67th Tobacco Science Research Conference

MONDAY MORNING, SEPTEMBER 16, 2013

Symposium

8:30 AM **WELCOME:** Michael Connor, Borgwaldt, 67th TSRC Chair

8:40 AM **SYMPOSIUM: "Constituent Lists: Reshaping Tobacco Science"**
Chair: Anthony Gerardi

8:45 AM **MONDAY**

1. TESTING & REPORTING HARMFUL AND POTENTIALLY HARMFUL CONSTITUENTS (HPHCS) IN TOBACCO PRODUCTS. Matthew R. HOLMAN; U. S. Food & Drug Administration, Rockville, MD USA

Despite the risks, approximately 46.6 million U.S. adults continue to smoke cigarettes. Smoking causes more than 440,000 deaths in the United States each year. The Federal Food, Drug, and Cosmetic Act (FD&C Act) requires FDA to establish and periodically revise a list of Harmful and Potentially Harmful Constituents (HPHCs). In guidance issued in January 2011, FDA has indicated its belief that the phrase 'harmful and potentially harmful constituent' includes any chemical or chemical compound in a tobacco product or in tobacco smoke: that is or potentially is inhaled, ingested, or absorbed into the body; and that causes or has the potential to cause direct or indirect harm to users or non-users of tobacco products. In April 2012, FDA established the HPHC list, which contained 93 constituents. Section 904(a)(3) of the FD&C Act requires tobacco product manufacturers to determine HPHC quantities in tobacco products and smoke and report those quantities to FDA beginning on June 22, 2012. Also in April 2012, FDA published a draft guidance document stating that, in order to comply with Section 904(a)(3), FDA does not intend to enforce the statutory requirement to provide quantities of all constituents identified by FDA as HPHCs by June 22, 2012, if manufacturers or importers complete testing and reporting for an abbreviated list of HPHCs. HPHC information can be very valuable to FDA in accomplishing its public health mission including informing tobacco products standards, marketing authorization decisions, and the public of the risks of tobacco use.

9:15 AM **MONDAY**

2. ISSUES, PARADIGMS AND PARADOXES THAT NEED TO BE ADDRESSED BY BOTH MANUFACTURERS AND REGULATORS IN THE MEASUREMENT AND INTERPRETATION OF CIGARETTE SMOKE YIELDS OF HARMFUL AND POTENTIALLY HARMFUL CONSTITUENTS (HPHCS). Stephen PURKIS; Imperial Tobacco Limited, Bristol, UK

Measurements of smoke components are made for a variety of reasons initiated by both manufacturers and regulators and can provide useful information. This paper discusses issues that need to be addressed to ensure that any smoke constituent measurements and interpretation are valid and meaningful.

There is a need for the development of internationally agreed and robust measurement standards to allow comparisons between products or verification against regulated yield limits. The ISO standardization process meets this requirement. Both manufacturers and regulators need a common understanding of the limitations of methodology used to measure smoke yields so that realistic measurement tolerances can be set. A forum is necessary for regulators and manufacturers to discuss methodological issues and to allow any apparent yield differences due to methodology to be discussed at an early stage and later as an on-going commitment during standards review.

It is hoped that addressing the issues raised in this paper will help to lead to future regulatory proposals based on sound science. On-going dialog leads to better understanding of both the intended and unintended consequences of regulation and helps to ensure that regulation meets intended objectives.

10:15 AM MONDAY

3. THE CHANGING ROLE OF THE CONTRACT RESEARCH LABORATORY IN THE TOBACCO INDUSTRY. Gene GILLMAN; Enthalpy Analytical, Durham, NC USA

The role of the contract research laboratory (CRO) in the tobacco industry is unusual compared to the role of CROs in other industries. Historically, there have been a limited number of tobacco CROs focused on smoke constituent measurement as typical projects were usually related to on-going product stewardship efforts or regulatory compliance programs. In the past, the tobacco industry worked with and relied on CROs to develop testing methods and quality control procedures to measure chemical constituents in their products. Since the industry has historically only had standardized methods for a few compounds, only a small number of CROs developed and implemented validated test methods to serve the tobacco industry. However, in the past few years, there has been a tremendous increase in the number of governmental bodies proposing regulations that would or do require measurement and testing of compounds in tobacco and mainstream smoke. Some of these new regulations include prescribed testing methodology while others greatly expand the number of compounds requiring measurement. This presentation gives a brief historical perspective, addresses the impact of these new requirements, and discusses the changing role of CROs in the tobacco industry.

10:45 AM MONDAY

4. DEVELOPMENT OF ROTARY SMOKING MACHINES IN THE LIGHT OF REGULATORY REQUIREMENTS. Nils ROSE; Borgwaldt, Hamburg, Germany

Smoking machines have been used for more than 50 years now. Their use was driven by product development and research puposes and evolved into the basis of routine smoking as a quality assurance tool. As their use spread around the world, it became obvious that standardization was essential to ensure consistant results independent of the location. In parallel to the first regulatory requirements of Tar analysis, CORESTA started developing the first methods for machine hamonization which was followed by ISO standards. This has further evolved over the years to include topics such as air flow harmonization, labyrinth seals, puff parameters and trapping systems, as well as related analytical methods. The

presentation starts with a short jouney through the histoy of rotary smoking machines and the first reported methods through to the requirements of today. It will take a look at the latest analytical needs as well as new product requirements and will give a brief look into possible future challenges.

1:00 PM POSTERS

5. ANALYSIS OF REFERENCE TOBACCO PRODUCT PREPARATIONS USED IN CELL CULTURE STUDIES. Eckhardt SCHMIDT and G. L. Prasad; R. J. Reynolds Tobacco Co., Winston-Salem, NC USA

Assessing the *in vitro* biological and toxicological effects are important components in biomarker research and evaluation of tobacco products. While researchers often utilize reference tobacco products such as 3R4F cigarettes and 2S3 moist snuff, information on the exposure dose is generally lacking for smoke extracts and smokeless tobacco preparations (TPP) and/or poorly characterized. Given the variability in smoking regimens, instrumentation and extraction conditions, the chemical composition of the dosing agent could vary, which could impact the observed biological and toxicological effects. In this research study, we report the analyses of selected chemicals in different batches of TPP. Whole smoke-conditioned medium (WS-CM) and gas-vapor phase conditioned medium (GVP-CM) were prepared from 3R4F cigarettes smoked under ISO conditions by bubbling the smoke through cell culture media, and total particulate matter (TPM) was prepared in dimethyl sulfoxide (DMSO). Smokeless tobacco extracts were prepared by extracting 2S3 moist snuff in complete artificial saliva (ST/CAS). Nicotine, TSNAs, PAHs, nitrate, nitrite, aldehydes and other select parameters were analyzed as appropriate for a given TPP in several different preparations. The measured quantities of nicotine (12-14 ug/ml) and TSNAs (< 1ng/ml) were generally in a narrow range in the WS-CM. GVP-CM, as expected, contained very little nicotine and other particulate phase constituents, but consisted of comparable amounts of vapor phase constituents present in WS-CM. On the other hand, 10% ST/CAS extracts contained 1.2-1.8 mg/ml of nicotine and higher amounts of TSNAs, indicating a batch- to- batch variability. Thus, establishing a range for the key constituents of TPPs is important to interpret the biological and toxicological effects due to the exposure of TPPs.

6. ANALYSIS OF THE POLYPHENOLS IN CURED TOBACCO LEAVES USING UPLC-ESI-MSM. John R. SHIFFLETT, Devin J. McNally and Dawit Z. Bezabeh; Alcohol and Tobacco Tax and Trade Bureau, Beltsville, MD USA

The U.S. Alcohol and Tobacco Tax and Trade Bureau (TTB) is responsible for determining proper tax classification of tobacco products. Tobacco products in the U.S. may fall into several taxable categories including cigars, cigarettes, snuff, chewing tobacco, pipe tobacco and roll-your-own. As significant components of tobacco, polyphenols are valuable for product characterization and differentiation.

The chemical changes that occur in tobacco leaves during curing have been studied extensively over the years and are well documented in the literature. The method of curing has a strong impact on the chemical profile of the processed tobacco. Chemical changes leading to color differences between flue-cured and air-cured tobaccos result

from chlorophyll decomposition, which contributes to yellowing in flue-cured tobaccos, and phenolic oxidation, which leads to browning in air-cured tobaccos. Typical phenolic content for flue-cured tobacco is 7.0% or more, while air-cured and fire-cured tobaccos generally contain less than 0.5%.

In the work described in this presentation, pressurized liquid extraction (PLE) and UPLC ESI-MS/MS were used to prepare and analyze air-cured, flue-cured, and oriental tobaccos supplied to the TTB laboratory. The combination of PLE and UPLC ESI-MS/MS provided levels of extraction efficiency and sensitivity that permitted our laboratory to study even minor polyphenolic components in samples where the total phenolic content was very low. The results from PLE extractions will be compared to results from samples prepared using ultrasonication, an established approach to polyphenol extraction from tobacco matrices.

7. BENZO[A]PYRENE IN TOBACCO PRODUCTS BY ULTRA-PRESSURE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION USING ITS DEUTERATED ANALOG AS AN INTERNAL STANDARD. Carrie SODEN and Fraser Williamson; Arista Laboratories, Inc., Richmond, VA USA

Benzo[a]pyrene (BaP) is a polycyclic aromatic hydrocarbon (PAH) classified by the International Agency for Research on Cancer (IARC) as a group 1 carcinogen. BaP is formed as a product of incomplete combustion and is found in tobacco smoke as well as tobacco, particularly dark-fire cured. There is much interest in BaP in tobacco products and its testing is required by regulatory bodies (*e.g.* Health Canada and the United States Food and Drug Administration) as well as manufacturers (*e.g.* Swedish Match's Gothiatek standard).

The objective of this study was to develop and validate an analytical method for BaP in various tobacco products as an improved alternative to an in-house GC-MS method, which required extensive sample clean-up and a long analysis time.

BaP was extracted from tobacco with methanol; shaken mechanically then centrifuged. An aliquot of supernatant was concentrated by a factor of 20 and filtered. Analysis was performed by ultra-pressure liquid chromatography (UPLC) with fluorescence detection using a Zorbax Eclipse PAH analytical column with an isocratic mobile phase consisting of water-acetonitrile. Quantitation was performed by the internal standard technique using deuterated d12-BaP. A range of individual leaf grades, reference and commercial products were evaluated by this method. CRP2, CRP3 and 3R4F gave results of 52.4, 39.5, 8.16 ng/g, respectively, which compared favorably to results obtained by the in-house GC-MS method. The limit of quantitation for BaP by this method is 0.1 ng/mL, equivalent to 0.1 ng/g.

The method was validated with acceptable linearity, accuracy, precision and selectivity to produce a robust method that utilized a simple extraction and shorter analysis time.

8. COMPARISON OF AUTO ANALYZER METHODS: DISCRETE VS CONTINUOUS FLOW. David THURSTON; Global Laboratory Services, Inc., Wilson, NC USA

Chloride, Sugar, Alkaloids, and Nitrate results were compared using a discrete analyzer and a continuous flow auto analyzer. The new generation discrete analyzers are more

automated than the continuous flow auto analyzers. In our initial assessment, the discrete analyzer yielded greater throughput and reliability than the continuous flow auto analyzer. We found the SmartChem 200 discrete analyzer performed comparably for methods such as chlorides and alkaloids. However, sugars and nitrates values were generally lower. For sugars, this is due to the discrete analyzer using an enzymatic method that is specific for sugars, while the continuous flow auto analyzer uses a potassium ferricyanide color reagent for determination that not only reacts with sugars, but also polyphenols and proteins. For nitrates, the discrete analyzer uses a cadmium coil reduction of nitrate to nitrite for nitrate determination, while the continuous flow auto analyzer uses a hydrazinium sulfate-copper sulfate reagent for nitrate reduction to nitrite. The exact reason for higher results from the continuous flow auto analyzer is unclear. Precision was found to be comparable on the two analyzers for chlorides and nitrates.

9. LIGHTER LIFE, TEMPERATURE AND INITIAL CO YIELDS. Ian TINDALL, Linda Crumpler, Shabir Moghal and Peter Jordan; Cerulean, Milton Keynes, UK

Coil lighters used to initiate smoke runs can be operated at different surface temperatures whether deliberately or unwittingly. This surface temperature is shown to be related to the pre-light time and the age of the lighter in terms of in use cycles and in use temperature. Lighter surface temperature decreases with repeated use and this is accelerated as the initial surface temperature is increased. It is possible to approximate the surface temperature of the lighter coil by reference to the number of lighting cycles employed and the pre light time. Using different lighter surface temperatures, achieved by altering the pre-light time, it is shown that different initial CO yields are achieved when smoking monitor test pieces under ISO conditions but that these differences are not statistically significant when smoking under a Health Canada Intense (HCI) regime. It is concluded that by understanding this effect modifications can be made to lighting temperature and this should be a consideration in maintaining consistent CO yields.

10. ORGANIC ACID INHIBITING GERMINATION AND GROWTH OF TOBACCO SEED AND SOIL MICROBIAL COMMUNITY. Maosheng WANG and Hancheng Wang; Guizhou Academy of Tobacco Science, Guizhou, China

Root exudates containing root-specific metabolites have critical ecological impacts on soil macro and microbiota as well as on the whole plant itself. Organic acids, as the most important component in plant root exudates, have been reported to accumulate in the continuous cropping system of tobacco. The aim of the present investigation was to assess their phytotoxic effects on the whole process of the flue-cured tobacco seedling. The germination experiments of tobacco seeds in organic acids including benzoic, p-hydroxybenzoic, salicylic and malic acids were conducted. The results showed that *p*-hydroxybenzoic and malic acids did not affect the germination rate, while the germination rate decreased with the concentration increase of benzoic and salicylic acids. Tobacco seedlings were grown in Hoagland nutrient solution with benzoic, *p*-hydroxybenzoic, salicylic and malic acids at concentration of 0, 100, 200, 400, 600 and 800 μM respectively. The dry and fresh weights of the whole plant showed increase firstly and decreased afterward with the concentration increase of benzoic, *p*-hydroxybenzoic and salicylic acids. The malic acids did not affect the dry and fresh weights of the plant. The microbial number of soil actinomyces was decreased with the concentration increase of benzoic and *p*-hydroxybenzoic acids, and increased

firstly and decreased afterward with the concentration increase of salicylic and malic acids. The microbial number of soil fungus was decreased with the concentration increase of benzoic, *p*-hydroxybenzoic, salicylic and malic acids. The microbial number of soil bacteria in different treatments of salicylic and malic acids, and the trend of which in different of Benzoic and *p*-hydroxybenzoic were first increased and then decreased.

11. WELL-CELLAR STYLE TRANSPLANTING OF FLUE-CURED TOBACCO (*NICOTIANA TABACUM* L.) IN CHINA. Yechun LIN, Yechun Lin, Wenjie Pan, Wei Chen and Weichang Gao; Guizhou Tobacco Research Institute, Guiyang City, China

Well-Cellar Style Transplanting (WCST) spread in China is an original transplanting method of flue-cured tobacco, and has obvious comparative advantages when compared to conventional transplanting (CT). Field and pot experiments were implemented to investigate primary environmental factors that affect growth of flue-cured tobacco in WCST and CT. The results showed that variation of air temperature in WCST ranged from 14.13°C to 30.52°C which was relatively stable as compared to CT (between 11.74°C and 44.22°C) in April. Furthermore, the similar variation of soil temperature was found between the bottom of the WCST and soil surface. Variation of air relative humidity in the WCST was between 64.28% and 100%, which was narrower than the soil surface in CT (from 25.71% to 97.75%). Otherwise, soil moisture at the bottom of the WCST varied from 24.92% to 36.63%, which was much higher than soil surface in CT (between 16.92% and 35.10%). Photosynthetically active radiation (PAR) of sunlight received in the WCST was lower than in CT; however, there were significant linear relationships between WCST and CT whether on a sunny day ($y = 0.95x-163.71$, $R^2=0.88$), a cloudy day ($y = 0.95x-163.71$, $R^2=0.88$) or a rainy day ($y = 0.36x-4.82$, $R^2=0.97$). According to fitted parameters of light response curves, light saturation point (LSP) and maximum net photosynthetic rate (P_{max}) in WCST were higher than in CT; nonetheless, initial quantum efficiency (α) was higher and light compensation point (LCP) and apparent dark respiration rate (R_d) in WCST were lower than in CT. Because of better hydrothermal conditions and moderate photosynthetically active radiation, well-cellar style transplanting promotes growth and development of flue-cured tobacco.

12. STRATEGY OF GM SCREENING BASED IN GENETIC ELEMENTS. Jing YU[1], Xiaolian Zhang[1], Jie Zou[1], Jiehong Zhao[1] and Dan Zhao[2]; [1]CNTC, Guiyang, China and [2]Guizhou University, Guiyang, China

Tobacco is a model plant widely used in transgenic research for years, but genetically modified (GM) tobacco was extremely limited in commercial applications. The ability to protect commercial tobacco products from transgenic pollution has become an important problem in the tobacco industry. At present, P-35S promoter, NPTII selective marker gene and T-NOS terminator are three common targets for GM tobacco screening, but they cannot cover all transgenic tobacco events, and some GM tobacco events with special transgenic elements will be missed. So, we need a new screening strategy which can cover the GM tobacco events as much as possible. We searched for articles on Google Scholar and PubMed by using key word “transgenic tobacco” or “GM tobacco”. And 229 related articles in recent 10 years were collected. By reading these articles, we investigated all types of transgenic elements and their use frequency in various tobacco transgenic events. We found that only 86% of these events can be detected by using the combination of P-35S,

NPTII, and T-NOS. In order to completely eliminate the possibility of missed detection, based on the information available for various transgenic elements in tobacco GM events, we proposed a more comprehensive set of transgenic elements as PCR amplified targets. It includes P-35S promoter, NPTII/HPT/Bar/aadA selective marker genes, GUS reporter gene and T-NOS terminator. These target sequences represent the most common elements in transgenic tobacco, thereby, nearly 100% of the transgenic events in tobacco can be detected by using these elements for screening target sequences. Also, a genetic elements of transgenic tobacco database was established concurrently with this study.

13. FURTHER REFINEMENT OF A MARGIN OF EXPOSURE (MOE) MODEL TO PRIORITIZE CIGARETTE SMOKE TOXICANTS FOR REDUCTION RESEARCH.
Damien BREHENY, Fiona H. Cunningham, Stacy Fiebelkorn, Debbie Dillon, Clive Meredith and Christopher Proctor; British American Tobacco Ltd., Southampton, UK

The US Food and Drug Administration (FDA) have outlined seven research priority areas relating to tobacco products. Research area three focused on "reducing toxicity and carcinogenicity of tobacco products and smoke". This has been coupled with an increased interest in characterising individual tobacco smoke toxicants from the perspective of regulatory frameworks and tobacco product development focused on selective toxicant reduction.

We previously described the Margin of Exposure (MOE) model as part of a quantitative risk assessment paradigm for individual tobacco smoke toxicants. Computed MOEs enable segregation of toxicants into high and low priority groupings for risk reduction research depending on their relationship to the critical MOE value of 10,000. Here we propose further segregation of tobacco smoke toxicants into bandings based on their MOEs as follows: 1–10 (top priority), 10–100 (very high priority), 100–1000 (high priority), 1000–10,000 (medium priority), 10,000–1,000,000 (low priority), >1,000,000 (very low priority).

We applied this approach to the WHO Study Group on Tobacco Product Regulation (TobReg) list of 18 toxicants for mandatory lowering and monitoring, resulting in the following classifications:

Top priority – Acrolein
Very high priority – Cadmium, Formaldehyde, Acrylonitrile
High priority – 1,3-butadiene, Acetaldehyde
Medium priority – Benzene
Very low priority – Benzo(a)pyrene

Ranking is based on the majority view for each toxicant. However, it is not always appropriate to apply these bandings. For 4-N-Nitrosomethylamino-1-(3-pyridyl)-1-butanone (NNK) and N-Nitrosonornicotine (NNN) typical MOE values lie both above and below 10,000, and for 2-aminonaphthalene there is only one MOE available. There were insufficient data to calculate MOE values for 7 remaining TobReg chemicals. While additional experimental data are needed, this tool provides valuable information for prioritization of toxicants for risk assessment purposes.

14. REVIEW OF THE IMPACT OF LIP REGULATION IN RELATION TO PUBLISHED FIRE STATISTICS. Steven COBURN, Chuan Liu and Kevin G. McAdam; British American Tobacco Ltd., Southampton, UK

At the 5th session of the Conference of the Parties (COP5) in Seoul (November 2012), members of the WHO's Framework Convention on Tobacco Control (FCTC) adopted the draft regulation on reduced ignition propensity (RIP) cigarettes into one of its Partial Guidelines under Articles 9 & 10 "Product Characteristics in Relation to Fire-Risk". The Partial Guideline recommends commercial cigarettes to be sold to meet a proposed performance standard as tested by the available standard methods (*e.g.*, ISO 12863 or ASTM E2187). In some jurisdictions RIP regulation has been in place for a number of years (*e.g.* since 2004 for New York). Fire statistics before and after the RIP regulation are available which may be used to evaluate the effectiveness of this regulation on cigarette-related fires in real life. At present there is a lack of agreement in the scientific literature on whether the effectiveness of the RIP regulation should be evaluated according to fire statistics on the number of fires or the number of fire fatalities. Without a fire, there can be no fire fatality. Mechanistically, however, RIP-compliant cigarettes are expected to display a reduced "ignition" probability in real life, *i.e.*, the modified properties of these types of cigarette may only be associated with fire initiation and not with fire propagation where fatalities may occur under the influence of a host of other factors. In this work, literature on cigarette-related fires will be reviewed to highlight the differences between the two approaches and the need to conduct post-RIP regulation impact assessment based on valid scientific principles.

15. QUANTITATIVE ANALYSIS OF EIGHT HETEROCYCLIC AROMATIC AMINES IN MAINSTREAM CIGARETTE SMOKE BY LIQUID CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY. Bhasha DESAI, Ulli Becker, Jonathan Wilkins and Ninitha Perumalla; Eurofins Lancaster Laboratories, Winston-Salem, NC USA

Heterocyclic aromatic amines (HAAs) are considered to be carcinogenic and are formed during the burning process of proteins and amino acids . The FDA has created a list of potentially harmful smoke constituents to be monitored in tobacco smoke that include 8 of these HAAs (AaC {2-Amino-9H-pyrido[2,3-b]indole}, Glu-P-1 {2-Amino-6-methyldipyrido[1,2-a:3',2'-d]imidazole}, Glu-P-2 {2-Aminodipyrido[1,2-a:3',2'-d] imidazole}, IQ {2-Amino-3-methylimidazo[4,5-f]quinoline}, MeAaC {2-Amino-3-methyl-9H-pyrido[2,3-b]indole}, PhIP {2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine}, Trp-P-1 {3-Amino-1,4-dimtheyl-5H-pyrido[4,3-b]indole}, and Trp-P-2 {1-Methyl-3-amino-5H-pyrido[4,3-b]indole}. Eurofins Lancaster Laboratories has developed and validated a method to determine the levels of these 8 HAAs in mainstream smoke by liquid chromatography and tandem mass spectrometry (LCMSMS). Various cigarettes and five smoking regimes were validated using a solid phase extraction (SPE) method for sample clean-up of the smoke pad prior to LCMSMS analysis.

16. A RAPID AND ACCURATE DETECTION METHOD FOR *RALSTONIA SOLANACEARUM* BASED ON LOOP-MEDIATED ISOTHERMAL AMPLIFICATION. Meng-ao JIA, Yi Cao, Xingjiang Chen, Ning Lu and Shenghua Shang; Guizhou Academy of Tobacco Science, Guiyang City, China

Tobacco bacterial wilt disease, caused by Ralstonia solanacearum, is one of the most important biotic stresses for tobacco production in Guizhou province, southwest China. It is necessary to develop a rapid and sensitive pathogen detection method for wilt disease management. Here we reported a new detection strategy based on Loop-mediated isothermal amplification (LAMP) for Ralstonia solanacearum diagnosis. Referring to the genome of Ralstonia solanacearum FQY4 (NCBI accession No. CP004013), which is the dominant strain in Guizhou province, aligned with the published sequences of other strains, the fliC gene which encoded the flagellar protein, was chosen as the detection target. We designed a set of 6 LAMP primers based on the sequence of the conserved region of fliC gene, and optimized the reaction condition as 60 min at 60°C. The amplification result, which reflected whether the pathogen existed or not, could be visual detected by colors identification, or could be presented as ladder-like bands pattern by the DNA product electrophoresis in agarose gel. Using this method, not only the Ralstonia solanacearum isolated colony but also the tobacco plant carrying pathogen could be identified rapidly and accurately.

17. QUANTITATIVE DETERMINATION OF VOLATILE NITROSAMINES IN SMOKE USING GAS CHROMATOGRAPHY-MASS SPECTROMETRY. Darius B. GRISSOM and Darren Steelman; Lancaster Laboratories, Inc., Winston Salem, NC

Lancaster Laboratories has done previous work which includes the analysis of VNA in smokeless and alternative tobacco products, carried out by GC/MS/MS. Although acceptable results have been ascertained using this GC/MS/MS method, routine testing of smoke samples require as streamlined and robust a method as possible. GC/MS/MS, while a very precise and sensitive technique, can produce throughput and method transfer issues, due to maintenance and instrument availability issues, for many routine testing laboratories.

This poster highlights the validation parameters used to successfully validate this method, while evaluating and optimizing quantitation techniques at very low levels in smoke matrices. The curve fit type and linear range across the calibration for this method were critical in producing accurate results. There was no loss of overall sensitivity when comparing the GC/MS data to the GC/MS/MS data. There was however, improved specificity and recoveries for some of the analytes with this new GC/MS method.

Chromatographic separation was achieved through an Agilent CAM column, 30m x 0.25mm x 0.25um. This column offered better resolution and specificity than Agilent DB-1701 and DB-Wax columns.

18. EFFECT OF POLYAMINES ON THE CULTURE OF TOBACCO EARLY EMBRYO *IN VITRO*. Jie ZOU, Jing Yu, Qiang Fu and Jie Hong Zhao; Guizhou Academy of Tobacco Science, Guiyang City, China

Polyamines(PA) are small polycationic moleculars exist in all living organisms, diamine putrescine(Put), the triamine spermidine(Spd) and the tetramine spermine (Spm) are the most abundant polyamines in organisms. Plant polyamines are preferentially presented in actively growing tissues and under biotic or abiotic stress. Polyamines have been shown to play an important role in cell division, reproductive development, fruit ripening, and stress signaling.

Recently, research has been carried out to study the polyamine's function in tobacco, however, studies on the role of polyamines in tobacco embryo development is still limited. Here, we set up a tobacco embryo *in vitro* culture system to investigate the role of polyamines during embryogenesis by adding different levels of polyamines to the culture medium.

The results show that the spermidine (100 μmol) can greatly stimulate the division and growth of the two-celled proembryo in the *in vitro* culture system. The other two polyamines (putrescine and spermine) had no obvious effect on the growth of two-celled proemmbryo. Further experiments revealed that spermidine (100 μmol) did not have significantly effect the growth of 16-32 celled proembryo. Moreover, spermidine(100μmol) did inhibit the growth of 64-celled globular embryo. This means spermidine has a stage dependent effect to the growth of tobacco embryo. Spermidine enhanced the growth of early proembryo (2-8cell), but displayed weaker and negative effects to the late embryo(16-64cell). Interestingly, we found that the effect of spermidine on embryo growth was similarl to 2-4D. This suggests a link between spermidine and auxin during the culture of early embyro *in vitro*.

19. TOBACCO PRODUCT PREPARATIONS DIFFERENTIALLY REGULATE HUMAN PBMC FUNCTIONS, INCLUDING T CELL AND NK FUNCTION. Subhashini ARIMILLI[1], Brad E. Damratoski[1] and G. L. Prasad[2]; [1] Wake Forest University School of Medicine, Winston-Salem, NC USA and [2]R.J. Reynolds Tobacco Company, Winston-Salem, NC USA

Natural Killer (NK) cells and T cells play essential roles in innate and adaptive immune responses in protecting against microbial infections and in tumor surveillance. Although evidence suggests that smoking causes immunosuppression, there is limited information whether the use of smokeless tobacco (ST) products affects immune responses. In this study, we assessed the effects of two cigarette smoke preparations, ST and nicotine on T cell and NK cell responses using Toll-like receptor-ligand stimulated human peripheral blood mononuclear cells (PBMCs). The tobacco product preparations (TPPs) tested included whole smoke conditioned media (WS-CM), total particulate matter (TPM) and a ST product preparation (ST/CAS). The PBMCs were stimulated with polyinosinic:polycytidylic acid (poly I:C) and lipopolysaccharide (LPS). A marked reduction of the expression of intracellular IFN-g and TNF-a was evident in NK cells and T cells treated with WS-CM and TPM. Consistently, attenuation of ligand induced secretion of cytokines (IL-1b, IL-10, IL-12 and TNF-a from PBMCs treated with WS-CM and TPM were observed. WS-CM and TPM also inhibited the cytolytic activity of human PBMCs. Significant suppression of perforin in PBMCs and in NK cells by WS-CM was detected. Although interference

from the vehicle confounded the interpretation of effects of ST/CAS, some effects were evident only at high concentrations. Nicotine treatment minimally impacted expression of cytokines and cytolytic activity. Data presented herein suggests that the function of NK cells and T cells is influenced by exposure to TPPs (based on equi- nicotine units) in the following order: WS-CM>TPM>ST/CAS. These findings are consistent with the hypothesis put forward by others that chronic smoking leads to immunosuppression, an effect that may contribute to increased microbial infections and cancer incidence among smokers.

20. A COMPREHENSIVE ANALYSIS OF TRANSCRIPTOME DYNAMICS IN TOBACCO (*NICOTIANA TABACUM* L.) LEAVES. Ruiyuan LI[1], Longjiang Fan[2], Yijie Gui[2], Yuewei Shi[1], Zhihong Wang[1], Shengdong Xi[1] and Xueliang Ren[1]; [1]Guizhou Academic of Tobacco Science, Guiyang City, China and [2]Zhejiang University, China

By using a custom-designed microarray containing 44,873 unigenes derived from public EST libraries, the transcriptome of tobacco leaves at different stalk positions and developmental stages was investigated and analyzed for three varieties planted in two plocations. ANOVA analysis identified 1862, 1530, 1915, and 1689 unigenes showing different expression levels between stalk positions, developmental stages, varieties, and locations, respectively. A total of 4129 unigenes showed differential expression in all combinations of the conditions. Gene rich analysis showed that the differentially expressed unigenes were involved in many GO categories and pathways including secondary metabolite pathways such as nicotine synthesis, but only a few GO categories showed significant differences in the number of unigenes between the four conditions. Together with gene expression pattern and network analysis, our results show that the inherent differences in gene expression and regulation patterns may play a very important role in leaf characteristics in tobacco varieties planted in different environments.

21. CLONING AND CHARACTERIZATION OF the CYSTEINE PROTEINASE INHIBITOR (CPI) GENE FAMILY IN TOBACCO (*NICOTIANA TABACUM* L.). Shi-feng LIN, Ren-gang Wang, Jie Zou, Qiang Fu, Jie-hong Zhao and Xue-liang Ren; Guizhou Academy of Tobacco Science, Guiyang City, China

In plants, cysteine proteinase inhibitors (CPI or cystatin) are implicated in biotic and/or abiotic stress responses and developmental regulation. Using the techniques of RT-PCR and SMART RACE, full-length cDNAs of four CPI genes (NtCPI1, NtCPI2, NtCPI3, and NtCPI4) were cloned for the first time from *Nicotiana tabacum* L. cv. K326. The four sequences have been deposited in GenBank, with accession numbers KF057988, KF057989, KF057990, and KF057991, respectively. Genomic DNA sequence analysis showed that NtCPI1 and NtCPI2 each have a single intron, while the other two have no intron. The four genes encoded predicted proteins of 98, 98, 120, and 123 amino acid residues, respectively. In addition to the typical inhibitory motifs, namely the central signature motif QXVXG, a GG doublet in the N-terminal region, and A/PW residues in the C-terminal part, these deduced amino acid sequences contained the PhyCys-specific LARFAV-like motif in the N-terminal region, of which an N-terminal signal peptide of 27 residues was found in both NtCPI3 and NtCPI4. Messenger RNAs specific to the four genes were detected in roots, stems, leaves, and buds by semi-quantitative RT-PCR, which indicated that they were broadly expressed in tobacco. This study lays the foundation for further exploration of the physiological functions of these cysteine proteinase inhibitor genes in plants.

22. ULTRA TRACE LEVEL QUANTIFICATION OF TOXIC ELEMENTS IN TOBACCO BY ICP-MS. Sharad K. MEHTA and B.J. Rajesha; ITC Limited, Bangalore, India

Element levels in tobacco depend upon factors such as soil and environmental conditions. Metals exist in tobacco and tobacco smoke in elemental and compound forms, both of which are harmful and potentially harmful to humans. The USFDA list of 93 HPHC constituents has shortlisted nine toxic elements, beryllium (Be), cadmium (Cd), chromium (Cr), cobalt (Co), lead (Pb), nickel (Ni), arsenic (As), selenium (Se), and mercury (Hg), as well as three radioactive elements (Uranium 235, Uranium 238, and Polonium 210). These constituent analyses have a direct impact on the cigarette business in the US market. This abstract describes Inductively coupled plasma mass spectroscope (ICP-MS) quantification of the trace multi-elements in tobacco.

Published methods in the literature use atomic absorption spectroscopy (AAS) and ICP-MS with the following limitations: AAS can detect a single element at a time, and detection is at the ppm level and is time consuming. The ICP-MS method uses a dynamic reaction cell with ammonia and methane as a reaction gas for a few elements, which will cause interference from other elements, and the sample preparation is laborious, time consuming, and costly. The method presented here uses ICP-MS with a kinetic energy discriminator to reduce polyatomic and isobaric interferences at ppt/ppb levels.

A simple, fast, and precise method developed using internal standards, a microwave-assisted digestion under controlled conditions, and small sample size reduces matrix interference and volatility of As and Se. The method was validated by analyzing a certified reference material by the Nuclear Chemistry Department, Poland having worldwide acceptance; there was an excellent agreement between the results of the certified reference material and the results of the titled method. Standard validation protocols *i.e.* limit of detection, limit of quantification, recovery, repeatability and reproducibility were used. Minimum recoveries of 87% Be, 97.2% Cr, 95.8% Co, 93.1% Ni, 92% As, 92.5% Se, 90.6% Cd, 102% Hg, and 92.9% for Pb were obtained with a linear regression coefficient of 1.000 at concentrations of 0.5-50 ppb of Be, Cr, Co, Ni, As, Se, Cd, and Pb, and a linear regression coefficient of 0.9998 at concentrations of 0.05-2.00 ppb of Hg were obtained. Limits of detection (LODs) were in the range of 3.5 ppb (ng/g) for most of the elements like Be, Co, As, Cd, and Hg. Limits of detection for Cr, Ni, Se, and Pb were 56.2, 73.2, 24.86, and 95.3 ppb respectively.

The titled method provides several advantages such as sensitivity, precision, simplicity, and accuracy. The method has been found suitable for rapid determination of multiple trace elements at ppt/ppb level. Several smokeless tobacco products and tobacco samples were analyzed.

23. SIMULTANEOUS QUANTIFICATION OF FDA-HPHC LISTED HETEROCYCLIC AMINES AND TOBACCO SPECIFIC NITROSAMINES IN MAINSTREAM CIGARETTE SMOKE USING LC-MS/MS. Sharad K. MEHTA, H.S. Raghu and N. Yamuna; ITC Limited, Bangalore, India

Heterocyclic amines [HA] are an important class of carcinogens present in cigarette smoke condensate, and some HAs are bacterially mutagenic and carcinogenic compounds. According to the IARC, the toxicity of HAs and tobacco-specific nitrosamines (TSNAs)

are CLASS 2A or 2B group and are known to be present at very low ppb levels. Accurate quantification of HAs are challenging because of their relatively low abundances and numerous chemical interferences that arise from cigarette smoke. TSNAs are well known carcinogens identified in tobacco and tobacco smoke. Because of strong carcinogenicity, only eight HAs and two TSNAs are on the FDA-HPHC list. There are separate methods in the literature for quantification of HAs & TSNAs involving tedious sample cleanup, so it was necessary to develop a simple, fast, and accurate method for simultaneous quantification of eight HAs and two TSNAs in a single run.

An LC-MS/MS method was developed for the quantification of these compounds in mainstream cigarette smoke. The method allows extraction of mainstream smoke particulate matter on a Cambridge filter pad with methanol containing isotopically labeled deuterated internal standards and subsequent analysis using LC-MS/MS. The Analytical conditions involve separation of all ten compounds using a C18 Column [15 cm x 4.6 µm x 5 µm] with buffer:methanol as mobile phase and a column flow rate of 0.7 ml/min.

The method has been validated and it exhibits excellent linearity (R2 >0.998) over a wide range of concentrations [0.1-20 ng/ml], and recovery varied from 80 to 105%. The limit of detection was 0.04 ng/ml and quantification was 0.15 ng/ml. The method is robust in a commercial laboratory environment and can be applied to analysis of the above compounds in mainstream cigarette smoke. The reported LC-MS/MS method is simple, fast, and accurate and does not involve any sample cleanup; none of the published methods have reported for >10 compounds analysis in a single run.

24. WITHDRAWN

25. ESTIMATION OF MEASUREMENT UNCERTAINTY OF MALEIC HYDRAZIDE RESIDUE IN TOBACCO BY ISO METHOD AND IDENTIFICATION OF THE DOMINANT CONTRIBUTORY COMPONENTS TO THE ESTIMATED UNCERTAINTY.
Masahiro MIYOSHI and Chikanori Kawakami; Japan Tobacco Inc., Tochigi, Japan

Measurement uncertainty (MU) is a quantitative indicator of the confidence in the analytical data and describes the range around a measured result within which the true value can be expected to exist with a defined probability. Generally, there are two principally different approaches available for estimating the MU associated with chemical analysis: top-down approach (estimation based on default values, the Horwitz Equation or method validation and/or proficiency testing) and bottom-up approach (estimation based on the function of uncertainty sources). In this study, the MU for maleic hydrazide (MH) residue in tobacco (Flue-cured; US, 2004) by the ISO method (4876-1980) using bottom-up approach was estimated and the dominant contributory components to the estimated MU were identified. As regards bottom-up approach, the following independent components were investigated: sample weighting, concentrations of calibration solutions (high and low levels), spiking of the calibration solutions and photometric absorbances of the calibration solutions and an analyzed sample solution. According to a mathematical model (two-level calibration model equation), the combined standard uncertainty was calculated by multiplying standard deviations of each component and sensitive coefficients obtained from partial derivative of the model equation with respect to every component. The estimated MU for MH in the tobacco sample was around 10% of the measured result with 95% confidence

level (coverage factor k=2). Either photometric absorbances of high level concentration calibration solution or the test sample solution were identified as dominant contributory components. It is suggested that to manage the MU of MH, reduction in variances of the absorbances of both the high level calibration solution and the analyzed sample solution was assigned as a critical control point.

26. WHICH REFERENCE MATERIALS SHOULD I USE? Patrick S. MILLER, Veni N. Lapko, Daryl D. Grafelman, Alan M. Dzerk, Jonathan O. Rathe, Ridha Nachi and Kirk E. Newland; Celerion, Lincoln, NE USA

It's a simply-stated question that requires multifaceted, serious consideration.

In order to quantify unknown concentrations of analytes, clearly calibrators having known concentrations of the analytes are required. However, "known" may be illusory, depending on the choice and usage of the reference materials from which the calibrators are prepared. The methods used to determine the purity of the reference materials will certainly impact the known concentrations of the calibrators and thus the accuracy of the interpolated concentrations of unknown samples. Moisture or solvents in a reference material (typically not quantified by purity testing) can significantly affect the potency. A critique of Certificates of Analysis from different suppliers, for tobacco-related compounds including N-nitrosoanatabine and N-acetyl o-toluidine, is presented.

Stable-labeled internal standards are preferred for bioanalysis by LC-MS/MS because they closely mimic their respective analytes with regard to separation chemistry and ionization efficiency. However, the choice of stable-label (*e.g.* 13C vs. 2H) for the internal standards can impact the accuracy and reproducibility in a method, especially for the fast and narrow peaks obtained from sub-2 μm particle sizes in UHPLC analytical columns. The separation can be so efficient that internal standards are partially or fully resolved from the analytes and are subjected to differential matrix effects. We have found, in general, that 13C-labeled internal standards are far less prone than deuterated internal standards to separate from their respective analytes by reversed-phase UHPLC. Exemplary cases for several mercapturic acid markers of exposure to tobacco, including 3-hydroxypropyl mercapturic acid (3-HPMA), 2-cyanoethyl mercapturic acid (CEMA), S-phenyl mercapturic acid (SPMA), and monohydroxy 3-butenyl mercapturic acid (MHBMA) are presented.

27. DETERMINATION OF COUMARIN IN TOBACCO AND ALTERNATIVE TOBACCO PRODUCTS. Nancy QIAN and Jonathan Wilkins; Lancaster Laboratories Inc., Lancaster, PA USA

A sensitive UPLC-UV method was developed, optimized and validated for the analysis of coumarin (2H-1-benzopyran-2-one) in tobacco blends and various alternative tobacco products (ATP). The validation parameters included precision/intermediate precision, linearity/range, spike/recovery/accuracy, LOD/LOQ, solution stability and specificity. Representative samples of different product category or matrix were used in the validation. The method had a linear range with R2 of 0.9990 from 0.05 μg/mL to 100 μg/mL. The method was developed using an Acquity UPLC system coupled with a TUV detector, monitoring the coumarin UV absorption at 274nm. The column used for the separation was a Waters Acquity UPLC BEH Shield RP18 1.7um, 2.1mm x 50mm and the separation

was obtained using isocratic conditions by water/acetonitrile 75/25 (v/v). The coumarin peak was detected in less than 3 minutes with a total run time of about 6 minutes. No significant interference was observed related to the blank sample. The sample preparation involved simple hydroalcoholic extraction using 1:1 ethanol:water at room temperature with no further clean-up. This method enabled the accurate measurement of coumarin at levels as low as 4 µg/g of material. It was used to quantitatively determine if any coumarin is present at a variety of sample matrices including 3R4F cut filler, tobacco blends and alternative tobacco products such as Orbs, SNUS, Moist Snuff, Sticks, Strips, and other oral tobacco products. The method was proven to be precise, accurate, specific, and robust.

28. THE TOBACCO-SPECIFIC NITROSAMINE 4-(METHYLNITROSAMINO)-1-(3-PYRIDYL)-1-BUTANONE (NNK) INDUCES MITOCHONDRIAL AND NUCLEAR DNA DAMAGE IN *CAENORHABDITIS ELEGANS*. G. L. PRASAD[1], Rakesh Bodhicharla[2] and Joel N. Meyer[2]; [1]R. J. Reynolds Tobacco Company, Winston-Salem, NC, USA and [2]Duke University, Durham, NC USA

The metabolites of the tobacco-specific nitrosamine NNK form DNA adducts in animal models. A previous report indicates that NNK could cause damage to the mitochondrial as well as nuclear genome in rats (Stepanov and Hecht, 2009 Chem. Res. Toxicol. 22: 406). Using a different DNA damage detection technology, we tested whether this could be repeated in the nematode *Caenorhabditis elegans*; we also evaluated whether mitochondrial function would be affected. We treated N2 strain (wild-type) nematodes with NNK in liquid culture. Quantitative PCR was applied to analyze NNK-induced nuclear and mitochondria DNA damage. This assay has the advantage of measuring all DNA lesions that inhibit the DNA polymerase, and normalizes results to mitochondrial DNA copy number. Our results confirm that NNK causes both nuclear and mitochondrial DNA damage, but surprisingly nuclear DNA damage was greater than mitochondrial DNA damage in *C. elegans*. To test whether the mitochondrial DNA damage was associated with mitochondrial dysfunction, we used a transgenic nematode strain that permits *in vivo* measurement of ATP levels and found lower levels of ATP in NNK-exposed animals when compared to the unexposed controls. To test whether the lower levels of ATP were due to the inhibition of respiratory chain components we investigated oxygen consumption in whole *C. elegans* and found reduced oxygen consumption in exposed animals when compared to the unexposed controls. Our data suggest a model in which NNK causes damage to both *C. elegans* nuclear and mitochondrial genomes, and support the hypothesis that the mitochondrial damage is functionally important. These results also represent a first step in developing this genetically tractable organism as a model for assessing NNK toxicity.

29. THE ESTABLISHMENT OF THE TILLING EXPERIMENTAL TECHNIQUES BASED ON CAPILLARY ELECTROPHORESIS. Qiang FU, Jie Zou, Ji-shun Zhang, Yi Wang and Xueliang Ren; Guizhou Academy of Tobacco Science, Guiyang City, China

TILLING (Targeting Induced Local Lesions in Genomes) is a general reverse-genetic strategy that provides an allelic series of induced point mutations in genes of interest. The CEL I enzyme that specifically cleaves the mismatch in DNA double strands is an important component of the TILLING experiment platform. Due to the expensive price of commercial CEL I, it was not conducive to large-scale TILLING experiments. Referring to published literature, the active crude extract of CEL I enzyme was obtained from celery

growing in the Guiyang, Guizhou province. In this study, an effective CEL I digestion system was established as follows: The CEL I enzyme effectively cut the mismatch DNA in the 15μL digestion reaction solution including 8μL PCR product, 4.5μL ddH_2O, 1.5μL 10× cleavage buffer (pH 7.5, 500mmol/ L KCL, 100mmol / L Tris-Cl, 15mmol / L $MgCl_2$) and 1μL of the 20-fold dilution of CEL I crude extraction after 30-min incubation at 42°C. We hope that the use of this reverse genetics resource will provide novel allelic diversity for tobacco functional genomics as a model organism.

MONDAY AFTERNOON, SEPTEMBER 16, 2013

SESSION A - Human Smoking/Clinical Studies/Biomarkers

2:30 PM MONDAY

30. CONSUMPTION PATTERNS AND BIOMARKERS OF EXPOSURE IN CIGARETTE SMOKERS SWITCHED TO SNUS, VARIOUS DISSOLVABLE TOBACCO PRODUCTS, DUAL USE, OR TOBACCO ABSTINENCE. George R. KRAUTTER, Peter X. Chen and Michael F. Borgerding; R. J. Reynolds Tobacco Company, Winston-Salem, NC USA

Camel SNUS and dissolvable tobacco Sticks, Strips and Orbs are smokeless products containing lower levels of most tobacco-related toxicants associated with cigarette smoke. This trial investigated short-term changes in product usage and biomarkers of exposure when smokers were switched for 5 days to consuming one of the four products exclusively, partially [Dual-use of cigarettes/SNUS (DU)], or were tobacco abstinent. Participants (167) were confined for a baseline day while smoking and randomized into one of 6 groups (n=25-30/group): DU smoked at 40% their baseline daily rate, self-regulating SNUS use; exclusive groups self-regulated their product usage; tobacco abstinent (TA) used no product. Matrices/biomarkers quantified: (24-hr urine) total nicotine equivalents, and metabolites of TSNAs, PAHs, aromatic amines, acrylamide, acrolein, benzene, ethylene oxide, crotonaldehyde, cyanide and mutagenicity; (blood) carboxyhemoglobin; (plasma) nicotine, cotinine and thiocyanate; (expired-air) carbon-monoxide (ECO).

Exclusive use of SNUS, Sticks, Strips, or Orbs averaged 6.1, 5.9, 13.5, and 8.5 units/day, respectively. DU reduced smoking from 19.2 to 7.6 cigarettes/day and averaged 3.2 SNUS pouches/day. DU's smoking behavior during intervention showed slight changes; shorter butt lengths and higher 'tar' and nicotine yields/cigarette. After 5 days intervention, daily exposure to nicotine declined in all groups; DU having the least (-32%) and TA the greatest (-98%) reductions. Substantial reductions of most biomarkers were observed in the exclusive use groups; lesser modest reductions were observed in DU. NNAL levels in tobacco-use groups were slightly reduced or not significantly different, potentially confounded by exposure route metabolic differences. No differences in 1-OH-pyrene were observed in any group. Results demonstrated that when smokers were switched to smokeless tobacco, exposures to cigarette smoke toxicants were greatly reduced, generally similar in magnitude to being tobacco abstinent.

2:50 PM MONDAY

31. BIOMARKERS OF TOBACCO EFFECT: LEUKOCYTE SUBTYPES AS POTENTIAL MARKERS OF INFLAMMATION IN CHRONIC TOBACCO CONSUMERS. G. L. PRASAD[1], Subhashini Arimilli[2], Peter Chen[1] and Bobbette A. Jones[1]; [1]R. J. Reynolds Tobacco Company, Winston-Salem, NC USA and [2]Wake Forest University School of Medicine, Winston-Salem, NC USA

Existing epidemiological data indicate that harm from smokeless tobacco consumption is significantly reduced compared to smoking, with no-tobacco-use being the least risky. Chronic cigarette smoking has been associated with increased inflammation and increase

in leukocyte (white blood cell or WBC) counts, and the numbers of total and some leukocyte subtypes have been proposed as potential biomarkers of effect. In this study, we investigated whether chronic consumption of smokeless tobacco, particularly moist snuff, is associated with inflammation, relative to smokers and non-tobacco consumers (NTC). Total leukocytes, peripheral blood mononucleocytes (PBMCs), and several leukocyte subtypes were fractionated from moist snuff consumers (MSC), smokers and NTC from subjects who participated in a cross sectional clinical study aimed at the discovery of tobacco-related biomarkers.

Total WBC, PBMC counts, T cells, monocytes and neutrophils were statistically ($p<0.05$) higher in smokers relative to the two non-smoking cohorts, with no detectable differences between MSC and NTC cohorts. While B cell counts were not statistically different between smokers and NTC, their counts were significantly lower in MSC (Smk> MSC), and were similar between MSC and NTC. Although no statistically significant differences were detected in Natural Killer (NK) cell numbers, the percent of NK cells were significantly lower in smokers compared to MSC cohort in the order of Smk<MSC<NTC. Collectively, these data suggest increased inflammation in smokers relative to MSC and NTC, with minimal differences between the two non-smoking cohorts.

3:10 PM MONDAY

32. PREDICTION OF SMOKE EXPOSURE FROM SMOKING TIME. Xavier CAHOURS[1], Stéphane Colard[2], Thomas Verron[1], Rémi Julien[1] and Stephen W. Purkis[2]; [1]SEITA, Imperial Tobacco Group, Fleury-les-Aubrais, France and [2]Imperial Tobacco Limited, Bristol, UK

We have recently shown that cigarette smoke yield (TNCO) depends linearly on the difference between the time of smouldering and the time of smoking using several machine smoking regimes (TSRC 2012), with the filter ventilation open or blocked. It is obvious that the smoker's exposure increases when the intensity of smoking increases, *i.e.* when the smoking time decreases. However, from our previous observations, we wanted to know whether human smoking yields could also be predicted through the measurement of human smoking time. For this purpose, a smoking behaviour controlled study was carried out to compare the human nicotine smoking yields obtained by both filter tip analysis and the cigarette burning time model. The results of our study show that i) smoke exposure, defined here as nicotine human smoking yield, can be assessed by measuring the smoking time and also ii) this smoke exposure is a linear function of the smoking time whatever the smoking behaviour.

In this presentation, the experimental set up and the results will be discussed as well as limitations and perspectives.

4:00 PM MONDAY

33. WORKSHOP ON FILTERED CIGARS, John H. LAUTERBACH; Lauterbach & Associates, LLC, Macon, GA USA

Filtered cigars are a growing segment of the tobacco marketplace with many of the brand-

styles on the market produced by small business tobacco manufacturers. However, very little has been presented or published on the routine and detailed chemistry and toxicology of filtered cigars. Since our initial collaboration with Labstat International, ULC, in 2011, we have led experimental work designed to understand the blend, wrapper, and filter rod chemistries of these products and the toxicology of the mainstream smoke from them. Study materials will not only include information from past publications and presentations, but will include experimental results not presented or published before.

4:00 PM MONDAY

34. DEVELOPMENT AND VALIDATION OF A BIOANALYTICAL ASSAY TO SELECTIVELY QUANTITATE AROMATIC AMINES (4-AMINOBIPHENYL, O-TOLUIDINE, 1-AMINONAPHTHALENE, AND 2-AMINONAPHTHALENE). Kirk NEWLAND, Veni Lapko, Alan Dzerk and Ridha Nachi; Celerion, Lincoln, NE USA

A new bioanalytical assay has been developed and validated that provides improved sensitivity, reproducibility, and selectivity for the analysis of total aromatic amines exposure. The determination of aromatic amines exposure has been identified as an important carcinogenic risk factor in regards to tobacco smoke exposure. It has been demonstrated that aromatic amines are metabolized through a variety of pathways to a broad number of possible metabolites. To establish the relative exposure levels of aromatic amines, measurements were made after various approaches at sample hydrolysis. Unfortunately, due to the lack of available purified metabolites, few assays have demonstrated adequate selectivity to determine the portion of metabolized aromatic amines that has been measured.

The new total aromatic amines assay developed and validated at Celerion incorporates a selective approach that has been demonstrated to consistently measure the aglycone, glucuronide, acetylated, and hydroxyl metabolites of the above listed aromatic amines. Additionally, chromatographic separation from structural isomers has been consistently demonstrated using a UPLC separation prior to LC-MS/MS detection.

4:20 PM MONDAY

35. THE STATISTICAL BENEFIT OF PERFORMING GLP BIOANALYSIS USING ASSAYS THAT HAVE REDUCED VARIABILITY. Raymond H. FARMEN, Kirk E. Newland, Donald W. Graff and Michelle L. Combs; Celerion, Lincoln, NE USA

Since the tobacco industry is moving into the area of regulated bioanalysis, we have noted that there is confusion surrounding the type of and regulatory standards required for analytical methods to be used for testing tobacco constituents and biomarkers in biofluids. This presentation will focus on demonstrating the benefits of performing GLP bioanalysis by discussing the following bioanalytical topics:

1. GLP – when is a study required to follow GLP guidelines and what constitutes a truly GLP study
2. The importance of assuring sample integrity and minimizing contamination
3. Critical elements of GLP method validation:
    a. Selectivity
    b. Sensitivity

c. Carry-over and Contamination
d. Accuracy & Precision
e. Stability

4. GLP standards are the cornerstone of bioanalytical chemistry. These principles are very different from clinical chemistry and GMP assays and include:
    a. Value of standards
    b. Relationship between standards and quality control samples
    c. Importance and timing of proper chromatographic integration
    d. Batch acceptance criteria
    e. Reporting sample concentrations
    f. Importance of incurred sample reproducibility

The benefit of performing GLP analysis for tobacco constituents and biomarkers is that the improved analytical precision has a direct impact on statistical analysis. Specifically, more precise analytical methods allow studies to dose fewer subjects to achieve the same statistical power.

4:40 PM MONDAY

36. DETERMINATION OF AROMATIC AMINES BIOMARKERS OF EXPOSURE TO CIGARETTE SMOKE IN HUMAN URINE USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY. Jingjing YU, Sheng Wang, Ge Zhao, Bing Wang, Li Ding, Fuwei Xie and Xiaobing Zhang; Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou, China

Many aromatic amines (AAs) are toxic compounds and/or suspected human carcinogens. Several AAs have been detected in mainstream cigarette smoke and environmental tobacco smoke, such as 1-naphthylamine (1-NA), 2-naphthylamine (2-NA), 3-aminobiphenyl (3-ABP) and 4-aminobiphenyl (4-ABP). What's more, 2-NA and 4-ABP are listed as Group 1 carcinogens by the International Agency for Research on Cancer (IARC), while 1-NA is listed as Group 3 carcinogen. AAs are metabolized to hemoglobin and DNA adducts in human urine. Here, a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method was developed for the determination of 4 AAs biomarkers in human urine, containing 1-NA, 2-NA, 3-ABP and 4-ABP. Urine samples were hydrolyzed in acidic condition in an 80°C water bath and purified with a molecularly imprinted polymers (MIPs) solid phase extraction column. The stable isotope labeled compounds of each AA were used as internal standards during the analytical procedure, which could decrease matrix effect effectively. Recovery experiments were conducted, and they are in the range of 88.7%-113.3%. The limits of detection and quantitation for 4 AAs are in the range of 0.34-0.95 pg/mL and 1.14-3.17 pg/mL, respectively. In addition, the precision of intra-day and inter-day are less than 8.9% and 9.9%, respectively. Urine samples of smokers and nonsmokers were analyzed using the developed method, and the results show that 24 hour metabolism amounts of 1-NA, 3-ABP and 4-ABP in the urine of smokers are several times higher than in urine of nonsmokers.

MONDAY AFTERNOON, SEPTEMBER 16, 2013

SESSION B - Materials/Manufacturing/Finished Product

2:30 PM MONDAY

37. DEGRADABLE CIGARETTE FILTERS: EVALUATING TIO2 ADDITIVES. Steven A. WILSON and Jeremy K. Steach; Eastman Chemical Company, Kingsport, TN USA

Cigarette filters are a common litter item which can create a negative visual impact and increase clean-up costs. In the outdoors, the persistence of cellulose acetate-based filters can be reduced by photo degradation with the addition of select TiO_2 additives. Most cigarette filters are composed of cellulose acetate fibers with titanium dioxide (TiO_2), which is added as a delustrant. TiO_2 is commercially available in two crystalline forms: anatase and rutile. Anatase TiO_2 crystals have long been known to be more photo active than rutile, but recent work has shown that mixed phases – anatase and rutile - are even more photo active. Although most TiO_2 is manufactured with an inorganic coating to facilitate particle dispersion, such coatings will hinder the TiO_2 photo activity and impact the rate of degradation. Since photo degradation takes place at the particle's surface, it is advantageous to utilize small and high surface area particles. Therefore to obtain a high rate of photo degradation, it is important to utilize small uncoated TiO_2 particles consisting of mixed phase crystals. Based on these findings, it was shown that filters containing mixed phase TiO_2 particles can be significantly degraded (~50% weight reduction) within a few months of outdoors exposure.

2:50 PM MONDAY

38. THE MIGRATION DURING STORAGE, TRANSFER TO SMOKE, AND FILTRATION EFFICIENCY OF NICOTINE, GLYCERIN, AND PROPYLENE GLYCOL IN CIGARETTES. F. Kelley ST. CHARLES[1], Serban C. Moldoveanu[2], H. D. (Buddy) Mills[2] and Norman P. Andresen[2]; [1]St.Charles Consultancy, Winston-Salem, NC USA and [2]R. J. Reynolds Tobacco Co., Winston Salem, NC USA

This study evaluated the migration during storage, transfer to smoke and filtration of nicotine, glycerin and propylene glycol (PG) for cigarettes with cellulose acetate/triacetin filters. Filtration efficiency (FE) and smoke yields were studied for two different types of experiments: 1) cigarettes with their original filters and 2) cigarettes with fresh filters inserted just prior to machine smoking. The behavior of PG was found to be much different than that of nicotine or glycerin presumably due a higher vapor pressure (which is about 2 times that of menthol). The migration of PG from the tobacco section to the filter increased linearly with time going from 15% (of total PG) at 76 days to 21% at 134 days for the whole filter and from 3.7% to 6.3% for the 10 mm mouth end of the filter. For nicotine and glycerin there was also some migration to the filter but it was less than 0.4% of that on the tobacco after 134 days. Nicotine showed a slightly greater FE than glycerin, but these two compounds behaved very similarly. PG filtration efficiency on fresh filters was about twice that of nicotine and glycerin. This indicates that another, equally important, mechanism must be taking place other than particulate filtration. Possibilities include direct vapor

absorption or a vapor transport mechanism from the particulate to the filter fibers. This could also be taking place with the nicotine and glycerin but to a much smaller extent.

3:10 PM MONDAY

39. STUDY ON THE CAPTURE OF SMOKING AEROSOL FLOWING PERPENDICULAR TO THE FIBER. Duanfeng LU[1], Wentao Wu[1], Wenkui Zhu[1], Hongsheng Wang[1], Chuanfang Yu[1] and Peixiu Sheng[2]; [1]Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou, China and [2]Nantong Cigarette Filter Co., Ltd., Nantong, China

The process of cigarette smoke aerosol being captured by a single fiber was observed with a microscope and CCD through an established micro observation channel. The images obtained were then binarized to investigate the influences of monofilament denier, particle concentration and gas flow rate on the coverage of the smoke aerosol particles on the surface of the monofilament. The results showed that: within the same time, (1) the coverage of particles on the surface of the fiber filaments increased with the decrease of the monofilament denier of the sectional profiled fiber, (2) the coverage of particles on the surface of the fiber filaments increased as the particulate concentration increased, and (3) the coverage of particles on the surface of the fiber filaments increased as the gas velocity increased within a certain range of gas flow rates.

4:00 PM MONDAY

40. COMPARISON OF THE VARIABILITY IN SMOKE YIELD DATA WHEN SMOKING CIGARETTE BRANDS ONTO A 44MM CAMBRIDGE FILTER PAD WITH A ROTARY SMOKING MACHINE. Mario MAYR and Paul Case; delfortgroup / Wattenspapier, Wattens, Austria

The objective of this study was to evaluate the variation in tar, nicotine and CO when smoking low-yield cigarette brands using a common and a modified Cambridge filter pad holder.

Several low yield cigarettes as well as cigarettes with target yields up to 10mg/cig were included in this study. All test pieces were FSC compliant cigarettes. Smoking was done with a Borgwaldt RM20H smoking machine according to ISO 4381 and the HCI-Regime. As a reference the Kentucky 1R5F was included. Two different types of Cambridge filter holders were used. Firstly, a conventional filter holder for filter pads with a diameter of 92mm; secondly, a modified filter holder for Cambridge filters with a diameter of 44mm. The only difference was the diameter of the Cambridge filter, while the construction of the filter holder stayed the same.

The volume of solvent used for extracting the Cambridge filter was changed when using the 44mm Cambridge filter pad:

44mm Cambridge filter with 20ml solvent (IprOH)
92mm Cambridge filter with 50ml solvent (IprOH)

The study was composed of 5 replicates of each sample with the ISO Regime as well as with the HCI-Regime using both types of filter holders and Cambridge filters with different

diameters. Results concerning yields with a special focus on the variation on the analysis data will be shown.

4:20 PM MONDAY

41. THE EFFECT OF REMAINING ENZYME ACTIVITY ON SUCROSE VARIABILITY DURING CURED LEAF STORAGE. Atsushi NAGAI; Japan Tobacco Inc., Yokohama, Japan

Sugars in cured tobacco leaves are one of the key compounds that affect the taste and aroma of cigarette smoke. Glucose, fructose, maltose, and sucrose are typical sugars found in cured leaves, and these undergo gradual changes during storage due to various factors. Investigation into this variability in sugars is important from a quality control standpoint. Sugars have been thought to be mainly changed by chemical reactions after curing. However, unlike reducing sugars, sucrose is chemically stable. It is difficult to understand how sucrose could be chemically decomposed under mild conditions such as low moisture content and temperatures below 40 degrees Celsius. In this study, the mechanism behind the reduction in sucrose was investigated in detail. We expected the cause for sucrose decrease to be enzymatic.

A crude enzyme solution was prepared from cured leaf tissue, and its reactivity with sucrose was investigated. Sucrose was converted mainly into glucose and fructose. A portion of the sucrose contributed to the formation of fructooligosaccharides. The prepared solution exhibited an optimum temperature of 55 degrees Celsius and an optimum pH of 5.0 in the hydrolysis of sucrose. It was also clarified that the reaction rate of sucrose hydrolysis followed enzymatic kinetics (Michaelis-Menten). The remaining activity, which hydrolyzes sucrose into fructose and glucose, was then quantified to investigate its relationship with the decrease in sucrose during cured leaf storage. A high correlation was found between the enzyme activity and the extent of sucrose decrease during cured leaf storage. These results suggest that sucrose is mainly changed by enzymatic reactions during cured leaf storage.

TUESDAY MORNING, SEPTEMBER 17, 2013

SESSION A - Agronomy

8:10 AM TUESDAY

42. INFLUENCE OF TILLAGE, CROPPING AND NITROGEN RATE ON BURLEY TOBACCO YIELD, NITROGEN UPTAKE, TSNAs AND ALKALOIDS. Congming ZOU, Robert C. Pearce, John H. Grove, Jack M. Zeleznik and Mark S. Coyne; University of Kentucky, Lexington, KY USA

Burley tobacco (*Nicotiana tabacum* L.) cured leaf yield and quality may be affected by tillage, crop rotation and nitrogen fertilization practices. To maintain yield and leaf quality, it is crucial to understand how agronomic management can affect cured leaf yield, nitrogen uptake and cured leaf TSNAs and alkaloids. Six burley tobacco tillage-rotation systems were established in 2007: continuous conventional tillage tobacco (TTT-CT); continuous no-tillage tobacco (TTT-NT); 2 yr sod and 1 yr conventional tillage tobacco (SST-CT); 2 yr sod and 1 yr no-tillage tobacco (SST-NT); no-tillage corn-soybean-tobacco (CST-NT); no-tillage soybean-corn tobacco (SCT-NT). In spring 2012, when all plots returned to tobacco, three N fertilization rates (0, 125, and 250 lbs N /acre as NH_4NO_3) were broadcast applied in split plots. We measured nitrogen uptake from lower, middle, and upper leaves and stalk, and investigated cured leaf yield by stalk position including flyings, lugs, reds, and tips, and analyzed TNSAs and alkaloids from the fourth cured leaf. Increasing nitrogen rate significantly increased every proportion of cured leaf yield and nitrogen uptake, and TNSAs and alkaloids. Conventional tillage also significantly increased every proportion of cured leaf yield, total nitrogen uptake, TNSAs and alkaloids relative to no-tillage. Compared to continuous tobacco, tobacco rotated with sod or other row crops had significantly increased total yield and nitrogen uptake, and slightly but not significantly decrease TNSAs and alkaloids. This data reinforces the concept that rotation has an advantage of higher yield and lower leaf chemical content even in the absence of disease.

8:30 AM TUESDAY

43. THE PATTERN OF BENZO [A] PYRENE AND TOBACCO-SPECIFIC NITROSAMINE ACCUMULATION IN FIRE-CURED TOBACCO. Anne JACK, Andy Bailey, Angela Schoergendorfer, Huihua Ji, Jade Singleton, Neil Fannin and Lowell Bush; University of Kentucky, Lexington, KY USA

The accumulation of benzo [a] pyrene (B[a]P) and tobacco specific nitrosamines (TSNAs) during fire-curing was studied in order to establish whether B[a]P and TSNAs can be reduced by modifying the firing regime. The dark fire-cured variety PD 7309 was grown and fire-cured with normal production practices. Eight samples were taken: one immediately before each of five firings, and at harvest, takedown and stripping. The first seven samples were analyzed as whole leaf; the eighth sample was separated into lamina and midrib and the data combined arithmetically. B[a]P increased very rapidly after the first firing (from 0 ppb to 169 ppb), with smaller increases thereafter to 262 ppb at takedown. Total and individual TSNAs showed much the same pattern of accumulation as B[a]P. Total TSNAs increased from 0 ppm to 4.6 ppm after the first firing, with smaller subsequent increases

to 5.4 ppm at takedown. Individual TSNAs at takedown were: N'nitrosoanatabine (NAT) 2.6 ppm, N'nitrosonornicotine (NNN) 1.9 ppm and [methylnitrosamino]-1-[3-pyridyl]-1-butanone (NNK) 0.8 ppm. The pattern of accumulation of both B[a]P and TSNAs was inconsistent with a previous study, where B[a]Ps increased exponentially with each firing, and the TSNA increase was linear. We speculate that the reason for both inconsistencies was the unusually hot first firing (174°F vs. the more usual 115°F, probably due to a different grade of sawdust). Nitrite nitrogen decreased from 9.6 ppm to 2.2 ppm from harvest to takedown. Both B[a]P and total TSNAs were much lower in the midrib than in the lamina (29 vs. 214 ppb and 0.5 ppm vs. 6.2 ppm, respectively). In fire-cured tobacco, the use of whole leaf rather than lamina results in lower B[a]P and TSNAs.

8:50 AM TUESDAY

44. EXPRESSION OF AN APOPLAST-DIRECTED, PHYLLOPLANIN-GFP FUSION GENE CONFERS FUNGAL RESISTANCE AGAINST PERONOSPORA TABACINA DISEASE IN A SUSCEPTIBLE TOBACCO. Antoaneta MIHAYLOVA-KROUMOVA[1], George G. Wagner[1], Dipak K. Sahoo[1], Indu B. Maiti[1] and Sumita Raha[2]; [1]University of Kentucky, Lexington, KY USA and [2]Feinberg School of Medicine, Northwestern University, Chicago, IL USA

Tobaccos and certain other plants secrete phylloplanin glycoproteins to aerial surfaces where they appear to provide first-point-of-contact resistance against fungal pathogens. These proteins can be collected by water washing of aerial plant surfaces, and as shown for tobacco and a sunflower phylloplanins spraying concentrated washes onto *e.g.*, turf grass aerial surfaces can provide resistance against various fungal pathogens, in the laboratory and field. These results suggest that natural-product, anti-fungal phylloplanins may be useful as broad-selectivity fungicides. An obvious question now is can a tobacco phylloplanin gene be introduced into a fungal-disease-susceptible plant to confer endogenous resistance. To our knowledge this is the first report describing the assessment of the impact of a phylloplanin over expression on a fungal disease. Here we demonstrate that introduction of a tobacco-phylloplanin gene - as a fusion with the GFP gene - targeted to the apoplastic space did confer increased resistance of disease susceptible host tobacco to infection by Peronospora tabacina, the blue mold pathogen, and that this resistance is stable in homozygous plants through at least the T4 generation. In addition, we argue that our study is also novel in that the effects of T-phylloplanin-GFP expression on fungal resistance were compared in transgenic plants where fusion proteins were targeted to the cell wall versus the cytoplasm. Here we report that wall targeting has advantages over cytosolic targeting in that it appears to confer higher and more stable resistance.

Endogenous protection against pathogenic fungi would reduce the need for applying chemical fungicides, thereby reducing the level of their residues, and contributing to risk reduction.

9:10 AM TUESDAY

45. TOBACCO PHYLLOPLANINS HAVE BROAD-SPECTRUM, ANTI-FUNGAL ACTIVITY IN THE LABORATORY AND FIELD. George J. WAGNER[1], A.B.M. Kroumova[1], D.W. Williams[1] and B.C. King[2]; [1]University of Kentucky, Lexington, KY USA and [2]FermSolutions, Inc., Danville, KY USA

A family of glycoproteins we have named T-phylloplanins are secreted to aerial surfaces of tobaccos by apparently specialized, short glandular trichomes. They can be recovered as leaf water washes (T-LWW), concentrated, and applied with spores to the leaf surface of the sensitive tobacco KY-14 to provide resistance to Peronospora tabacina, the blue mold pathogen. Expression of the gene encoding T-phylloplanins in *E. coli* produces a trace level of soluble T-phylloplanin protein that inhibits germination of blue mold spores, in situ. Knockdown of the T-phylloplanin gene using RNAi renders a normally blue mold resistant tobacco susceptible to this disease (see, Shepherd, R and G.J. Wagner. Fungi and leaf surfaces: A Review. In: "Plant Fungal Interactions," D. Southworth, Ed., pp 131-154, 2012, Wiley-Blackwell Press). And, overexpression of a T-phylloplanin-GFP fusion gene in a blue mold sensitive tobacco renders this tobacco resistant (Kroumova *et al.*, submitted).

Here we report that T-LWW containing T-phylloplanins confers resistance to a broad range of fungal pathogens including at least one representative of 4 major groups of fungi/fungi-like organisms, the oomycota, ascomycota, basidiomycota, zygomycota. Resistance was demonstrated in the laboratory using hyphal extension inhibition assays, and on turf grasses in the laboratory and the field, the later under natural infection conditions (King and Wagner, Crop Sci. 51:2829-2839,2011, and, MS in preparation). Thus, natural product T-phylloplanins, and other phylloplanins (*e.g.*, from sunflower) have potential to serve as "natural" fungicides in agriculture (US Patent# 8227573).

In the context of risk reduction, T-phylloplanins have potential to reduce the need for applying chemical fungicides, thereby reducing the levels of chemical- fungicide-residues on commercial tobacco.

9:30 AM TUESDAY

46. DIVERSITY AND HERITABILITY OF THE TOBACCO RHIZOSPHERE MICROBIOME UNDER FIELD CONDITIONS. Hancheng WANG; Guizhou Academy of Tobacco Science, Guiyang City, China

Tobacco rhizosphere is a critical interface supporting the exchange of resources between plants and their associated soil environment. Rhizosphere microbial diversity is influenced by the physical and chemical properties of the rhizosphere, some of which are determined by the genetics of the host plant. However, within flue-cured tobacco species, the impact of genetic variation on the composition of the microbiota is poorly understood. Here, we characterized the rhizosphere bacterial diversity of major modern tobacco cultivars possessing exceptional genetic diversity grown under field conditions, including healthy, black shank diseased and bacterial wilt diseased tobacco rhizosphere soils. PCR-DGGE method was utilized based on 16S rDNA/rRNA, we observed substantial variation in bacterial richness, diversity, and relative abundances. Tobacco rhizosphere soils had

different bacterial varieties, highest was noted by black shank diseased soil, followed by healthy soil, the last was bacterial wilt diseased soil. When at the same place of the same kind of tobacco cultivar, soil bacterial variety was corrected with healthy tobacco. The black shank healthy and unhealthy tobacco soils had some similarity in bacterial variety, with Cs value of 79.4, while not for bacterial wilt healthy and unhealthy tobacco soil. The results of this study should facilitate expanded studies to identify robust heritable plant-microbe interactions at the level of individual polymorphisms, so that plant-microbiome interactions can ultimately be incorporated into plant breeding.

SESSION A - Method Development/Tobacco & Smoke Chemistry

10:20 AM TUESDAY

47. DETERMINATION OF TEN AGROCHEMICAL RESIDUES IN TOBACCO USING MATRIX SOLID-PHASE DISPERSION AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY. Jeong-Min LEE, Hye-Jung Min and Gi-Chul Jang; KT&G Research Institute, Daejeon, Korea

A matrix solid-phase dispersion (MSPD) method was developed for extracting and cleaning-up ten selected agrochemicals in tobacco using gas chromatography-mass spectrometry with selected ion monitoring (GC/MS-SIM). Different parameters of the method were investigated and optimized, such as the type of solid-phase (alumina, C18, and florisil), the amount of solid-phase and eluent (acetone, acetonitrile, ethyl acetate, and n-hexane). The best results were obtained using 0.5 g of tobacco sample, 1.0 g of C18 as dispersant sorbent, 1.0 g of florisil as clean-up sorbent, and acetonitile saturated with n-hexane as eluting solvent. The method was validated using tobacco samples fortified with ten agrochemicals at different concentration levels. This method gave good linearity for the selected agrochemicals ranging from 0.01 μg/mL to 0.1 μg/mL. The limits of detection and quantification fully satisfied the requirements of the CORESTA GRL. Recoveries of the ten selected agrochemicals in tobacco yielded more than 80%, and reproducibility was found to be better than 10% RSD. These results suggested that the analytical procedure including the MSPD method in combination with GC/MS could be applied to the rapid determination of ten selected agrochemicals in tobacco, and has a number of advantages such as low solvent consumption, flexibility, selectivity, and the possibility of performing extraction and clean-up in one step.

10:40 AM TUESDAY

48. COMPARATIVE ANALYSES OF CIGARETTES BY ELECTRONIC NOSE AND GC/MS. Yelin LEE; KT&G R&D Headquarters, Daejeon, Korea

An Electronic Nose(E-Nose) and Gas Chromatography/Mass Spectroscopy(GC/MS)are meanwhile conventional techniques to analyze volatile materials in many industries (*e.g.*, food, medicine, environment) and have also found broad acceptance in the analysis of tobacco products.

In this study, an experiment where tin oxide gas sensor array responses and GC/MS profiles are used to characterize the volatile compounds of different cigarettes at the same time

is performed and the measurements of two instruments are statistically correlated for cigarette samples with a known chemical information.

E-Nose and GC/MS were employed to differentiate and match flavored cigarettes with commercial tobacco flavoring agents such as menthol, vanillin and *etc.* For verifying reliability of two systems, the analyses were conducted in terms of amount of flavors in each cigarettes using partial least squares (PLS) and both measurements showed good linearity in proportion to concentration. From the principal components analysis (PCA), various chemical sensor and GC/MS data was reduced into two principal factors (PC1, PC2) and all samples were clearly distinguished with visualized regions.

The gas sensor responses were then coupled with specific individual components values obtained from GC/MS at the same measurement session and provided great correlation between two analytical devices for obtaining technical and interpretational advantages for sample evaluations.

11:00 AM TUESDAY

49. INCLUSION OF TWO NEW HPHC ANALYTES TO THE AROMATIC AMINE DETERMINATION BY GC/MS IN MAIN STREAM CIGARETTE SMOKE. Ulli BECKER and Ninitha Perumalla; Eurofins Lancaster Labs, Winston-Salem, NC USA

In March 2012, the Food and Drug Administration published a list of 93 compounds as the Harmful and Potentially Harmful Constituents (HPHC) in Tobacco Products and Tobacco Smoke list. The list contains several aromatic amines, some of which (aminonaphthalenes and aminobiphenyls) have been determined in the tobacco industry routinely for several years using GC-MS.

Two new analytes on the HPHC list are o-toluidine and o-anisidine, also in the class of aromatic amines. We have added these two analytes to the list of aromatic amines that are routinely determined in our lab. The method for aromatic amines now determines 6 compounds simultaneously. The method utilizes sample purification via solid phase extraction and determination on a GC-MS with chemical ionization detection. The validated method was robust, specific and reliable and shows good repeatability and reproducibility across a wide range of smoking regimes and tar deliveries for traditional cigarette products as well as HNB products and e-cigs.

11:20 AM TUESDAY

50. IMPACT OF USING A METAL SHEET AS AN "ALTERNATIVE SUBSTRATE FOR ASTM E.2187" ON SE PERFORMANCE. Mario MAYR and Huub Vizee; delfortgroup / Wattenspapier, Wattens, Austria

The objective of this study is to demonstrate the impact on SE performance when using different material than filter paper as a possible substrate. NIST proposed to perform these tests on a thin metal sheet (0,2mm) with 1 Layer of filter paper. As the ISO 12863 method allows using an alternative substrate, all ASTM tests have been conducted on Whatman No.2- and LIPCan filter papers. NIST proposed to use a metal sheet of type "AISI 302" with

one layer of substrate paper. Two different cigarette / test pieces have been chosen for this study. To see if this proposal would be in contradiction with the aims of the regulators, we also included an old design cigarette without any FSC technology on the cigarette paper. The regulators consider the old design cigarettes as dangerous in relation to fires caused by these cigarettes. When the proposed metal plate is used for testing, the results should be compared and be in line with results from existing test methods with ten layers of substrate paper. The conclusion of this internal study would show if this effect can be compared to the actual oxygen transfer through the filter paper. Does a metal plate really match the results from tests with substrate paper?

11:40 AM TUESDAY

51. STUDY OF CORRELATION BETWEEN VOLATILE CARBONYLS IN CIGARETTE MAINSTREAM SMOKE AND CHEMICAL CONSTITUENTS IN TOBACCO LEAVES.
Zhao-liang GENG[1], Yong-gang Feng[1], Zhang-min Xiang[1], Jie Zhang[1], Yong-hui Ge[1], Kai Cai[1] and Xian-Ling Zhu[2]; [1]Guizhou Academy of Tobacco Science Research, Guiyang, China and [2]Research Institute of Tobacco and Health, University of Science and Technology of China

In order to study the correlation between volatile carbonyls in cigarette mainstream smoke and chemical constituents in tobacco leaves, chemical constituents (soluble sugars, organic acids, sterols, solanesols, polyphenols, alkaloids, and other routine chemical components) in flue-cured tobacco leaf samples from 5 areas in southwest China were analyzed to evaluate their effect on volatile carbonyls (formaldehyde, acetaldehyde, acetone, acrolein, propionaldehyde, crotonaldehyde, butyl aldehyde and 2-butanone) in cigarette mainstream smoke. The results showed that various secondary metabolism substances in tobacco leaves from different areas varied considerably. Among them, organic acids, polyphenols and alkaloids were relatively high, sterols were moderate, and main chemical components were well coordinating in general. For released volume of most volatile carbonyls in cigarette mainstream smoke, tobacco leaf stalk position's order was the middle leaves > the upper leaves. In terms of different areas, tobacco leaves from area 1 and 2 released more volatile carbonyls in mainstream smoke than those from other area leaves, tobacco leaves from area 5 released the lowest volatile carbonyls in cigarette mainstream smoke, while tobacco leaves from area 3 and area 4 were placed in the middle. The correlation analysis demonstrated that both leaf carbonaceous compounds and nitrogen compounds were important precursors of volatile carbonyls in cigarette mainstream smoke. The emission of important volatile carbonyls in cigarette mainstream smoke shows a positive correlation with the contents of soluble sugars, some organic acids, sterols, potassium and starch in tobacco leaf, but negative correlation with the contents of total organic acids, solanesols, polyphenols, alkaloids, proteins and total nitrogen in tobacco leaf.

TUESDAY MORNING, SEPTEMBER 17, 2013

SESSION B - Method Development/Tobacco & Smoke Chemistry

8:10 AM TUESDAY

52. MODELING FDA HARMFUL AND POTENTIALLY HARMFUL CONSTITUENT SMOKE YIELDS. Michael MORTON and Jingzhu Wang; Altria Client Services, Richmond, VA USA

In 2012, the US Food and Drug Adminstration (FDA) required cigarette manufacturers to test all cigarette products for eighteen harmful and potentially harmful constituents (HPHC) in smoke under both ISO and Health Canada Intense smoking regimens, and for six HPHCs in cigarette filler.

It has long been known from benchmarking and market mapping studies that many smoke constituents are well-correlated to tar and/or carbon monoxide (CO) yield, and that the correlation can often be improved further by incorporating cigarette filler information and/or cigarette design features.

In this study we examined the 146 cigarette products tested in 2012 for HPHCs by Philip Morris USA (PM USA) for FDA reporting. All of the smoke HPHCs were statistically significantly correlated to tar and/or CO yield. Some of the constituent correlations, such as nicotine and the tobacco-specific nitrosamines NNN and NNK, were greatly improved by incorporating the concentration of the corresponding filler constituent. Because of these correlations, it is possible to test a subset of the products for HPHCs and to predict the HPHCs of the remaining products. This was demonstrated using a subset of 31 of the 146 products.

8:30 AM TUESDAY

53. NON-TARGETED ANALYSIS OF SELECTED HOFFMANN TOXICANTS IN SMOKE CONDENSATE BY CRYOPROBE 1H NMR. Jana TICHA[1], Adrian Charlton[2] and Jasper Van Heemst[1]; [1]British American Tobacco, Southampton, UK and [2]The Food and Environment Research Agency, York, UK

Tobacco smoke constituents have been routinely analysed using either single analyte methods or methods for the determination of a class of substances with very similar physico-chemical properties. To enable their quantitative determination at sometimes very low levels (parts per billion), the analytical approach may comprise multiple sample preparation and clean-up steps in order to remove unwanted matrix artefacts.

As well as methods for the quantitative determination of cigarette smoke constituents, for example the FDA list of Harmful and Potentially Harmful Constituents, the availability of screening techniques for multiple classes of constituents is desirable for research purposes and to facilitate the development of quantitative methods, which can be effort intensive. Furthermore, screening techniques have potentially high value as independent

or orthogonal methods of measurement confirmation for a wide range of substances of different physico-chemical characteristics.

We are evaluating the capability of Nuclear Magnetic Resonance (NMR) spectroscopy and liquid chromatography / high resolution mass spectrometry. Thirty three constituents (anticipated to be compatible with NMR) representing different chemical classes were selected in order to evaluate the potential of NMR. A database of these constituents was created and used for their identification and semi-quantification in mainstream tobacco smoke extracts. Of these 33, 20 were identified and their approximate Limits of Quantification were determined.

8:50 AM TUESDAY

54. PROFILING OF VARIOUS TOBACCO PRODUCTS USING HEADSPACE - SOLID PHASE MICROEXTRACTION - GAS CHROMATOGRAPHY MASS SPECTROMETRY (HS-SPME-GCMS). David LI, Peter Joza and William Rickert; Labstat International ULC, Kitchener, ON, Canada

The aim of this research was to develop a rapid screening method that could be applied to the detection of flavors currently found in tobacco products. HS-SPME-GCMS was the chosen technique since this approach has been used previously in the qualitative and quantitative analysis of processed tobacco and tobacco smoke. The approach is particularly useful for the analysis of a wide range of volatile and semi-volatile compounds when optimized to give very low chromatographic background. Identification of the chromatographic peaks is achieved from the mass spectral information. These analysis parameters represent the compromise made to assure method suitability across the diverse range of tobacco products being tested. 0.5 grams of tobacco product were placed into a 10mL headspace vial. The addition of a 2mL saturated KCL solution provided a consistent response. Extraction of the headspace onto a PDMS-DVB fiber occurred at 50°C, with desorption onto the GC column occurring at 250°C. Chromatographic separation was achieved using a 60 m × 0.25 mm ID × 0.25 µm DB-5MS column, over a 60 minute run time. The mass spec was operated in the range from 35-500 m/z. A series of tobacco products was analyzed using this technique. Profiles of tobacco filler, pipe tobacco, cigar tobacco, smokeless products, hookah ("shisha") tobacco and more "novel" products will be presented and the technique's profile interpretation discussed.

9:10 AM TUESDAY

55. SELECTED AROMATIC AMINES BY GAS CHROMATOGRAPHY MASS SPECTROMETRY: CHALLENGES OF MAINSTREAM CIGARETTE SMOKE. Alexandra MARTIN; Arista Laboratories, Inc., Richmond, VA USA

Six of the 93 compounds currently included on the US FDA's established list of harmful and potentially harmful constituents in tobacco products and tobacco smoke (HPHCs) are primary aromatic amines (PAAs): 4-aminobiphenyl, 1- and 2-aminonaphthalene, o-anisidine, 2,6-dimethylaniline, and o-toluidine. A seventh PAA, 3-aminobiphenyl, is also required for Health Canada and ANVISA Brazil reporting regulations. These compounds are routinely found in mainstream tobacco smoke and are typically analyzed

by gas chromatography mass spectrometry (GC-MS). The method described here is used routinely in a high-sample throughput laboratory and demonstrates the successful GC-MS analysis of PAAs in tobacco smoke using a combination of ion-exchange and non-retentive solid phase extraction (SPE) clean-up steps.

Primary aromatic amines are found in the particulate fraction of mainstream tobacco smoke and can be collected using a 44 mm Cambridge filter pad (CFP). Once smoking is complete the CFP is extracted with 1.6 N HCl for 30 minutes using mechanical shaking. The sample extract is passed through a MCX ion exchange SPE cartridge where the aromatic amines are retained. The cartridge is washed with further acid, the pH is adjusted and then the aromatic amines are eluted using dichloromethane. The dichloromethane eluate is cleaned further using non-retentive silica SPE prior to derivatization with pentafluoropropionic acid anhydride and analysis by GC-MS using negative chemical ionization.

The lower limits of quantitation for each compound ranged from 0.09 – 2.5 ng/cigarette (ISO regime) & 0.15 – 4.2 ng/cigarette (Canadian Intense regime). Results obtained for 3R4F ranged from 1.2 – 46.7 ng/cigarette and 2.8 – 98.3 ng/cigarette when smoked under ISO and Canadian Intense regimes, respectively.

9:30 AM TUESDAY

56. EVALUATION OF THE LEVEL OF SEVERAL MINOR ALKALOIDS IN TOBACCO WITH THE SEPARATION OF R- AND S-NORNICOTINE. Serban MOLDOVEANU; R. J. Reynolds Tobacco Co., Winston-Salem, NC USA

The chiral separation of minor alkaloids from tobacco is of interest because R and S isomers of these compounds have differences in their physiological activity. This difference is also reflected in the physiological properties of tobacco specific nitrosamines (TSNAs), in particular that of nitrosonornicotine. This compound results mainly from nornicotine N-nitrosation. The previously reported analytical techniques for the enantiomer separation of minor alkaloids have various shortcomings, such as the need for bidimensional chromatography or poor enantiomer separation. A new method for the analysis of nornicotine, anabasine and anatabine has been developed, based on an original derivatization and a simple GC/MS analysis. The minor alkaloids containing active hydrogens in their molecule were derivatized with isobutyl chloroformate (isobutyl chlorocarbonate). The method allows separate quantitation of S-nornicotine and R-nornicotine, and the analysis of anabasine and anatabine (without isomer separation). The procedure has been very favorably compared regarding the accuracy with a technique commonly used for minor alkaloids analysis (without enantiomers separation). It was found that the proportion of S-nornicotine in the total nornicotine present in tobacco varies, depending on the tobacco type, between 52.6% for an offshore flue-cured tobacco to 91.4% for an offshore burley. Green tobaccos (freeze dried) showed lower levels of minor alkaloids and the S-nornicotine is present between 31.6% to 43.8% from the total nornicotine (in the analyzed samples).

10:20 AM TUESDAY

57. A VALIDATED METHOD FOR THE ANALYSIS OF POLYCHLORINATED DIBENZODIOXINS (PCDDS) AND POLYCHLORINATED DIBENZOFURANS (PCDFS) IN MAINSTREAM CIGARETTE SMOKE. Charles J. NESLUND and Nelson Risser; Eurofins Lancaster Laboratories, Lancaster, PA USA

Many studies have reported over the years on the levels of PCDDs and PCDFs detected in mainstream smoke and materials (tobacco, paper, filter) used in the manufacture of cigarettes. Most of the studies report the PCDD/PCDF content detected in terms of TEQ, the Toxicity Equivalency as determined, in most cases, by the World Health Organization (WHO) TEF values. To calculate these values, the investigator needs to determine the concentrations of the individual congeners of PCDDs/PCDFs in cigarette smoke. Reference is often made to a lab contracted to perform the analysis by a standard EPA methodology, typically either 1613b or 8290A. The results are reported as TEQ with less consideration given to the individual congeners.

A review of these methods indicates that they have been written for application to waters and soils with performance criteria based on same. The extensive column clean-ups are also geared towards broad ranging attributes that could be associated with various kinds of water and soils. How well does this apply to the tobacco matrix, in particular to mainstream smoke? Does the smoke matrix collected on Cambridge pads present a matrix that the broader EPA approach can handle? Do the performance criteria in the EPA methods apply to the analysis of mainstream smoke? What kind of precision and accuracy can be expected from the application of such to mainstream smoke. The results of a method evaluation and validation will be presented that address these concerns.

10:40 AM TUESDAY

58. THE ANALYSIS OF FURAN AT PART PER BILLION CONCENTRATIONS IN A WIDE VARIETY OF MATRICES. Charles J. NESLUND and Tim Trees; Eurofins Lancaster Laboratories, Lancaster, PA USA

Furan is an aromatic volatile organic chemical with a boiling point of 31.3°C. It is used as a starting material in a number of chemical synthesis processes and chemical manufacturing, and a furan resin is used in the manufacture of some thermoset plastics. Furan also occurs in low levels in some foods that are heat-treated or otherwise subjected to thermal degradation. The International Agency for Research on Cancer (IARC) has classified furan as a possible human carcinogen.

Since furan was placed on FDA's Harmful and Potentially Harmful Compound list, the need for an analytical approach to quantify furan in tobacco and alternate tobacco products was created. This presentation will cover the development of an analytical approach for the analysis of furan that is specific, sensitive, and amenable to a wide variety of tobacco-related sample matrices.

11:00 AM TUESDAY

59. QUANTITATIVE DETERMINATION OF DIBENZO POLYCYCLIC AROMATIC HYDROCARBONS IN SMOKE USING GAS CHROMATOGRAPHY-MASS SPECTROMETRY. Darius B. GRISSOM and Joseph Kennaday; Eurofins Lancaster Laboratories, Inc., Winston Salem, NC USA

Based on the Harmful and Potentially Harmful Constituents (HPHC) list from the FDA, Dibenzo Polycyclic Aromatic Hydrocarbons (PAHs) need to be carefully monitored in smoke. This proposed FDA list includes many PAHs that have not been typically monitored by the Tobacco industry. Also, within this list are analytes that pose well documented specificity issues, especially in tobacco sample matrices.

This presentation will highlight the parameters used to successfully validate this method, while achieving substantial chromatographic separation of all HPHC analytes. Isolation and matrix cleanup were achieved through solid phase extraction (SPE). The SPE cartridge used for validation was the SupelMIP SPE- PAH cartridge. These molecularly imprinted polymers (MIPs) target PAHs with very high selectivity.

Chromatographic separation was achieved through an Agilent DB-EUPAH column, 20m x 0.18mm x 0.14um. This column is specifically designed to handle difficult to resolve critical isomer pairs and offer adequate quantitation of high-boiling PAHs, like Dibenzos.

11:20 AM TUESDAY

60. QUANTITATIVE ANALYSIS OF 15 SEMIVOLATILE ORGANIC COMPOUNDS IN MAINSTREAM CIGARETTE SMOKE BY GAS CHROMATOGRAPHY MASS SPECTROMETRY. Norman FRALEY and Rachel Cone; Eurofins Lancaster Laboratories, Inc., Winston Salem, NC USA

FDA requirements indicate the addition of many new compounds for monitoring in cigarette smoke. The majority of these compounds are in significantly lower levels than those historically monitored. Trace analysis of low molecular weight molecules in complex smoke matrix is a challenge. Quantifying 15 semivolatile compounds in a single method is even moreso. Eurofins Lancaster Laboratories has developed and validated a method using single quadrupole GC mass spectrometry (GCMS) applied for the quantitative analysis of selected semivolatile organic species in cigarette mainstream smoke, namely, propylene oxide, vinyl acetate, acrylonitrile, nitromethane, benzene, benzofuran, toluene, 2-nitropropane, pyridine, ethylbenzene, styrene, urethane, acetamide, acrylamide, nitrobenzene and quinoline. The 3R4F and 1R5F research cigarettes, E-cig and heat-not-burn cigarettes were investigated under the HCA and ISO regimens. Using commonly available impinger collection techniques and robust chromatography we have validated a method to quantify these 15 analytes in a single run from a single smoking.

11:40 AM TUESDAY

61. EVALUATION OF HYDROGEN PEROXIDE GENERATED FROM POLYPHENOLS AND AROMATIC AMINES WITH YIELDS AS IN CIGARETTE SMOKE. Yuichiro TAKANAMI, Akihito Shimazu and Nobumasa Kitamura; Japan Tobacco Inc., Yokohama, Japan

Cigarette smoke generates reactive oxygen species including hydrogen peroxide. While hydroquinone and catechol have been thought to be the source compounds of hydrogen peroxide in cigarette smoke, the two compounds, at concentrations similar to that observed in cigarette smoke, generated less hydrogen peroxide in model reactions than was found in cigarette smoke. We have already shown, using model solutions, that seven polyphenols found in cigarette smoke generated hydrogen peroxide. In addition, we have found that aniline enhanced the generation of hydrogen peroxide from catechol but not from hydroquinone. In this study, an electrochemical detector with higher sensitivity was used in the analysis of hydrogen peroxide. The model experiments were re-evaluated using the compounds at concentrations similar to that found in cigarette smoke. Hydroquinone and methylhydroquinone were analyzed using UPLC-Fluorescence detection, while the other five polyphenols were analyzed using LC-MS/MS. Anline and o-, m- and p-toluidines were analyzed using GC/MS (NCI). The yield of hydrogen peroxide from the solution containing the seven polyphenols was approximately 1/10 compared with that from cigarette smoke extract. Adding aniline and toluidines did not increase the yield of hydrogen peroxide. The result suggested that some other compounds would be involved in the mechanisms generating hydrogen peroxide in the aqueous extract of cigarette smoke.

TUESDAY AFTERNOON, SEPTEMBER 17, 2013

SESSION A - Regulation/Quality/Toxicology

1:30 PM TUESDAY

62. ESTABLISHING A PROFICIENCY TESTING PROGRAM AT THE UNIVERSITY OF KENTUCKY. Orlando CHAMBERS, John Geary and Huihua Ji; University of Kentucky, Lexington, KY USA

The University of Kentucky has provided reference tobacco products for scientific research since 1968. Reference cigarettes are used as a standard for tobacco smoke and tobacco product analysis worldwide. The FDA requirement that tobacco companies report data on harmful and potentially harmful constituents (HPHCs) known to be in tobacco products is limited by a lack of established analytical capability and consensus on appropriate analytical methods. Reference tobacco products have been characterized in regard to many HPHCs and thus are a necessary component for emerging tobacco product regulation that requires reporting of HPHCs. Information will be presented on new reference tobacco products that cover the ISO tar band. In addition, specific information on progress to establish a program for laboratory proficiency testing based on reference cigarettes will be presented.

1:50 PM TUESDAY

63. VARIABILITY IN TOXICANT YIELDS FROM SELECTED PRODUCTS. Alison ELDRIDGE and Kevin McAdam; British American Tobacco, Southampton, UK

The FDA have mandated the reporting of HPHC in tobacco products. Regulations recommend 'appropriate sampling techniques' to provide 'reproducible results based on multiple measurements' that are 'representative of your product as marketed' with a recommendation for reporting the mean and standard deviation. In order to inform a suitable testing regime and contextualise measurement data, it is important to understand the variation of HPHC emissions from representative or typical commercial cigarette products across time.

A study was conducted to provide information on the variability in mainstream smoke yields of HPHC at Health Canada intense (HCI) and ISO smoking regimes, from three commercial products across multiple time points from a single market. Tobacco blend chemistry and cigarette physical measurements were also included. The three large volume commercial cigarette products from Germany were sampled monthly over 10 consecutive months in 2010-11. Control data from the 3R4F Kentucky Reference cigarette was also collected at the same interval.

Two main sources of variation were investigated – analytical variation (*e.g.* from instrument, operator or chemical standards) and cigarette product variation (*e.g.* from blend composition, product design or manufacturing variation).

Generally variation in HPHC measurements was < 15% coefficient of variation (CV) over the period evaluated. A practical lower limit of variation for a reasonably controlled

analysis was identified. Also identified were HPHC which demonstrated larger than average analytical variation and would benefit from improvement in analytical methods.

The increase in variation over time with these commercial products was identified. The mean variation per HPHC will be presented along with an estimate for relative uncertainty that uses 2 x σ as an estimate for a 95% confidence limit. This estimate provides a within laboratory 'tolerance' around a single measured value for each HPHC.

2:10 PM TUESDAY

64. EFFECTS OF PRODUCT FORMAT ON NICOTINE AND TSNA EXTRACTION FROM SNUS POUCHES. Nathan GALE, Graham Errington and Kevin McAdam; British American Tobacco, Southampton, UK

Understanding the quantity of product constituents extracted by snus consumers during use is an important step in estimating exposure. Different characteristics of pouched snus products, such as pouch size and water content, may potentially influence exposure. The objective of this study was to examine whether these factors have an effect on snus constituent extraction during normal use.

Eighteen volunteer pouched snus-users took part in a central-location trial in Sweden, each attending six sessions and using three different non-commercial snus test-pieces (developed for this study) per session. Each test-piece possessed a unique combination of water content and pouch size. Used and unused portions were analysed for nicotine and tobacco-specific nitrosamines (NNN and NNK); the differences in measured quantities between used and unused portions provided an absolute and percentage measure of extraction from the pouch by the user.

Increasing pouch size increased absolute extraction but decreased percentage extraction of nicotine, NNN and NNK. In contrast to our recently-published study which showed that, at fixed pouch size, constituent extraction was proportional to its content; this suggests a specific pouch size effect. Increasing portion water content increased absolute extraction of NNN, NNK and, to a lesser extent, nicotine, and resulted in a significant increase in percentage extraction of all three of these constituents.

In conclusion, exposure of snus users to tobacco constituents such as nicotine, NNN and NNK is measurably influenced by both pouch size and water content.

2:30 PM TUESDAY

65. MAKE YOUR OWN (MYO) CIGARETTES. WHAT YOU SEE ON YOUTUBE IS NOT ISO 15592. John H. LAUTERBACH; Lauterbach & Associates, LLC, Macon, GA USA

Among the comments the US Food and Drug Administration ("FDA") received on regulations on Substantial Equivalence ("SE") and Hazardous and Potentially Hazardous Constituents in Tobacco and Smoke ("HPHC"), there were two that suggested that the mainstream smoke ("MSS") from smoking articles made from roll-your-own ("RYO") products (RYO includes tobaccos, rolling papers, and tubes with and without filters

already incorporated in them) be tested for HPHC. Perhaps the writers envisaged such requirements could be bad for producers of RYO products given the known difficulties and costs of getting even MSS "tar" and nicotine determined on RYO products. Perhaps they also considered the difficulties in using the applicable ISO Standards (ISO 15592-1:2001, 15592-2:2001, 15592-3:2008) to fabricate smoking articles from RYO products available to most US consumers. ISO 15592 was developed with tubes and tobaccos used in Europe. Typical US tubes are larger in diameter (~8.1 mm versus ~7.2 mm, both 70 mm tobacco column) and some popular US RYO contain high amounts of expanded tobacco ("ET", as determined by solvent flotation) while the tobacco blends used to develop the ISO standard did not contain ET. Based on the formula given in ISO 15592-3:2008, US tubes require a tobacco weight of 950 mg versus 750 mg required for the European tubes (tobaccos conditioned at 75% RH, calculated tobacco density ~ 265 mg/cc). Several different filter tubes were evaluated using a Top-O-Matic maker along with four types of tobacco [high ET RYO (sold with rolling papers), tube-cut containing ET, cigarette-cut without ET, and traditional European fine-cut (sold with rolling papers). Smoking articles complying with ISO 15592 could not be made reliably with the high ET RYO blend.

3:20 PM TUESDAY

66. ALTERNATE TEST SUBSTRATE FOR ASTM TEST METHOD E2187-09. Joseph WANNA; SWM Intl., Alpharetta, GA USA

The ASTM test method E2187-09 "Standard Test Method for Measuring the Ignition Strength of Cigarettes" lists Whatman #2 filter paper as the substrate to use in testing the ignition propensity of cigarettes. Tests are performed on 15, 10, and 3 layers of filter paper. As cigarette ignition propensity regulations are expanding worldwide, there is a need to include other filter papers or an alternate substrate. The target would be a substrate that is uniform, reproducible, can be easily sourced, low cost, and reusable, if possible. NIST tested and is proposing to use a stainless steel grade 302 sheet with one layer of filter paper. Test results at NIST yielded results close to those obtained on 10 layers of Whatman #2 filter paper. This presentation will discuss properties of grade 302 stainless steel plate. Test cigarettes with different levels of self-extinguishments were also tested according to the ASTM test method on 10 layers of Whatman #2 filter paper and also tested using the same method but substituting the 10 layers of filter paper with one sheet of stainless steel covered by one layer of filter paper. Findings will be reviewed and compared.

3:40 PM TUESDAY

67. FITNESS FOR PURPOSE OF TEST METHODS FOR THE REPORTING OF CONSTITUENTS OF CIGARETTE SMOKE. Christopher WRIGHT, Nicholas Timms, Graham Errington and Derek Mariner; British American Tobacco, Southampton, UK

Regulatory organisations in different regions have requested the reporting of constituents of cigarette smoke but have not fully defined the chemical tests to be used or specified the analytical performance required. At present there are few International Standards for the measurement of constituents of cigarette smoke and the methods used to report data to authorities have undergone method validation and inter-laboratory harmonization to varying degrees.

A comparison of the analytical precision of published methods with those predicted by the Horwitz equation indicates that for some methods analytical precision may be less than optimal.

The presentation will illustrate the performance and stability of test methods in current use and will discuss approaches, including the establishment of proficiency studies, that can reduce the impact of data variability on interpretation, especially in a regulatory context.

4:00 PM TUESDAY

68. Workshop: RECENT DEVELOPMENTS IN NATURAL PRODUCTS PATENT LAW.
G. David McCLURE; Middleton Reutlinger, Louisville, KY USA

The *Myriad Genetics* case has cast doubt on the patentability of natural products, including isolated DNA sequences. This workshop will review the development of natural products patent law with emphasis on recent developments and a view toward application to natural products derived from tobacco. Patent strategies in light of *Myriad Genetics* will be discussed.

4:00 PM TUESDAY

69. DO CIGARETTE SMOKE YIELDS FROM A SINGLE SMOKING REGIME FIT WITH CURRENT REGULATORY OBJECTIVES?
Stéphane COLARD[1], Thomas Verron[2], Rémi Julien[2], Xavier Cahours[2] and Stephen W. Purkis[1]; [1]Imperial Tobacco Limited, Bristol, UK, [2]SEITA, Imperial Tobacco Group, Fleury-les-Aubrais, France

Many regulations worldwide require the reporting of tar, nicotine and carbon monoxide (TNCO) and set limits on their yields measured following the ISO smoking regime (ISO3308, 2012). The intention of FCTC Art. 9 is to characterise and monitor cigarettes, and in the USA, the FDA has to make testing data publicly available in an understandable, non-misleading way. The introduction or recommendation for an additional more intense smoking regime with filter ventilation blocked has been made within this regulatory context. However, this raises a number of analytical issues and does not make the data less misleading. On the basis of a cigarette burning model presented previously (TSRC 2012), investigations were conducted on 10 products with different designs in order to understand the burning process when different machine smoking regimes were applied. The relationship between yields and the difference between smouldering and smoking time, with filter ventilation open or blocked, is described by a straight line passing through the origin for all designs tested. These studies showed that a single smoking regime would fit with the FCTC Art. 9 and FDA purposes. The reporting of i) ISO TNCO yields with puff numbers, ii) filter ventilation and iii) cigarette dimensions, characterizes the products as well as data from two smoking regimes, and it provides valuable data for cigarette monitoring. In addition, the association of the burning time derived from the number of puffs with the yields would be appropriate to communicate understandable and not misleading data.

4:20 PM TUESDAY

70. A POWERFUL TOOL FOR QUALITY CHECK OF CIGARETTES AND FILTER-RODS - WITH AND WITHOUT AROMATIC CAPSULES AND SEGMENTS. Andre TEWS; TEWS Elektronik GmbH & Co. KG, Germany

During the last several years, numerous important product innovations occurred in cigarette- and filter-technology. Eventually, those developments resulted in products that called for appropriate technologies to optimize product quality control.

Microwave resonator technology had become a well-recognized and effective tool to analyze the various unique quality characteristics of the products spawned by those new developments. In particular, the tobacco industry is familiar with the ability of the microwave resonator technology to precisely determine tobacco moisture, weight and cut position of cigarettes independently of the tobacco-sort density (*e.g.* to prevent loose-end tobacco fall out). Such devices require pre-calibration, and their measurements are not affected by time, user, and location. Hence, they provide the advantage that they can readily be used for benchmarking against the same organization-wide standard (transferability of calibrations across devices derived from a single master calibration).

A new development is the use of microwave technology to verify and monitor the quality and characteristics of both filter rods and cigarettes that contain aromatic capsules or have multi-segmented filter tips, and of cigarette filter tips that contain either charcoal or mono-acetate segments. The position and the content of each capsule are now measureable, and cigarettes or filter rods with incorrect positions or incompletely filled capsules can thus be ejected.

The microwave resonator technology is now able to measure the edge-positions of the segments in multi-segment filters or cigarette filter-tips. In segments which contain carbon, the charcoal content and the distribution of the granules can now be measured as well, and cigarettes or filter rods with incorrect edge-positions or charcoal content can be ejected. In further developments, it is anticipated that foreign body detection and ejection of cigarettes will be possible. This application can likely be extended to in-line high speed quality checks for control for filter rod machines.

TUESDAY AFTERNOON, SEPTEMBER 17, 2013

SESSION B - Method Development/Tobacco & Smoke Chemistry

1:30 PM TUESDAY

71. NON-TARGETED ANALYSIS OF TOBACCO SMOKE CONSTITUENTS BY TOF-MS. Justin FROSINA and Jasper van Heemst; British American Tobacco, Southampton, UK

Tobacco smoke is a complex aerosol which contains a large number of chemical constituents. Recent reviews indicate the presence of over 6000 identified compounds in tobacco smoke, and suggest the total number of compounds may approach 100,000. The introduction of the FDA's list of harmful and potentially harmful constituents (HPHC) has identified 93 analytes that require reporting, comprising of ~1.5% of the known constituents that make up cigarette smoke. By creating targeted analysis methods to identify and quantify the HPHC list, the remaining 98.5% of the known constituents remain unaccounted for. Developing targeted methods for 6000+ constituents is not practical and therefore there is a need for a more holistic, non-targeted approach to chemical profiling of tobacco smoke constituents.

Previous efforts to analyse for the chemical profile of smoke in a non-targeted way used quadrupole mass spectrometers (Q-MS) in scan mode. Although this did provide a profile of the volatile and semi-volatile compounds in smoke, limitations in the scan speed, sensitivity, resolution and data processing software left the technique suitable only for the higher abundance constituents. Recent advances in time-of-flight mass spectrometers (TOF-MS) and more sophisticated deconvolution algorithms have improved upon the limitations previously encountered with Q-MS.

The presentation will examine non-targeted applications we have developed for the analysis of smoke constituents. Gas chromatography and liquid chromatography have been coupled to high-resolution TOF-MS to enable the generation and comparison of the chemical profile of tobacco smoke constituents. Additionally we have been utilising comprehensive gas chromatography (GC×GC) coupled to a TOF-MS for the separation and identification of components in tobacco smoke. Future refinements of sample collection and introduction techniques will allow for a more holistic chemical profile of tobacco smoke.

1:50 PM TUESDAY

72. EFFECT OF HYDRATION ON THE EXTRACTION EFFICIENCY OF SELECTED TOBACCO CONSTITUENTS. Carol GOSS and Laura Ashmore; British American Tobacco, Southampton, UK

Hydration of plant material by the addition of water ensures that the cell walls are fully turgid before extraction. This sample preparation technique has been used to improve the efficiency of extraction of residues from tea for the analysis of pesticides. We have studied the effects of hydration on the efficiency of extraction of selected analytes from Virginia tobacco.

Tobacco constituents were chosen to study the effect of hydration when an organic solvent is used for extraction. Without hydration of the tobacco matrix, organic solvents may only be in contact with part of the matrix and will therefore not fully penetrate the matrix. This would result in incomplete extraction of the analytes from the tobacco. A standardised approach has been used for the application of water to ensure the volume, mixing and length of equilibration time was optimal. The effects of hydration on the analysis of selected alkaloids, benzo(a)pyrene and tobacco specific nitrosamines were assessed.

It was found that the use of hydration for the extraction of tobacco samples increased the extraction efficiency and improved precision for the analytes of interest. Extraction efficiency is key in obtaining reproducible and accurate results for an analytical method.

2:10 PM TUESDAY

73. AN IMPROVED HIGH PERFORMANCE ION CHROMATOGRAPHY SUPPRESSED CONDUCTIVITY DETECTION METHOD FOR THE DETERMINATION OF AMMONIA IN TOBACCO AND TOBACCO SMOKE SAMPLES. Jingcun WU, Bill Rickert, Andrew Masters and Peter Joza; Labstat International ULC, Kitchener, ON Canada

An improved HPIC method has been developed and validated for ammonium analysis using a high efficiency analytical column (smaller particle size, 5.5 um and narrower inner diameter, 3 mm). Compared to the widely used Health Canada HPIC method, the improved method is faster (25 min per sample instead of 50 min per sample), simpler (two mobile phases of water and MSA needed instead of three mobile phases as in HC method) and 10 times more sensitive with the limit of detection at 0.004 ug/mL and limit of quantification at 0.013 ug/mL. Both linear and quadratic calibration methods were evaluated with correlation coefficients larger than 0.99. Another significant improvement of the method is that it can completely separate ammonium peak from interfering peaks of organic amines and therefore, better accuracy of the results can be achieved. The method can be applied to ammonia analysis in a wide range of tobacco sample matrices including cigarette smoke samples, cigarette filler, tobacco-related extracts and smokeless tobacco samples. Examples of the applications will be presented using different reference tobacco samples such as Kentucky reference cigarettes KY 3R4F and KY 1R5F, CORESTA Monitoring CM 6 and CORESTA reference for smokeless tobacco CRP-3, as well as commercial cigarette smoke samples.

2:30 PM TUESDAY

74. DETERMINATION OF MYCOTOXINS IN TOBACCO AND SMOKELESS TOBACCO PRODUCTS BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY. Jingcun WU; Labstat International ULC, Kitchener, ON Canada

A LC-MS/MS method was developed for the determination of mycotoxins (aflatoxins B1, B2, G1, G2 and ochratoxin A) in tobacco products. A tobacco sample was spiked with commercially available internal standards (U-[13C17]-AFB1 and U-[13C20]-OTA), and then extracted with methanol:water solution. After appropriate dilution with a phosphate buffered saline (PBS), sample clean up was achieved using an immunoaffinity cartridge. Three mass transitions for each analyte were evaluated to enhance the selectivity

and accuracy of the method. The method exhibits good linearity (R2 = 0.999) over a concentration range of 0.05 to 50ng/mL for all the analytes. Kentucky reference KY 3R4F cigarette filler and CORESTA reference products for smokeless tobacco CRP-2, CRP-3 and CRP4 were tested during validation, no aflatoxins were detected in these samples, but low contents of ochratoxin A were found. Method accuracy and precision was assessed using laboratory fortified matrix samples at three concentration levels with recoveries ranging from 83.4 to 116% across all analytes tested. A simple direct LC-MS/MS method was also validated for fast Aflatoxin B1 analysis and screening without sample clean up, using 3R4F, CRP2, CRP3 and CRP4 reference samples as examples for its applications.

SESSION B - E-Cigarettes

3:20 PM TUESDAY

75. COMPARATIVE YIELDS OF SELECTED SMOKE CONSTITUENTS FROM CONVENTIONAL CIGARETTES AND E-CIGARETTES. Tony McCORMACK and Michael J. Taylor; Filtrona Technology Centre, Jarrow, UK

The increasing use of e-cigarettes has led to great interest in the performance of these products and previous work has studied the effect of smoking parameters on their particulate matter and nicotine yields. However, the levels of minor constituents found in the vapour produced by e-cigarettes – particularly those compounds that may be potentially harmful – has been, and remains, the subject of some controversy. The present work studies the comparative yields of a number of compounds found in the mainstream smoke of conventional cigarettes (in this case the K3R4F product) with those found in the vapour of a commercially available e-cigarette. These compounds quantified include phenols, minor alkaloids, carbonyls, volatile organic compounds and tobacco specific nitrosamines. All smoking was carried out using a 55ml/2 second profile, with e-cigarettes being smoked according to previously-described comparable protocols.

The present paper also gives details of the analytical procedures used and precautions that should be observed when testing and comparing conventional cigarettes and e-cigarettes.

3:40 PM TUESDAY

76. AEROSOL PRODUCTION AND CHEMICAL ANALYSIS OF ELECTRONIC CIGARETTES USING A LINEAR SMOKING MACHINE. Matt S. MELVIN, Gene Gillman and Kathy E Humphries; Enthalpy Analytical, Inc., Durham, NC USA

A survey of four electronic cigarettes that were commercially available on the world market was conducted at the laboratories of Enthalpy Analytical, Inc. The aerosol production of these devices was determined using traditional smoking regimes (ISO and Canadian Intense) as well as a regime optimized for aerosol production. The aerosol was analyzed to determine nicotine, propylene glycol, and vegetable glycerol yield over a 250 puff aerosol collection series. Data will be presented as yield summation over 25 puff blocks and compared to device weight loss over the same 25 puff blocks. Carbonyl generation was studied for the aerosol produced by the devices as well as their presence in the native liquid. The aerosol was collected using the optimized regime with 2,4-dinitrophenylhydrazine

(DNPH) derivatization and the liquid was also analyzed after DNPH derivatization. The results of these studies will be reported.

4:00 PM TUESDAY

77. DEFINING THE SMOKING REGIME VARIABLES THAT EFFECT THE TMP YIELD OF E-CIGARETTES. Michael CONNOR; Borgwaldt, North Chesterfield, VA USA

The purpose of this study was to identify if the TPM delivery of e-cigarettes is consistent or independent of each e-cigarette design and the smoke regime under which the TPM is collected.

Because of the lack of burning coal each e-cigarette brand was smoked to a fixed number of puffs irrelevant of the actual total puff capability of each specific e-cigarette brand.

Five different e-cigarette brands and suppliers were selected and puffed under the normal ISO 3308 smoke parameter setup as a basis of comparison.

The smoke runs were repeated with stepped changes in volume, duration and interval as well as puff shape. The TPM yield for each brand through each progression was recorded and then compared on a singular brand singular parameter basis, to identify the underlying yield effect of each specific parameter.

Brands are then compared to identify if the magnitude of the puff parameter changes are brand independent or specific to the designs of each e-cigarette.

Lastly, a comparison of multiple parameters is also compared to demonstrate the interaction of the parameters and if they are cumulative of interdemendant.

4:20 PM TUESDAY

78. COMPARISON OF MAINSTREAM CIGARETTE SMOKE PH WITH MAINSTREAM E-CIGARETTE AEROSOL PH. John H. LAUTERBACH; Lauterbach & Associates, LLC, Macon, GA USA

While the determination of the pH-value of mainstream cigarette smoke (MSS) does not fit the classical definition of pH, techniques have been reported in the literature for the determination of MSS pH-values and several regulatory agencies have specified methods. Some authors have pointed to the importance of carbon dioxide (CO_2) both in the MSS gas-vapour-phase and dissolved in water entrained in the particulate phase as important factors in keeping MSS pH-values acidic (Lauterbach *et al.*, 2010; Ingebrethsen, 2001, Dong *et al.*, 2000). However, there is no reason to expect CO2 in e-cigarette aerosols. We used Health Canada Method T-113 (a puff-by-puff method based on the technique reported by Sensabaugh and Cundiff in 1967) and calculations with the Health Canada Intensive ("HCI") smoking regimens. We used the Kentucky 3R4F Reference Cigarette to represent conventional cigarette products. We used a two-part e-cigarette to generate the e-cigarette aerosols. Both acidified (reportedly contained 3% malic acid) and non acidified commercial e-liquids (both PG-based, no water in ingredient statements) with a reported

nicotine content of 1.2% were used. The MSS pH for the 3R4F was 5.92 (14 puffs) and 5.96 (11 puffs) with the usual fall-off in pH-values of the latter puffs. For the e-cigarette using acid-free e-liquid, the aerosol pH was 6.68 (12 puffs); and for an e-liquid whose ingredient statement included malic acid, the aerosol pH was 6.21 (14 puffs). Another e-liquid with a reported nicotine content of 1.8% (glycerin, water, citric acid preservative) gave an aerosol pH 6.73 (14 puffs). These results show the importance of CO_2 in reducing the pH of MSS and the higher pH-values for e-cigarette aerosols.

WEDNESDAY MORNING, SEPTEMBER 18, 2013

SESSION A - Method Development/Tobacco & Smoke Chemistry

8:50 AM WEDNESDAY

79. PUFF-BY-PUFF ANALYSIS OF MAINSTREAM SMOKE CONSTITUENTS OF NON-LIP AND LIP CIGARETTES (2). Stefan BACHMANN, Maria Gleinser, Irene Rohregger, Huub Vizee and Dietmar Volgger; Papierfabrik Wattens GmbH & Co KG, Wattens, Austria

As presented at the CORESTA congress 2012, a puff-by-puff profile of LIP cigarettes showed differences in CO and O2 between a puff taken on the banded area and a puff on the band spacing. The cigarettes for the investigation of CO and O2 were smoked according to ISO 3308 with a puff volume of 35 ml.

In continuation of the study presented in 2012, effects were studied for larger puff volumes. Cigarettes were produced with different base paper parameters and bands with different diffusion capacities. Cigarette papers with permeabilities in a range of 50-125 CU and burn additive levels between 1-2% were used for the investigation of the base paper parameters. Band diffusion capacities varied between 0.05-0.15 cm/s. A puff-by-puff profile of the sample cigarettes with fully blocked filter ventilation was taken by simulating the puff volume of 55 ml of the Canadian Intense smoking regime. Changes in mainstream smoke yields of CO, O2, tar and nicotine will be presented.

A single channel smoking machine (Borgwaldt RM1), a mass analyzer (Airsense Compact), a GC-FID and GC-TCD were used for the determination of mainstream smoke yields. This study compared smoke yields of non-LIP and LIP cigarettes with different base paper parameters (*e.g.* permeability, burn additives) and different band diffusion capacities.

9:10 AM WEDNESDAY

80. ANALYTICAL STRATEGIES FOR THE DETAILED ANALYSES OF SHISHA TOBACCO. John H. LAUTERBACH[1] and Deborah. A. Grimm[2]; [1]Lauterbach & Associates, LLC, Macon, GA USA and [2]Tulane University, New Orleans, LA USA

Commercial shisha tobacco formulations represent a new challenge to the analytical scientists specializing in tobacco products. One expert described shisha formulations as tobacco ranging anywhere from 10-12% up to 30%, glycerin in excess of 30-35%, added sugars (invert sugar, for example) in excess of 20%, and exotic flavors in excess of 5%. Physically, commercial shisha products appear to be a sticky mass of chopped or coarsely ground tobacco. When slurried in water, some products give a cloudy supernatant, likely indicating lipophilic flavors. While one could think of numerous multistep methods of analysis, could there be an easier way? Our first approach was to slurry the sample in hexafluoroisopropanol (HFP) and perform GC-MS analyses on a DB-5-MS column. Five different shisha samples from three different manufacturers were analyzed. All five samples could be differentiated by this technique both in terms of semivolatile flavor components

and endogenous tobacco analytes. The results of other simple approaches to the analysis of shisha samples will also be presented.

9:30 AM WEDNESDAY

81. AIR FLOW, TURBULENCE AND SMOKE YIELDS. THE UNEXPECTED CONSEQUENCES OF MACHINE DESIGN. Ian TINDALL, Linda Crumpler and Peter Jordan; Cerulean, Milton Keynes, UK

An important but little understood factor in the determination of mainstream smoke yield is the impact of air flows during the smoking process. Although the ambient air velocities surrounding cigarettes in an analytical smoking machine during the smoking process are defined in ISO3308, and in turn referenced by the Health Canada Intense method, the specification lacks detail concerning vectors and stability of air flow. These are considered to contribute to both the absolute yields obtained during smoking but also to the repeatability of measurements. A series of experiments were undertaken to understand the origins of the air consumed during smoking, where the smoke generated goes and how seemingly simple changes within the smoke hood can change yields. In particular user exposure to smoke , as evidenced by CO exposure, was examined and it was found that the user is not exposed (less than 1ppm CO measured compared with OSHA PEL based on an 8hr TWA 50ppm or the ACGIH TLV of 29ppm) provided overall extraction was maintained. It was observed that adding a barrier for ETS has the unexpected consequence of increasing yield variability. This can be explained by examining the detailed path whereby air impinges on the smoked cigarette. Some methods of controlling air flow were investigated including the use of various "air straightening" systems which were used to reduce smoke hood edge effects, reduce turbulence in the smoke path and how these efforts changed yields and variability of yields.

9:50 AM WEDNESDAY

82. EFFECT OF DRYING METHOD ON FRESH FLUE-CURED TOBACCO LEAVES MORPHOLOGICAL, COLOUR AND CHEMICAL COMPOSITION. Huina ZHAO, Bo Lei, Wenjie Pan, Fuzhang Ding, Kai Cai and Zhu Ren; Guizhou Academy of Tobacco Science, Guiyang, China

A drying method was explored for keeping fresh tobacco leaf inclusions to a max and detecting chemical composition accurately. The effect of kill-enzyme torrefaction and freeze-drying on leaf morphology and chemical composition was investigated by half leaf method. The results showed that there was a significant difference of tobacco sample color, size, dry weight , volatile semi-volatile substances (aroma) and non-volatile substances (polyphenols) content between kill-enzyme torrefaction and freeze-drying.

Freeze-drying was a sample drying method which was suitable for detecting chemical composition accurately while it could keep inherent color, structure and inclusions of fresh flue-cured tobacco leaf. The color of fresh tobacco leaf was green by freeze-drying while the color was yellow brown by kill-enzyme torrefaction. At the same time, the tobacco leaf size by freeze-drying was 3.25 times of that by kill-enzyme torrefaction with the same weight. In the process of kill-enzyme torrefaction, 9 kinds of maillard reaction production and 8 kinds

of carotenoid degradation production were generated by polyphenols and carotenoids, 11 kinds of small molecular aromatic components were lost, such as 3-methyl-butanol, 2-pentyl alcohol, 3-pentenal *et al* for the influence of the outside temperature stress and body water dissipation. At last, it was lower than freeze-drying, and the difference was 4.37% of dry substance content.

10:10 AM WEDNESDAY

83. QUALITATIVE AND QUANTITATIVE ANALYSIS OF LEAF PROTEOME IN TOBACCO (*NICOTINA TABACUM* L.) PLANTS IN TWO DIFFERENT ECOLOGICAL REGIONS USING ITRAQ. Chengsong LIAO, Bo Lei, Wenjie Pan, Fuzhang Ding and Zhu Ren; Guizhou Academy of Tobacco Science, Guiyang, China

Increasing evidence suggests that tobacco grown in different ecological regions can have different aromas. However, the underlying proteomic mechanism remains poorly understood. [Method] In present study, the 11st leaves of tobacco grown in two typical ecological regions were collected ar four key stages, and their constitutive proteomes were analyzed by isobaric tags for relative and absolute quantitation (iTRAQ). The results showed that the expression of 291 out of 2005 proteins were significantly different in tobacco leaves between two typical ecological regions, including 64 proteins in the rosette stage, 65 proteins in the bud stage, 90 proteins in the physiological maturity stage, and 72 proteins in the pre-baked stage. Functional annotation of these proteins showed that many of them were related to the catalytic activity and binding functions. The proteins were located in the cell, cellular components, and organelles, mainly involved in metabolic processes, cellular processes, and response to external stimulations. Interestingly, proteins involved in the carbon fixation pathway and photosynthesis and secondary metabolite biosynthesis showed marked differences in expression between the two different ecological regions. Therefore, our results imply that different ecological regions may lead to differences in protein expression patterns in tobacco leaves. Importantly, differences in protein expression involving the carbon fixation pathway and photosynthesis and secondary metabolite biosynthesis may be one of reasons for the formation of different aromas in different ecological regions.

WEDNESDAY MORNING, SEPTEMBER 18, 2013

SESSION B - Agronomy

8:50 AM WEDNESDAY

84. IDENTIFICATION OF VOLATILES WITH NEMATICIDAL ACTIVITIES FROM SPHINGOBACTERIUMNEMATOCIDA ZY-71-1 AND CONTROL EFFICIENCY TO *MELOIDOGYNE INCOGNITA*. Jiaqin XI[1], Xi Jia Qin[1], Deng Bin Lin[2], Yin Qi Sheng[1], Song Ji Zhen[1], Mo Ming He[3], Xue Chao Qun[1], Wang Guang Shan[1] and Guo Jian Hua[1]; [1]Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou, China, [2]Guangxi Tobacco Industrial Co. Ltd, Nanning, China and [3]Yunnan University, Kunming, China

Chemical pesticide is an effective way in controlling tobacco disease, but it caused some problems such as environmental pollution, ecological balance destruction and chemical residues. So biological control of tobacco disease attracts more and more attention. The usage of antagonistic microbes or microbial metabolites is an important approach in controlling nematodes. *Sphingobacteriumnematocida* ZY-71-1, a endophyticbacterium isolated from tobacco leaves, could produce volatile substances with nematicidal activity. The biocontrol effect of the bacterium towards *Meloidogyne incognita* was evaluated under Petri dish test and greenhouse assay. The mortality rate to the nematode caused by *S. nematocida* ZY-71-1 was 100% and the inhibition rate to the hatch of the eggs of the nematode was also 100%. Additionally, nematicidal bioactivity materials produced by *S. nematocida* ZY-71-1 were determined by GC/MS analysis and pure chemical confirmation. Five volatile chemicals with nematicidalactivity against juveniles and eggs of *M. incognita* were identified from *S. nematocida* ZY-71-1, including benzeneacetaldehyde, 2-nonanone, decanal, 2-undecanone and dimethyl disulfide. This study provided an effective way for biological control of nematodes and established a foundation on controlling the nematodes.

9:10 AM WEDNESDAY

85. PHOSPHOROUS ADSORPTION, DESORPTION AND PHOSPHORUS FRACTIONS IN RESPONSE TO LONG-TERM APPLICATIONS OF CHEMICAL FERTILISERS AND MANURE IN THE TOBACCO SOIL. Xiang LI, Yanxia Liu and Junxiong Shi; Guizhou Academy of Tobacco Science, Guiyang City, China

This paper describes the effects of chemical fertilisers and pig manure application on the characteristics of soil phosphorus (P) adsorption-desorption and changes of soil organic P (Po) and inorganic P (Pi) fractions following 4 years of tobacco–maize rotation in Southeast China. The experiment was designed according to the local crop rotation and management system, consisting of a control treatment with no fertiliser application, a treatment with chemical nitrogen (N), P and potassium (K) fertilizers (NPK), and a treatment with chemical N, P and K fertilisers plus pig manure (NPKM). The results suggest that the characteristics of soil P adsorption-desorption were changed remarkably under different patterns of fertilizer application, The affinity constant (k) of soil P adsorption, the maximal P adsorption (Q) and the buffering capacity of P adsorption (MBC) of the NPKM treatment were lower than that of the NPK treatment. As for various forms of organic P under different fertilization in soil, labile organic P (LOP) in NPKM treatments was higher than that of the NPK treatment. In the treatment of no P application, the contents of

moderately labile organic P (MLOP) decreased. The decreasing rate was in order of NPKM >NPK> CK. The combination of organic and inorganic P fertilizers had stronger effects on iron phosphates (Fe-P) and occluded P (O-P) than on aluminum P (Al-P) and calcium P (Ca-P). Compared with the NPK treatment, soil inorganic phosphorous (Pi) in NPKM treatment decreased by 8.14%. Our results indicated combined application of organic and inorganic fertilizers could decrease the P fixation, enhance P mobility more effectively and promote P use efficiency.

9:30 AM WEDNESDAY

86. GENOME-WIDE SELECTION AND IDENTIFICATION OF SUITABLE REFERENCE GENES FOR QUANTITATIVE GENE EXPRESSION STUDIES IN TOBACCO (*NICOTIANA TABACUM*). Yushuang GUO, Ruiyuan Lie, Qingyuan Chen, Zhixiao Yang, Yi Wang, Jiehong Zhao and Xueliang Ren; Guizhou Academy of Tobacco Science, Guiyang City, China

The real time quantitative reverse transcription PCR (qRT-PCR) is becoming increasingly important to measure gene expression levels. Accurate and reproducible results are dependent on the correct choice of the reference genes for data normalization. Tobacco (*Nicotiana tabacum*) is an important model plant in studies of gene expression, but only a couple of genes that can be used as reference genes were reported till date. In this study, we developed a rapid method using microarray combined with qRT-PCR to screen suitable reference genes for the real-time RT-PCR research. The expression stability of all tobacco genes (ESTs) was measured using a custom-designed microarray in a diverse set of 54 tissue samples representing 3 varieties, 3 tissues and 6 developmental stages. The gene stability in the 54 tissue samples were analyzed by two software program: geNorm and Normfinder. The most stable 20 genes were selected as new candidate reference genes and were validated by real time PCR with two traditional housekeeping genes (L25 and EF-1a) which were proved the highest expression stability. The data were also calculated by geNorm and Normfinder software and our results showed that the tobacco phosphoribosyltransferase gene (Accession NO: AB038494.1) exhibits highest expression stability followed by the digalactosyldiacylglycerol synthase gene (Accession NO: AY651024.1), both of which showed more stable than L25 and EF-1a. These results confirmed that tobacco phosphoribosyltransferase and digalactosyldiacylglycerol synthase genes were now the best choice for reference genes in real-time RT-PCR study, providing a foundation for the more accurate and widespread use of real-time RT-PCR in tobacco. Our method can be used in other kinds of plants to screen the reference gene in real-time RT-PCR study.

9:50 AM WEDNESDAY

87. EFFECTS OF CARBON NANOPARTICLES ON SEED GERMINATION, NUTRIENT ABSORPTION, AND YIELD OF TOBACCO. Taibo LIANG, Tai-Bo Liang, Qi-Sheng Yin, Yan-Ling Zhang, Bao-Lin Wangi, Wei-Min Guo and Jian-Ping XIE; Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou, China

Unlike macromaterials, nanomaterials have some particular properties such as surface effects, volume effects, quantum size effects, *etc.* In recent years, the biological and agricultural applications of nanomaterials have become of increasing interest. Some studies have shown

that nanomaterials can enhance crop seed germination and promote plant growth. Also, the rate of chemical fertilizer utilization can be increased by nanomaterial application to soil. A recent study showed that carbon nanotubes induce growth enhancement of tobacco cells. Tobacco is an important economic crop in China, however, to date, little attention has been paid to the growth and nutrient absorption of tobacco plants in the presence of nanomaterials.

This study was conducted to determine the effects of carbon nanoparticles on seed germination, nutrient accumulation, and yield in tobacco. Carbon nanoparticles (10-100nm) in two forms (sols and powder) were used in this study. The results showed that carbon nanoparticle sols can increase the root hair length and number, and promote growth of the radicle and embryo in a wide range of concentrations (5-40 mg/L), which promoted tobacco seed germination. In water cultivation, carbon nanoparticles at 5-20 mg/L promoted tobacco plant growth and increased root biomass and dry matter accumulation. Also, application of carbon nanoparticles enhanced the absorption of nitrogen and potassium in tobacco plants, increasing the nutrient content and accumulation amount. Under field conditions, carbon nanoparticle fertilizer increased the yield and value in tobacco production. It also increased the potassium content of tobacco leaves, which improved the tobacco quality. Therefore, carbon nanoparticles are suitable for use as a new fertilizer in tobacco production.

10:10 AM WEDNESDAY

88. THE BIOCONTROL MECHANISMS OF BIOORGANIC FERTILIZER TO CONTROL TOBACCO BACTERIAL WILT IN SOIL MICROORGANISM PERSPECTIVE.
Yanxia LIU, Xiang Li and Junxiong Shi; Guizhou Academy of Tobacco Science, Guiyang City, China

Bacterial wilt caused by *Ralstonia solanacearum* (Rs) is one of the most serious tobacco diseases worldwide. An organic fertilizer was secondly solid fermented by two antagonists, making it a bio-organic fertilizer (BOF). The BOF was made up of BOF25 fermented by strain L-25 and BOF9 fermented by strain L-9 respectively in a 1:1 (w:w) proportion. Field experiments were conducted in Anhui Province for two years to investigate the biocontrol efficacy of BOF. Results showed that the control efficacies of the BOF treatment were up to 75.2% for the first year and 95.4% for the second year. The results of scanning electron microscope (SEM) observations showed that there was viscous material deforming and blocking vessels in the vascular bundles of wilted tobacco, while the vascular bundles of healthy tobacco and those treated with BOF grew well with a normal shape. The populations of cultivated bacteria, actinomycetes and antagonists were significantly higher than in the control, while the number of fungi significantly decreased. BOF depressed the colonization of Rs on tobacco roots when Rs was labeled by green fluorescent protein. The functional diversity of the microbial community, as determined by the Shannon Index, Simpson Index, and Mclntosh Index of the soil microbial community, were significantly higher in the BOF treatment than in the control. The DGGE patterns of bacteria and fungi from the BOF soil and control treatments belonged to two corresponding clusters, suggesting that bacterial species increased and fungal species decreased with the application of BOF. These results suggest that the tobacco-specific BOF application can effectively improve the micro-ecology in the rhizosphere, and is thus a potentially promising treatment for the control of tobacco bacterial wilt disease.