplification process. This could result in false negative results if inhibitors are present in a sample.

B. Post Analysis Questions

1. **How do you know there is tobacco plant DNA in the sample?** As the target gene amplifies during the real time-PCR, the fluorescence, from the probes used, will grow in intensity. Once the fluorescence reading hits the threshold determined by the ABI 7500 the sample will be determined positive and a Ct value will be recorded.
2. **Which probe dyes are used to target the tobacco genes of interest?** FAM is used for NT1 and CY5 is used for NtNir.

3. **What quality controls were used to assure the analysis performs as intended**? Three controls are used:
   a. Negative Template Control (NTC): IPC (+) / Targets (-)
   b. Negative Amplification Control (NAC): IPC (-) / Targets (-)
   c. Positive Tobacco Control (PC): IPC (+) / Targets (+)
4. **How many sample replicates are needed?** Three sample replicates to show consistent replication and detection (Ct values).
5. **What reagent control is used to determine if inhibition has occurred during an analysis and what steps are taken to rule out false negatives due to inhibition?** A TaqMan® Exogenous Internal Positive Control (IPC) Reagent is used in the Master Mix to determine if inhibitors are present by either not showing any amplification or amplifies very late with an abnormal curve (high Ct). If any inhibition occurs, samples are to be diluted 1:10 in an either nuclease-free water or Tris-EDTA (TE) Buffer. Rerun the original sample template along with the diluted template and include the appropriate controls.

6. Document Change History

Revision #	Status* (D, I, R)	Date	Author Name and Title	Approving Official Name and Title
00	I	See QMiS InfoCard	LMEB	LMEB

* - D: Draft, I: Initial, R: Revision

7. Change History

Revision #	Change
00	New document