65th Tobacco Science Research Conference

MONDAY MORNING, SEPTEMBER 19, 2011

Symposium

9:00 **WELCOME:** Anne Jack, University of Kentucky, 65th TSRC Chair and Nancy Cox, Associate Dean for Research, University of Kentucky

9:10 **SYMPOSIUM: "Challenges in the Development of Biomarkers of Smoking Exposure and Effect"** Chair: Ed Robinson, Lorillard Tobacco Company

9:15 AM MONDAY

1. BIOMARKER DISCOVERY AND VALIDATION USING GLOBAL METABOLOMICS. Mike MILBURN; Metabolon, Durham, NC, USA

The search for biomarkers is a hotly studied and active pursuit in a variety of industries. In fact, the future of personalized health interventions rests squarely on the ability to discover and validate new biomarkers. The biomarkers themselves can be nearly anything that distinguishes one individual from another. They can be based on a diagnostic test (*e.g.*, glucose or cholesterol measurements), physical characteristic (*e.g.*, BMI), genetics (*e.g.*, SNPs) or any other distinguishing characteristic (*e.g.*, age, diet). Unfortunately, general screening methods for the discovery of new biomarkers have been very challenging with few success stories. The challenges are mathematical, technological, and/or limited availability of proper sample types and number. One recent promising technology with potential to overcome a number of these issues is metabolomics.

9:45 AM MONDAY

2. SMOKING RELATED BIOMARKERS OF POTENTIAL HARM/ EFFECT: CHALLENGES AND OPPORTUNITIES. G. L. PRASAD; Clinical Studies, R & D Department, R. J. Reynolds Tobacco Co., Winston-Salem, NC, USA

Chronic cigarette smoking has been associated with several diseases such as lung cancer, COPD, cardiovascular disease and oral cancer in some smokers. While smoking has been known to adversely affect multiple cellular and physiological processes, further research is necessary to understand the aberrant physiology that leads to disease in susceptible individuals following decades of smoking. Appropriate biomarkers indicating smoking effects may enhance this understanding. Ideally, the biomarkers of effect would be able to predict harm from smoking in healthy consumers, and thus identify the at-risk individuals. Further, putative biomarkers might be useful in determining whether consumption of potentially reduced exposure products (PREPs) or modified cigarettes could reduce harm, or allow comparison of tobacco product categories. Therefore, these assessments should be carried out in short-term cell culture assays, animal models and clinical studies. Notwithstanding their promise, very few potential biomarkers of effect are currently available, and several challenges remain. For example, the biomarkers need further qualification, and the methods for their quantification need development and validation. In addition, testing the biomarkers in appropriate experimental models and disease-specific

clinical studies will be necessary to rigorously validate them prior to their integration into health assessments.

Current cutting-edge discovery technologies such as transcriptomics, epigenomics and metabolomics, together with an understanding of chemistry and biological consequences of smoking hold promise in the discovery and characterization of biomarkers of effect. Given the complexity of smoking-induced biological changes, a diverse array of biomarkers of effect may emerge. A "fit-for-purpose" strategy may be appropriate for qualifying biomarkers and validating methods to expedite the discovery and characterization of smoking-related biomarkers of effect/harm, and their application for assessing risk to individual consumer and evaluating PREPs.

10:15 AM ***Break***

10:45 AM MONDAY

3. UTILITY OF BIOMARKERS IN ASSESSING EXPOSURE TO CIGARETTE SMOKE CONSTITUENTS IN ADULT SMOKERS. Mohamadi SARKAR; Altria Client Services, Richmond, VA, USA

There are thousands of constituents in cigarette smoke, which makes assessment of total systemic exposure to cigarette smoke challenging. Exposure to cigarette smoke constituents can occur through many other sources in addition to cigarette smoke. Measurement of a tobacco constituent or metabolite in a biological fluid (biomarker of exposure – BOE) as a surrogate of classes of smoke constituents is a practical approach for assessing exposure. Observations from a stratified, multi-center, cross-sectional study of 3,585 adult smokers and 1,077 non-smokers as well as open-label, parallel design clinical studies in adult smokers (n=411) will be shown. Exposure to tobacco specific constituents was measured from 24-hour urinary excretion of nicotine and five of its metabolites (nicotine equivalents, NE), total NNAL (metabolites of NNK), total N'-nitrosonornicotine (NNN) and plasma cotinine. In addition exposure to the non-tobacco specific constituents – benzene (S-phenyl mercapturic acid), aromatic amines by urinary 4-aminobiphenyl (ABP), 4-ABP hemoglobin adducts, o-toluidine, 2-aminonaphthalene, carbon monoxide (blood Carboxyhemoglobin), acrolein (3-hydroxypropyl mercapturic acid), 1,3-butadiene (MHBMA and DHBMA) and PAHs (total 1-hydroxypyrene and 3-hydroxy benzo(a)pyrene) was also measured. In addition, it was also observed that some of the biomarkers have poor discriminatory power (*e.g.* DHBMA, *o*-toluidine and urinary 4-ABP) and would not be suitable for estimating exposure in adult smokers. Based on this analysis it can be concluded that NNN and 3-OH BaP have limited utility as biomarkers for estimating exposure to TSNAs and polycyclic aromatic hydrocarbons respectively. It can also be concluded that based on the analysis of the relationships with the different biomarkers, NE may be considered as a surrogate biomarker of total cigarette smoke exposure.

11:15 AM MONDAY

4. A SCIENTIFIC FRAMEWORK FOR ASSESSING MODIFIED RISK TOBACCO PRODUCTS. Christopher PROCTOR and Martin Ward; British American Tobacco (Investments) Limited, Group Research and Development, Southampton, UK

Given that tobacco is a leading cause of morbidity and mortality the development and assessment of potential reduced exposure products (PREPS) or modified risk tobacco products (MRTPs) is a research imperative. To assess the potential impact of a MRTP on both individual and population health risks will require a wide range of pre-clinical, clinical and pre- and post marketing studies, with evaluations staged over time. Pre-clinically this would involve chemical, physiochemical and biological characterisation (for example, through *in vitro* models of disease) and behavioural studies. The clinical phase would assess the impact of product switching to biomarkers of exposure and of biological effect, while a pre-marketing phase would assess likely tobacco user and non-user reaction to the product and any claim made. PMS would evaluate both the longer term effect on individual health status and population dynamics of users and non-users. At each stage a weight-of evidence approach should be adopted. This paper describes the current state of science in being able to undertake such an assessment and identifies research needs.

11:45 PM ***Lunch***

1:00 PM **POSTERS**

5. A CELL CULTURE MODEL FOR CHRONIC EXPOSURE TO CIGARETTE SMOKE. Subhashini ARIMILLI[1], Brad E. Damratoski[1] and G. L. Prasad[2]; [1]Department of Microbiology & Immunology, Wake Forest University School of Medicine, Winston Salem, NC, USA and [2]R.J. Reynolds Tobacco Company, Winston Salem, NC, USA

A wide range of experimental models, including cell culture systems, have been developed to investigate the cytotoxic and biological effects of cigarette smoke and its constituents, individually and by chemical class. For example, cell culture studies involving short-term exposure to Total Particulate Matter (TPM; commonly known as cigarette smoke condensate) at cytotoxic doses have provided important information on cellular responses to cigarette smoke. However, smoking-related pathophysiological changes typically manifest after many years of exposure. Hence a comprehensive investigation into the long-term effects of exposure and cellular responses to sub-toxic levels of cigarette smoke is necessary.

Here, we describe the development of a cell culture model to investigate the effects of long-term systemic exposure to cigarette smoke. This model utilizes HL60 cells, a widely studied promyelocytic leukemia cell line, and exposure to non-cytotoxic levels of TPM. Initial studies were performed to determine the EC_{50} (50% cell death values in a 24h exposure assay) for TPM exposure. Continuous exposure of HL60 cells at 30% EC_{50} (0.46 ug/ml nicotine units) of TPM for 4 months was performed. Cell survival and adaptation to the TPM was assessed by examining cell proliferation, cell death (live/dead assay, late apoptosis measured by caspase-3), cell cycle regulation, and double stranded DNA damage (γ-H2AX staining). TPM-adapted HL60 cells displayed similar growth properties and comparable levels of DNA damage as the untreated cells. These initial studies suggest that HL60 cells survive and adapt to exposure to TPM in long-term culture, and exhibit similar growth properties as the untreated cells. Thus, this cell culture model may be a useful tool for investigation of the long-term effects associated with exposure to TPM.

6. DETERMINATION OF THEOBROMINE IN PROCESSED TOBACCO PRODUCTS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY. Mingliang BAO; Labstat International ULC, Kitchener, ON, Canada

Theobromine is the primary methylxanthine found in chocolate, a common cigarette additive. A simple and reproducible method has been developed and validated for the analysis of theobromine in processed tobacco by using high performance liquid chromatography (HPLC). Theobromine is extracted from tobacco sample with water by sonication. The aqueous extract is analyzed by HPLC with a propyl-linked pentafluoropheny (PFP) column using methanol/water/acetic acid (10/89/1, v/v/v) as the mobile phase at 0.8 mL/min and a ultra-violet (UV) detector at 280 nm. The utilization of a PFP column allows for better separation of tobacco matrix interference from the theobromine peak, compared to the most commonly used C18 reverse phase column. The recovery of theobromine from spiked tobacco sample is 97% with relative standard deviation under 6%. The detection limit of theobromine is 0.38μg/g in tobacco. This method was applied to several commercial tobacco products, of which the concentration range of theobromine varied from below the detection limit to 86.5 μg/g.

7. ANALYSIS OF MULTIPLE CLASSES OF CIGARETTE SMOKE CONSTITUENTS BY COMPREHENSIVE TWO-DIMENSIONAL GAS CHROMATOGRAPHY WITH TIME OF FLIGHT MASS SPECTROMETRY (GCxGC-TOFMS). Elizabeth HUMSTON-FULMER, Joe Binkley and Mike Riley; LECO Corporation, Saint Joseph, MI, USA

The Food and Drug Administration now has the authority to regulate cigarettes, cigarette tobacco, and smokeless tobacco products. Among the areas of regulation is the monitoring of tobacco smoke constituents. It is well known that tobacco smoke is a very complex mixture of chemical compounds including benzene, nicotine, phenols, polyaromatic hydrocarbons (PAHs), and tobacco specific nitrosamines (TSNAs), to name a few. The complexity of the smoke matrix causes the need for multiple gas chromatography (GC) methods to accurately determine monitored constituents. Currently, there are separate methods used for different classes of compounds, including methods specific to volatiles, semi-volatiles, PAHs, and TSNAs.

This work will show the development of a comprehensive two-dimensional gas chromatography method coupled with time of flight mass spectrometry (GCxGC-TOFMS) for the analysis of smoke extracts from Kentucky reference cigarettes. The goal was to limit the need for multiple methods of analysis. GCxGC offers increased chromatographic resolving power, highly structured two-dimensional chromatograms, and increased detectability through the cryofocussing effects of thermal modulation. The data shows that the capabilities of GCxGC-TOFMS facilitate the ability to perform a comprehensive analysis across several compound classes.

8. MAINSTREAM SMOKE CHEMISTRY OF SAMPLES FROM THE 2009 U.S. CIGARETTE MARKET. J.A. BODNAR, P.A. Murphy, W.T. Morgan and M.W. Ogden; R.J. Reynolds Tobacco Company, Winston-Salem, NC, USA

A survey of selected mainstream smoke analytes from commercially marketed U.S. cigarettes was conducted in 2009. The U.S. cigarette market was stratified into thirteen (13) strata

based on Cambridge Filter Method (CFM) "tar" category and cigarette design parameters (length and circumference). Menthol and non-menthol cigarettes were included. Sixty-one cigarette (61) brand styles were chosen to represent the market. Another thirty-four (34) brand styles of interest were included in the survey along with a Kentucky 3R4F reference cigarette. Eighteen mainstream smoke constituents were evaluated using the Health Canada smoking regimen. By weighting the results from the 61 brands the constituent means and medians of the U.S. cigarette market wwere estimated.

For nicotine, catechol, hydroquinone, benzo(a)pyrene and formaldehyde the mean yields increased with increasing "tar" yields. Analyte yields for the ultra-low "tar" and low "tar" cigarettes were not significantly different for most other analytes as vent blocking defeated any filter air dilution design features. In contrast, normalization per mg nicotine provided an decrease in cigarette yields per increase in "tar" yield. Menthol cigarette analyte means were within the range of the non-menthol cigarettes of similar "tar" categories.

9. A FUNDAMENTAL UNDERSTANDING OF THE FILTRATION OF VOLATILE TOXICANTS IN CIGARETTE SMOKE BY ACTIVE CARBONS. Peter J. BRANTON[1], Kevin McAdam[1], Chuan Liu[1], Martin Duke[1], Michele Mola[1], Maria Curle[1],Christopher Proctor[1] and Robert Bradley[2]; [1]British American Tobacco Group Research & Development Southampton, UK and [2]MatSIRC Ltd., Carbon Technology, Penrith, Cumbria, UK

The ability of two very different active carbons, a polymer-derived carbon (with ultramicropores and supermicropores and a large volume of 'transport' pores) and a coconut shell-derived carbon (predominantly ultramicroporous), to reduce the levels of volatile toxicants in cigarette smoke has been measured and compared. The polymer-derived carbon was found to be approximately twice as effective in removing the majority of measured smoke vapour phase toxicants compared to the coconut-shell derived carbon in 3 different cigarette formats and with 2 different smoking regimes.

By applying adsorption first principles to equilibrium isotherm data and also measuring dynamic breakthrough times and volumes, we have established criteria by which active carbon performance for removing various toxicants from challenge streams (characterized by relatively high flows per weight of carbon and short contact times) can begin to be understood. This approach identifies some of the key factors which influence dynamic toxicant adsorption.

Single component dynamic breakthrough experiments were conducted with benzene, acrylonitrile and 2-butanone at 298 K for beds of each carbon under dry (0%RH) and wet (60% RH) conditions. Longer breakthrough times were found with the polymer-derived carbon, and breakthrough times recorded under wet conditions were found to be up to 20% shorter than those obtained under dry conditions. Correlations were found between micropore volume, dynamic adsorption volume and filter bed breakthrough time.

10. REDUCTION OF ALDEHYDES AND HYDROGEN CYANIDE YIELDS IN MAINSTREAM CIGARETTE SMOKE USING AN AMINE FUNCTIONALISED ION EXCHANGE RESIN. Peter J. BRANTON, Kevin McAdam, Chuan Liu, Martin Duke, Dinah Winter and Christopher Proctor; British American Tobacco Group Research & Development, Southampton, UK

Active carbon has been shown to be an effective material for the physical adsorption of many of the smoke volatile species. Volatile species that are less well physically adsorbed include acetaldehyde, formaldehyde and hydrogen cyanide. Alternative methods for the removal of these from cigarette smoke are therefore of interest. A macroporous, polystyrene based ion-exchange resin (Diaion®CR20) with surface amine group functionality has been investigated for its ability to react with aldehydes and HCN in an aerosol stream, and thus selectively reduce the yields of these compounds (in particular formaldehyde) in mainstream cigarette smoke.

Resin surface chemistry was characterized using vapour sorption, XPS, TOF-SIMS and 15N NMR. Diaion®CR20 was found to have structural characteristics indicating weak physisorption properties, but sufficient surface functionalities to selectively remove aldehydes and HCN from cigarette smoke. Using 60mg of Diaion®CR20 in a cigarette cavity filter gave reductions in smoke formaldehyde greater than 50% (estimated to be equivalent to > 80% of the formaldehyde present in the smoke vapour phase) independent of a range of flow rates. Substantial removal of HCN (>80%) and acetaldehyde (>60%) was also observed. The performance of Diaion®CR20 was found to be consistent over a test period of 6 months. The overall adsorption for the majority of smoke compounds measured appeared to follow a pseudo-first order approximation to second order kinetics.

This study has shown that Diaion®CR20 is a highly selective and efficient adsorbent for formaldehyde, acetaldehyde and HCN in cigarette smoke. The reductions for these compounds were greater than those achieved using an active carbon. The results also demonstrate that chemisorption can be an effective mechanism for the removal of certain vapour phase toxicants from cigarette smoke.

11. AN INTER-LABORATORY COMPARISON OF URINARY 3-HYDROXYPROPYLMERCAPTURIC ACID MEASUREMENT DEMONSTRATES GOOD REPRODUCIBILITY BETWEEN LABORATORIES. Francis CHEUNG[1], Emmanuel Minet[1], Graham Errington[1], Mike McEwan[1], Gerhard Scherer[2], Kirk Newland[3], Mehran Sharifi[4] and Brian Bailey[5]; [1]British American Tobacco GR&D, Southampton, UK, [2]ABF GmbH, [3]Celerion, [4]Labstat International Inc., and [5]Covance Laboratories Ltd.

Background - Biomarkers have been used extensively in clinical studies to assess toxicant exposure in smokers and non-smokers and have recently been used in the evaluation of novel tobacco products. The urinary metabolite 3-HPMA, a metabolite of the major tobacco smoke toxicity contributor acrolein, is one example of a biomarker used to measure exposure to tobacco smoke. A number of laboratories have developed liquid chromatography with tandem mass spectrometry (LC-MS/MS) based methods to measure urinary 3-HPMA; however, it is unclear to what extent the data obtained by these different laboratories are comparable.

Findings - This report describes an inter-laboratory comparison carried out to evaluate the comparability of 3-HPMA measurement between four laboratories. A common set of spiked and authentic smoker and non-smoker urine samples were used. Each laboratory used their in-house LC-MS/MS method and a common internal standard. A comparison of the repeatability ('r'), reproducibility ('R'), and coefficient of variation for 3-HPMA demonstrated that within-laboratory variation was consistently lower than between-

laboratory variation. The average inter-laboratory coefficient of variation was 7% for fortified urine samples and 16.2% for authentic urine samples. Together, this represents an inter-laboratory variation of 12.2%.

Conclusion – The results from this first inter-laboratory comparison for the measurement of 3-HPMA in urine demonstrate a reasonably good consensus between laboratories. However, some consistent measurement biases were still observed between laboratories, suggesting that additional work may be required to further reduce the inter-laboratory coefficient of variation.

12. AN EXAMINATION OF THE BIOLOGICAL EFFECTS OF REDUCED TOXICANT PROTOTYPE CIGARETTES USING *IN VITRO* MODELS OF SMOKING-RELATED DISEASES. Ian M. FEARON, Mark Taylor, Tony Carr, Natalia Cockcroft, Geoff-Foss Smith, Katherine Hewitt, Linsey Haswell, Emma Bishop and Gary Phillips; British American Tobacco Group R&D, Southampton, UK

INTRODUCTION: A potential approach to reduce the harm associated with cigarette smoking is to reduce smoke toxicant levels. *In vitro* models of smoking-related diseases may provide screening tools for cigarettes with altered smoke toxicant levels and data from these models used to provide support for the reduced harm potential of novel cigarettes. Here, we describe *in vitro* models of cardiovascular disease, chronic obstructive pulmonary disease (COPD) and oxidative stress in which we have examined the effects of smoke extracts from conventional and novel reduced toxicant prototype (RTP) cigarettes.

METHODS: Cells were exposed to cigarette smoke particulate matter (PM) derived either from commercial controls or from RTP cigarettes in which several smoke toxicant yields are reduced. The migration of cultured endothelial cells was used as a vascular damage repair assay with relevance to cardiovascular disease development. In the bronchial epithelial H292 cell line, we monitored both cellular antioxidant (GSH) levels as a secondary measurement of oxidative stress and the secretion of COPD-related inflammatory mediators.

RESULTS: PM caused a dose dependent inhibition of migration by the endothelial cells, an effect which was reduced in magnitude when using PM derived from an RTP. Similarly, in H292 cells PM from conventional cigarettes caused depletion of cellular GSH levels and the secretion of various inflammatory mediators such as the vascular endothelial growth factor (VEGF). Both of these responses were mitigated when using PM derived from RTP cigarettes. CONCLUSIONS: Cigarette smoke extracts induce changes in *in vitro* models of smoking-related diseases and these effects can be mitigated by modifying the smoke chemistry. Further studies are required to investigate the clinical implications of reducing cigarette smoke toxicants.

13. DETERMINATION OF A WIDE RANGE OF N-NITROSO-COMPOUNDS IN TOBACCO SMOKE THROUGH THE USE OF TANDEM MASS SPECTROMETRY COUPLED TO GAS PHASE CHROMATOGRAPHY. Gene GILLMAN, T. D. Daniels, K.A Wilkinson, K.E Humpries and S.S. Brown; Enthalpy Analytical, Durham, NC, USA

The number of N-nitroso compounds of regulatory interest in tobacco and tobacco smoke has increased in recent years. The US FDA Tobacco Products Scientific Advisory Committee

(TPSAC) proposed list includes six volatile or semi-volatile N-nitroso compounds in addition to tobacco specific nitrosamines (TSNA). Unfortunately, the widely used LC-MS/MS method for the analysis of TSNA is not easily adapted for the analysis of volatile N-nitroso compounds given the low molecular weight and volatility of these compounds. The objective of this study was to develop a single analytical method that was capable of determining as many compounds as possible in one analytical run. We chose to use GC-MS/MS as an alternative to LC-MS/MS. In brief, tobacco smoke particulate matter was collected on Cambridge filter pads which were extracted with alkaline water and methylene chloride. The organic layer was separated, filtered and injected without any further sample preparation. The N-nitrosoamines were separated on an Agilent 7890 using an HP-5MS column with subsequent detection on an Agilent 7000 QQQ tandem mass spectrometer. The mass spectrometer was operated in positive chemical ionization mode using ammonia as the ionization gas. Compounds were determined in daughter ion mode (MRM). In general, all compounds gave excellent levels of detection with most compounds giving LODs of less than 1 ng/mL. Calibration and other analytical details will be presented for all compounds. One compound, N-nitrosodiethanolamine (NDELA), proved difficult to analyze and required significant method development work. We will present additional information on NDELA as well as the results from several reference cigarettes.

14. FOLIAR NUTRITION FOR IMPROVING TOBACCO LEAF QUALITY: INSIGHTS FROM AGRONOMIC AND MOLECULAR STUDIES. Mahavishnan KARUPPAN, Ambika P Upadhyay and Navin K Sharma; ITC R&D Centre, Bangalore, Karnataka, India.

Soil chemistry plays an important role in influencing the leaf quality of FCV tobacco. For example, in India FCV tobacco grown in black cotton soils (*vertisols*) tend to have significantly lower leaf K^+ content and higher starch content mainly due to interference of soil constituents such as high Ca^{++} which hinders K^+ availability & its uptake. In tobacco, K^+ is an important leaf quality parameter as it affects leaf colour, grade, texture and burning qualities. Attempts to enhance K^+ in leaf through soil interventions did not result to enhanced leaf K^+ content due to high degree of soil complexity in the crop growing region. Here we report a unique intervention to stimulate tobacco plant to take up K^+ from such soils by foliar application of FA. Tobacco plant treated with FA at 45 and 60 days after planting resulted in enhanced lamina K^+ content. The finding also revealed that apart from enhanced K^+, it also resulted in improved volatile flavour compound, cured leaf yield and reduction in starch content.

Additionaly, to identify the mechanism of action of FA, molecular tools like Quantitative PCR and Next generation sequencing were used. Results indicate enhanced K^+ was due to overexpression of transporters (HAK, KUP), starch reduced due to downregulation of ADP glucose pyrophosphorylase gene and overexpression of starch degrading enzymes (amylases, glucanwaterdikinases and glucosidases). Improvement in leaf yield was possibly due to upregulation of genes involved in C&N fixation (RuBisCO, Nitrate reductase), The aforementioned agronomic and molecular evidences reveal that foliar application of FA is a viable approach to improve the leaf quality of tobacco.

15. COMPARISON OF THE VOLATILE FLAVOR COMPOUNDS IN DIFFERENT TOBACCO TYPES BY DIFFERENT EXTRACTION METHODS. Jang-Mi LEE, Jeong-Min Lee, Chang-Gook Lee, Jin-Young Bock and Keon-Joong Hwang; KT&G Research Institute, Daejeon, South Korea

Traditional simultaneous distillation extraction(SDE) and solid-phase micro extraction(SPME) methods using GC/MS were compared for their effectiveness in the extraction of volatile flavor compounds from different tobacco leaves types(flue-cured, burley, oriental). The major volatile flavor compounds of flue-cured and burley tobacco were similar such as neophytadiene, solanone, megastigmatrienone isomers, β-damascenone and β-ionone. On the other hand, volatile flavor compounds such as norambreinolide, sclareolide were specifically identified in oriental tobacco. Each method was used to evaluate the responses of some analytes from real samples and standards in order to provide sensitivity comparisons between two techniques. Among three types of SPME fibers such as PDMS(Polydimethylsiloxane), PA(Polyacrylate) and PDMS/DVB (Polydimethylsiloxane/ Divinylbenzene) which were investigated to determine the selectivity and adsorption efficiency, PDMS/DVB fiber was selected for the extractions of the volatile flavor compounds due to its effectiveness. Besides parameters that might affect the SPME, such as the duration of absorption and desorption, temperature of extraction. The qualitative analysis showed that the total amount of volatile flavor compounds in SDE method (130 species) was much more than that in SPME method (85 species). SPME method was more efficient for all the highly volatile compounds than SDE method, but on the other hand, low-volatile compounds such as fatty acids or high-molecular hydrocarbons were detected in SDE method. SPME method based on a short-time sampling can be adjusted to favor a selected group compounds in tobacco. Furthermore this results could be used to estimate the aroma characteristics of cigarette blending by using a different type of tobacco with more effectiveness and convenience.

16. CLINICAL DEVELOPMENT OF BIOMARKERS OF BIOLOGICAL EFFECT FOR PRODUCT ASSESSMENT STUDIES. Frazer J LOWE[1], Audrey Richter[1], Karsta Luettich[1] and Antonella Bassi[2]; [1]British American Tobacco, Southampton, UK and [2]British American Tobacco, Rome, Italy

Further tools are needed to understand how consumers use novel tobacco products, how much of the chemical yield is absorbed by the body, and how to subsequently evaluate the biological effects and health risks of novel product use. Biomarkers of biological effect (BOBE) are being developed to provide an initial evaluation of the body's response to smoke constituent challenge. Previously, candidate BOBE were evaluated in a heterogeneous population to distinguish smokers from non-smokers (1). Further pilot studies were conducted to confirm previous data (Twins study) and to evaluate BOBE reversibility in participants enrolled on a smoking cessation programme (Reversibility study).

The Twins study was of cross-sectional design. Blood and 24-hour urine samples were collected from 22 pairs of Italian monozygotic twins, discordant for smoking. Several candidate BOBE were significantly elevated in the smoking twin, compared to their non-smoking counterpart including: urinary 11-dehydrothromboxane B2, 2,3-dinorthromboxane B2, 8-epi-prostaglandin F2α, plasma fibrinogen, white blood cell count, neutrophil and lymphocyte counts and heart rate (all $p<0.05$). These data confirmed

the findings of the previous study by Lowe *et al.* (1), and gave a sound rationale to investigate these candidate BOBE further in a reversibility study.

The Reversibility study was of longitudinal design. Blood and 24-hour urine samples were collected from 50 healthy smoking volunteers over a 6 month period of smoking cessation in Messina, Italy. 47 quitters maintained their non-smoking status throughout the study. Samples were analysed for numerous BOBE and white blood cell, neutrophil and eosinophil counts were all significantly different compared to baseline after 6 months ($p<0.05$). We therefore propose that these BOBE would be suitable candidates to complement the current BOBE panel proposed by Hatsukami *et al* (2).

References

1. Lowe FJ, Gregg EO, McEwan M. Evaluation of biomarkers of exposure and potential harm in smokers, former smokers and never-smokers. Clin Chem Lab Med. 2009;47(3):311-20.
2. Hatsukami DK, Benowitz NL, Rennard SI, Oncken C, Hecht SS. Biomarkers to assess the utility of potential reduced exposure tobacco products. Nicotine Tob Res. 2006;8(4):600-22.

17. THE USE OF CIGARETTE SMOKE DILUTION BY GLYCEROL AS A MEANS OF REDUCING SMOKERS EXPOSURE TO SMOKE TOXICANTS. Kevin G. McADAM[1], E.O. Gregg[2], C. Liu[1], D.J. Dittrich[1], M.G. Duke[1] and C.J. Proctor[1]; [1]GR&D Centre, Southampton, UK and [2]Consultant – ENI Limited, Towcester, UK

The Institute of Medicine encouraged the pursuit and development of potential reduced exposure products, tobacco products that substantially reduce exposure to one or more tobacco toxicants and can reasonably be expected to reduce the risk of one or more specific diseases or other adverse health effects. One possible approach for reducing exposure of smokers to cigarette smoke toxicants is to dilute mainstream smoke with glycerol.

We describe the development of a novel glycerol containing "tobacco-substitute" sheet, and its inclusion in experimental cigarettes at levels up to 60% w/w. Analysis of mainstream smoke from these experimental cigarettes showed reductions in the yields of most measured constituents. *In vitro* toxicological tests showed reductions in the activity of smoke particulates in proportion to the % smoke glycerol content. Human exposure to nicotine and some particulate phase compounds were reduced by 14% to 29%, as determined by filter studies and 24h urinary biomarker analysis. These results demonstrate that reducing exposure to some smoke toxicants is possible through incorporation of a glycerol bearing tobacco-substitute sheet in cigarettes.

18. REDUCTION OF NITROGENOUS AND PHENOLIC CIGARETTE SMOKE TOXICANT YIELDS THROUGH THE USE OF A TOBACCO TREATMENT PROCESS. Kevin G. MCADAM[1], C. Liu[1], Y. DeGrandpré[2], A. Porter[3], A. Griffiths[1], R. Voisine[2], F. Côté[2] and C. Proctor[1]; [1]GR&D Centre, Southampton, UK, [2]Imperial Tobacco Canada Ltd, Montreal, Quebec, Canada and [3]Montreal, Quebec, Canada

A tobacco treatment process has been developed to remove proteins and polyphenols from cut Virginia tobacco without affecting its bulk physical structure. Proteins and polyphenols

in tobacco leaf are thought to be precursors of some nitrogenous and phenolic smoke toxicants respectively. The first step of the process involves aqueous extraction of cut tobacco. The aqueous extract and tobacco fibre are then separated mechanically. The aqueous extract is treated with adsorbents (bentonite to remove proteins, and polyvinylpolypyrollidone to remove polyphenols) and then concentrated. The tobacco fibre is treated with a protease solution to reduce residual protein levels. Rinsing followed by a high-temperature treatment removes and deactivates the enzyme residue. The concentrated tobacco extract is then added back to the fibre and the resulting tobacco is dried to a moisture content suitable for cigarette manufacturing.

Compared with the untreated tobacco blend, a treated flue-cured blend had reduced levels (w/w) of protein nitrogen (59%), chlorogenic acid (33%), rutin (79%), scopoletin (78%) and caffeic acid (53%). Nicotine levels were found to be reduced by 12% while sugar levels increased by 16%. The treated tobacco was made into cigarettes using single segment cellulose acetate filters and with tar yields of 9 mg (ISO/FTC smoking conditions). Mainstream yields of 43 smoke toxicants were measured under ISO smoking conditions. Compared with control cigarettes, cigarettes with treated tobacco gave lower yields of tar (16%), nicotine (17%) and carbon monoxide (20%). Of the 43 toxicants analysed there were significant reductions for some nitrogeneous and phenolic components and also some increases for other constituents.

19. QUANTIFICATION OF MENTHOL PERCEIVED BY HUMAN ON CIGARETTE SMOKING. Sharad K METHA, Manoj K.Singh and S.V.Dhalewadikar; ITC R&D Centre, Peenya, Bangalore, India

Few methods are available in literature for determination of menthol in cigarette smoke by trapping on Cambridge filter pad (CFP) and subsequent extraction with solvent and further analysis by GC-FID/GC-MS. However none of the methods describe about the amount of menthol available in vapor phase and volume of trapping solvent.

A new method has been developed by trapping the mainstream smoke directly in isopropyl alcohol (IPA) with an internal standard in Dewar flask cooled at oC. Sample of cigarettes were smoked under ISO regime *i.e.* puff volume-35 ml, puff duration - 2(s) and puff interval-60(s).Quantification was done using in-house validated and accredited method for cigarette as per ISO 17025 using GC-FID with Carbowax column (30m*0.25mm id*0.25µm).

All method validation parameters are performed in mainstream smoke without Cambridge filter pad mimicking human smoking. There is excellent linearity over a concentration range (7.9-29 mg/Cig) with regression coefficient of 0.989 confirms good recoveries. Limit of detection - (0.13 mg/Cig), Limit of quantification - (7.9mg/cig). Different mentholated brands were analyzed using method and average menthol perceived by a smoker is 4.7-16.4% in a cigarette.

The method provides information about the working concentration range and to optimize the volume of trapping solution.

20. AN *IN VITRO* COMPARATIVE STUDY OF [^{14}C]-EUGENOL AND [^{14}C]-METHYLEUGENOL ACTIVATION AND DETOXIFICATION KINETICS IN HUMAN, MOUSE, AND RAT LIVER FRACTIONS. Emmanuel MINET[1], Gentile Daniela[2], Clive Meredith[1] and Eian D. Massey[1]; [1]British American Tobacco, Group R&D, Southampton, UK and [2]Charles River Laboratories, Edinburgh, UK

Introduction - Eugenol is a natural alkenylbenzene compound used in a variety of consumer products including Kretek cigarettes. There is limited evidence for the carcinogenicity of eugenol to experimental animals. However, *in vitro* tests for the genotoxic potential of eugenol have on occasion reported a positive result. In contrast, the structurally related alkenylbenzene methyleugenol is consistently reported as genotoxic and carcinogenic *in vitro* and *in vivo*. The absence of unequivocal translation of toxicity data obtained from animal models to human is a limiting factor for eugenol toxicity assessment.

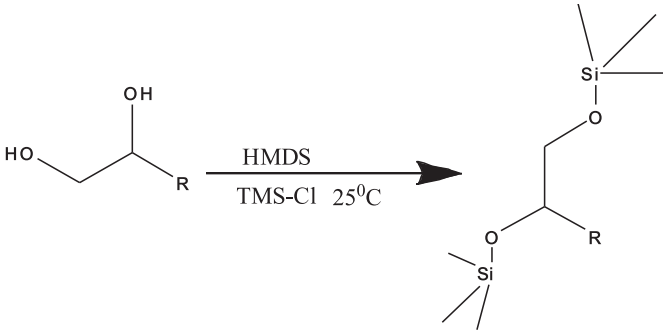
Objective - We decided to compare the bioactivation and detoxification kinetics of eugenol and methyleugenol in human, rat, and mouse to further assess their potential toxicity.

Method - The formation of a 1'-hydroxy proximate carcinogen, and a cytotoxic quinone methide, two key toxic metabolites for eugenol and methyleugenol, were quantified in hepatic microsomes and S9 fractions. Kinetic constants appKm and appVmax were measured for these reactions.

Results - We report that methyleugenol generates a significant amount of the 1'-hydroxy proximate carcinogen while eugenol glucuronidation prevents the formation of both 1'-hydroxyeugenol and the quinone methide. Comparative kinetics highlighted key metabolic differences between human, mouse, and rat, providing a mechanistic insight into the bioactivation and detoxification of alkenylbenzenes.

21. QUANTIFICATION OF 1,2 PROPYLENE GLYCOL, GLYCEROL, AND SORBITOL IN TOBACCO AND TOBACCO PRODUCTS. N. PALANI, Devaraj Sathish Kumar, Deepa Pillai and T.K. Dinesh; ITC R&D Centre, Peenya, Bangalore, India

Humectants have been largely used in Tobacco and Tobacco products for various applications and there are number of methods available in literature for quantification of these humectants (1). Although there are quiet number of methods, because of the complex matrix of Tobacco and Tobacco products the interference of other compounds present in the matrix makes the methods quiet difficult for quantification. In order to overcome the above difficulties there has been a lot of efforts in quantifying humectants using derivatization techniques. We have developed a simple derivatization technique using hexamethyldisilazane and trimethyl silylchloride for simultaneous quantification of all three humectants (2).



Our method is very simple and does not involve any sample preparation and clean up procedures. The recoveries are more than 90% for all the humectants.

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22. CELL TRANSFORMATION ACTIVITY OF CIGARETTE SMOKE CONDENSATE IN Bhas 42 ASSAY. Kamala PANT[1], Shannon W. Bruce[1] and C. Gary Gairola[2]; [1]BioReliance Corp Rockville, MD USA and [2]University of Kentucky Medical Center, Lexington, KY, USA

Cell transformation assays have been commonly used to predict chemical carcinogenicity. Using v-Ha-ras transfected BALB/c3T3 (Bhas42) cells, Sasaki and coworkers (2010, 2011) have recently validated a sensitive short-term assay that simulates the two-stage animal tumorigenesis model and measures tumor initiating and promoting activities of chemicals. In the present study, we used this assay to determine the cell transformation activity of cigarette smoke condensate (CSC) in Bhas42 cells. DMSO and water extracts of CSC (CSC-D & CSC-W, respectively) from the University of Kentucky reference cigarettes were prepared and tested at concentrations of 2.5μg to 40μg/ml in the initiator or promoter assay formats. The results of the initiator assay showed up to 3.5- fold increase in transformed foci at 40μg/ml of CSC-D but none by CSC-W. In contrast, a robust dose response (up to 14-fold increase) was observed in the promoter assay between 5μg to 40μg/ml of CSC-D and 20μg to 40μg/ml of CSC-W. Pre- and co-incubation of Bhas42 cells with selenium (100nM) significantly reduced CSC activity in initiator assay suggesting a role of oxidative stress in CSC-induced cell transformation. Co-treatment of cells with a sub-toxic dose of arsenate significantly enhanced cell transformation activity of CSC-D in promoter assay suggesting a synergistic interaction. The results suggest i) the presence of both water soluble and insoluble tumor promoters in CSC, ii) a role of oxidative stress in CSC-induced cell transformation, iii) a potential interaction of smoking with environmental metal pollutants in influencing neoplastic changes, and iv) a potential for application of Bhas-42 assay in initiator/promoter activity assessment of CSCs from different cigarettes.

23. THE EFFECTS OF MAINSTREAM CIGARETTES SMOKE COMPONENTS ON GENE EXPRESSION IN NCI-H292 CELLS. Takashi SEKINE, Yoko Sawamoto and Yasuo Fukano; Japan Tobacco Inc., Kanagawa, Japan

Cigarette smoke is a complex mixture of more than 4,000 components, and many different *in vitro* tests are currently used to assess the biological effects of cigarette smoke. The present study was carried out to determine the mainstream cigarette smoke component(s) associated with the expression of four genes: interleukin-8 (IL-8), heme oxygenase-1 (HO-1), matrix metalloprotease-1 (MMP-1) and the airway mucin MUC5AC.

First, the correlation factor was calculated using the correlative analyses between each value of gene expression relative to the vehicle control induced by 9 different kinds of sample cigarettes and the amount of each component in the smoke. Benzene, benzopyrene and catechol in PP and carbonyl components in GVP had a high correlation with the induction of the four genes. Next, we estimated the contribution of particulate phase (PP) and gas vapor phase (GVP). GVP induced IL-8, HO-1 and MMP-1 gene expressions more than PP. By contrast, MUC5AC was more strongly induced by PP than GVP.

When additional experiments were carried out on each individual chemical component by treating with each candidate component in the amount equivalent to that in test solution, the 3 candidate chemicals in PP didn't induce any of the four genes. In contrast, the mixture of carbonyls and acrolein alone induced each of the four genes

In summary, although we suggested that carbonyls are one component of cigarette smoke associated with the gene expression, they were only part of the effects of cigarette smoke. Furthermore, as there were differences in the contribution of PP and GVP in regards to the expression of each gene, it suggests the presence of the specific components that contribute to the expression of each gene.

24. A CUSTOMIZED SOFTWARE SOLUTION FOR TRACKING BIOLOGICAL SAMPLES IN CLINICAL AND BIOMARKER RESEARCH. Angela M. SLATER, T. Ryan Meadows, Bobbette A. Jones and G.L. Prasad; R.J. Reynolds Tobacco Company, Winston-Salem, NC, USA

Sample management is a critical step in clinical research. Because of the time, cost and complexity of conducting clinical studies, the integrity of the sample management process is vital. Accurate information on sample types, location and aliquot availability is important for effective sample management.

In clinical studies where biomarker discovery is a goal, various biological specimens (blood, urine, saliva, buccal cells) are collected from subjects as multiple aliquots. Subsequent analyses may occur in a phased manner at different bioanalytical laboratories. In a recent clinical study, a simple, cost-effective, customized software solution for tracking clinical samples (Track'Em™, RVB Systems Group) was developed and implemented.

Track'Em™, a barcode-based tracking system, was deployed at three locations: the clinical site, a collaborating academic laboratory, and RJRT. The clinical site enrolled 120 subjects into the study. Each subject was assigned a uniquely numbered lab kit (containing 58 bar-

coded vials and containers for collecting specimens). Collected samples were shipped to the academic laboratory. Both site and lab utilized scanners to electronically track the bar-coded vials/containers to verify sample collection, movement between locations, and storage locations. The three non-networked sites relayed information through emailed text files. These text files were imported into a Microsoft Access database. The master database, housed at RJRT, integrated data from all locations.

By study conclusion, approximately 7000 sample containers were successfully tracked. Track'Em™'s updatable feature verified accurate chain of custody between locations, allowed precise sorting of numerous sample types, and documented final storage location for efficient retrieval prior to shipment.

The customized Track'Em™ software effectively managed this critical sample inventory, allowing RJRT to store large numbers of biological specimens for current and future biomarker research analysis.

25. HIGHER YIELD PRODUCT SMOKING, ARE THERE YIELD ERRORS INHERENT WHEN USING A MECHANICAL SMOKING MACHINE? Ian TINDALL and Tim Mason; Ceulean, Milton Keynes, UK

Cigars when smoked using CRM#64/65 commonly give greater yields than cigarettes smoked under the corresponding ISO regime. Analytical cigar smoking is characterised by larger puff counts and an overall longer smoking time than the cigarette equivalent. It has been posited that pulling air and vapour phase through the Cambridge filter pad as it becomes increasingly loaded can result in reduced trapping efficiencies of the pad and so reduce the apparent yield of the product under test.

An examination of "breakthrough" was made for different yield products using two Cambridge filter pads in series. The influence of pad area on trapping efficiency and retention was further examined using the same system and the reduction in vapour loss through use of a small diameter pad, relative to the larger pad, plotted against nominal yield.

The effect of puffing (clearing puffs) on a pre-loaded Cambridge filter pad was examined for different loadings, different puff volumes and different pad diameters.

A recommendation for modification of the method used when smoking cigars is presented with an estimate of the apparent impact this will have on measured TPM. The consequences and applicability of these changes for Canadian method intense smoking are explored.

26. SINGLE-NUCLEOTIDE POLYMORPHISM FREQUENCY BETWEEN NICOTIANA TABACUM CULTIVARS. Yuri UM, Yi Lee and Yeong-Seon Seok; Department of Industrial Plant Science & Technology, Chungbuk National University, Chungbuk Cheongju, South Korea

We analyzed 163,000 genomic DNA sequences downloaded from Tobacco Genome Initiative database and assembled to 31,370 contigs and 60,000 singletons. Using relatively long contigs, we designed primer sets for PCR amplification. We amplified 61 loci from 24

cultivars and sequenced 57,660 bp of genomic DNA sequence of each variety. We found 7 significant single-nucleotide polymorphisms (SNPs) and 1 insertion-deletion (INDEL) from the sequences and we could classify the 24 cultivars to 10 groups. Group I has Hicks, BY4, Walker's Broadleaf, Lizard Tail Turtlefoot, Shirev, Greenwood Dark, and Little Crittenden, group II has PV-08, MSKF0102-37, Xanthi, and Goose Creek Red, group III contains Korean local varieties, Mokgicho and Hyangcho, and closest to group II. Burley varieties, KB103, VA528, and White Burley, were grouped together (group V) and South American varieties, Chilean tobacco and Perique tobacco, were grouped together with Iranian tobacco (group VII). Interestingly, Orient varieties, Xanthi, Basma, and Ismir, were not grouped together. SNP frequency of tobacco, 1/8,237 bp, was very low in comparison with those of other plant species, between 1/46 bp and 1/336 bp. For exact identification of tobacco cultivars, much more SNP markers should be developed. This study is the first try to identify SNP markers from tobacco cultivars.

27. DETERMINATION OF OXIDES OF NITROGEN (NOx) IN ANALYTICAL SMOKING PART 1: COMPLEXITIES IN CALIBRATION METHODS. James VINCENT, Tim Mason and Ian Tindall; Ceulean, Milton Keynes, UK

Regulatory demands for reporting of larger numbers of analytes in tobacco and tobacco smoke has rekindled interest in quantification of oxides of nitrogen (NO_x) in tobacco smoke. Confounding measurement accuracy is the shortened lifetime of free NO within the tobacco smoke vapour phase matrix and the relationship between experiment equipment design and the calibration process.

Various arrangements have been devised where by standard smoking machines have been converted to capture and analyse NO_x using bag systems but this approach has inherent compromises between accuracy and simplicity. In contrast the design of commercially available analytical smoking engines in a puff by puff manner were unsuitable for truly accurate measurements of NO_x so an optimised smoking platform was devised and built using the chemiluminescent method for NO/NO_x analysis.

The accuracy of measurement of calibration gas introduction at three points, at analyser, through vapour path and through whole smoke path were compared for a clean system and one where smoking had been conducted. The influence of vapour phase condensate within the system on the calibration and by inference the analysis of smoke vapour was investigated and an estimate formed of the error introduced by calibration by these three different paths.

28. COMPARISON OF THE TRANSCRIPTIONAL EFFECTS OF TWO HARM-REDUCED CIGARETTES. Michael J. WOLKOWICZ, Tatyana Kotova and Michael Timko; University of Virginia, Charlottesville, VA, USA

Tobacco use causes many deleterious effects on the human body, including lung cancer, the primary cause of death in the United States, and chronic obstructive pulmonary disease (COPD), the second ranked cause of U.S. death. Pharmacologically however, nicotine plays a minor role in the etiology of smoking-induced diseases and instead results in addiction to smoking and the reinforcement of the behavior. Due to increased public awareness and societal pressure over the harmful effects of cigarettes both governmental and private sectors

have initiated policies and products to lessen the deleterious effects of smoking or to aid in quitting. Two potential reduced-exposure products (PREPs) marketed to aide in smoking cessation are the supposed less hazardous "lite" cigarettes and infrequently studied, non-tobacco, nicotine-free herbal cigarettes. We examined the transcription of specific targeted groups of genes involved in either Signal Transduction or Stress and Cytotoxicity in normal cultured lung epithelial cells in response to two reduced-harm cigarettes. Lung epithelial cells were exposed to functionally equivalent smoke dosages from either a low-tar, reference tobacco cigarette (1R5F) or a non-nicotine cigarette manufactured from cocoa bean husks. Respective isolated mRNAs were utilized to assay any changes in gene transcription versus air-treated control cells in both cases. We discovered that while transcription of many genes remained similar, the two disparate types of cigarettes also generated changes in different genes. Specifically, the cocoa bean cigarette demonstrated decreased p53 pathway-associated TP53I3 gene transcription while the survival pathway-associated FN1 gene was up-regulated. In addition, at half the equivalent dose levels the oxidative and metabolic stress-related HMOX1 gene was induced two-old greater in the herbal cocoa bean cigarette.

29. APPLICATION RESEARCH ON FLUE-CURED TYPE CIGARETTE TECHNOLOGY BY TWICE CASING. YAN Keliang, Wu Yi, Zeng Xiaoying and Hu Weiyao; Technology Center of Hongyun Honghe Tobacco (Group) Co., Ltd., Kunming, PR, China

In order to research the application on flue-cured cigarette technology by twice casing (functional casing and brand casing flavor), based on obtaining of functional casing and technology optimization of addition by functional casing, the function mechanism on addition of functional casing was researched, meanwhile, application experiment and industrial verification on flue-cured cigarette technology by twice casing were investigated. The results showed that functional casing can be obtained by mixing three biological reagents at ratio of 2:1:2. Furthermore, orthogonal experiment showed that the effective is signature when the conditions were as follows: addition amount of functional casing is 0.07%, temperature is 20°C, 18% of moisture and time of storage tobacco leaf for 24h. In addition, addition of functional casing can degrade some substance on the surface of tobacco leaf by the enzyme in them to improve the quality of tobacco leaf. In the optimal conditions, comparing with one casing technology, the results showed that twice casing technology can not only improve comfort, reduce irritation and odor, but also effectively enhance the quality of cigarette.

30. MICROARRAY-BASED GENE EXPRESSION PROFILES OF ORAL CAVITY CELLS EXPOSED TO DIFFERENT TOBACCO PREPARATIONS. Wolfgang ZACHARIAS[1], Hong Gao[1] and G. L. Prasad[2]; [1]Dept. of Medicine, J.G. Brown Cancer Center, Univ. of Louisville, Louisville, KY and [2]RJ Reynolds Tobacco Co., Winston-Salem, NC, USA

Global gene expression profiles were determined for a panel of oral cavity cells in response to treatment by different tobacco preparations. Two human oral squamous cell carcinoma cell lines (101A, 101B) and normal gingival epithelial cells (HGEC) were treated with standardized cigarette smoke condensate (total particulate matter; TPM), smokeless tobacco extracted with complete artificial saliva (ST/CAS), or whole-smoke conditioned media (WS-CM). Doses of 30% cytotoxicity at 24 h treatment (EC-30) were applied to 3 biological replicates for each agent, using the respective solvents (DMSO, CAS, or media) as controls. Global transcriptome profiles were collected with Affymetrix HG-U133A v2.0

microarrays, and data analyzed with Partek and Ingenuity software.

Data mining using Ingenuity PathwayAssist revealed that the most prominently affected pathway was cell cycle, followed by cancer, cell death, DNA replication/repair, and cell movement. Venn diagrams were constructed to identify differential effects on gene expression in each cell caused by combustible (TPM, WS-CM) but not by smokeless (ST/CAS) tobacco products (101A, 65 genes; 101B, 116 genes; HGEC, 941 genes). Similarly, differential effects were identified for each agent elicited in malignant but not normal cells (TPM, 71 genes; ST/CAS, 1 gene; WS-CM, 13 genes), or elicited in normal but not malignant cells (TPM, 95 genes; ST/CAS, 45 genes; WS-CM, 224 genes). Top-ten gene lists based on fold-changes were diverse and showed little overlap across the different agents and cell types.

This comprehensive set of expression profiles is the basis for developing a mechanistic understanding of the effects and elucidating biological relevance of different tobacco preparations on oral cavity cells, and will be further interrogated to identify new candidates for biomarkers of exposure/effect to combustible or smokeless tobacco products.

MONDAY AFTERNOON, SEPTEMBER 19, 2011

SESSION A ***Session Chair: Florian Perini***

2:20 PM MONDAY

31. EFFECTS OF THE NUMBER OF LABORATORIES AND REPLICATES ON THE PRECISION OF MEASUREMENT METHOD. Thomas VERRON[1], Xavier Cahours[1], and Stephen W. Purkis[2]; [1]SEITA, Imperial Tobacco Group, Fleury-les-Aubrais, France and [2]Imperial Tobacco Limited, Bristol, UK

In an inter-laboratory comparison, different laboratories measure particular characteristics of a product. For cigarettes, smoke analyte yields in one or more homogeneous samples are measured under ISO-defined conditions. Inter-laboratory comparisons are used by groups intending to produce an analytical method to be used in a broader environment, such as international standards for a commercial interface; methods approved by regulatory bodies; official methods produced by reference committees or other types of international organisations, in this case the terms collaborative study or collaborative trial is currently used.

The required final results are the precision of the method in terms of repeatability and reproducibility. A sufficient number of participating laboratories is required to provide robust values. However, different organizations recommend different numbers of participating laboratories. For example ISO standards (ISO 5725-1, 1994) recommend 8–15 laboratories; IUPAC specify an absolute minimum number of 5 and AOAC recommend a minimum of 8. Different organizations also have different approaches towards the replication of experiments, for example, ISO does not specify the number of replicates.

Additional smoke constituent testing and regulation is likely in the future and further awareness and understanding of the statistical interpretation of generated data is required to facilitate testing based on sound scientific foundations. In this paper, we described the effects of the number of laboratories and replicates on the estimate of precision. We also examined the effect of the ratio SDR/SDr on the estimated confidence intervals in order to investigate the contribution of number of replicates on the reproducibility. We demonstrated that for a high ratio the minor improvement of within-laboratory precision, obtained by increasing the number of replicates, is totally diluted in the between-laboratory precision.

2:40 PM MONDAY

32. THE EFFECTS OF TRAPPING SOLUTIONS ON RECOVERIES OF CARBONYL COMPOUNDS IN MAINSTREAM CIGARETTE SMOKE. Kevin A. WILKINSON, Kristin H Bounds, Kathy E. Humphries and I. Gene Gillman; Enthalpy Analytical, Inc., Durham, NC, USA

The most commonly used method to determine carbonyl compounds in mainstream cigarette smoke involves derivatization using an acidic trapping solution of dinitrophenylhydrazine

(DNPH). The acid is later neutralized to form a stable hydrazone. These hydrazones are strong absorbers of UV light and are quantified using HPLC-UV/Vis.

Although this method is widely used to determine carbonyl compounds in smoke it is known that certain hydrazones, especially acrolein-DNPH, are unstable in trapping solution that is not neutralized. This could result in underreported but consistent measurements of carbonyl compounds in tobacco smoke if the time between sampling and neutralization is consistent from run to run.

In order to improve trapping efficiency and make the timing of neutralization less likely to interfere with measurement, we tested the stability of hydrazones in three different trapping solutions over one hour by neutralizing aliquots of the solution at regular time intervals after smoking. The trapping solutions were (1) DNPH in acidified acetonitrile, (2) solution 1 diluted in varying amounts in water, and (3) an acidic aqueous DNPH solution overlayed with toluene which allows for continuous liquid-liquid extraction of hydrazones into toluene.

We will present our recommendation for a trapping solution that gives both good recovery and stable hydrazones.

3:00 PM MONDAY

33. SETTING THE RECORD STRAIGHT – A REPLY TO GOODPASTER *ET AL.* – THE REAL CHEMISTRY AND TOXICOLOGY OF NOVEL SMOKELESS TOBACCO PRODUCTS. John H. LAUTERBACH[1] and Deborah A. Grimm[2]; [1]Lauterbach & Associates, LLC, Macon, GA USA and [2]Coordinated Instrumentation Facility, Tulane University, New Orleans, LA, USA

The assessment of the toxicological properties of contemporary and novel smokeless tobacco products (STP) on the US market can be challenging. The responses of the extracts of such products in common *in vitro* assays are generally marginal, if indeed, a positive response is obtained at all. Levels of the so-called GothiaTek analyses are often close to, if not below the levels found in Swedish snus. Therefore, any assessment of the toxicological properties of such STP requires a more complete knowledge of the product composition than can be obtained from routine analyses alone. One such analytical scheme was recently reported by Goodpaster *et. al.* [*J. Agric. Food Chem.*, 2011, 59 (6), pp 2745–2751]. Goodpaster used several GC-MS techniques to analyze semivolatiles and nicotine in novel STP; however, he only found a few of the compounds present in the samples. Moreover, he incorrectly used the Henderson-Hasselbalch equation to estimate the percent of nicotine in the products that was not protonated, and his assessments of the toxicological properties of the analytes he found were less than correct. We will present the results of our detailed GC-MS analyses of novel STP products including some studied by Goodpaster using techniques we reported at the CORESTA Smoke Science and Product Technology meeting in 2009 (SSPT 11). Our results showed that Goodpaster missed compounds of potential toxicological interest. We will also show why the Henderson-Hasselbalch equation is not satisfactory for use with novel STP, and we will provide a critique of Goodpaster's toxicological assessments of the ingredients he found in them.

3:20 PM *Break*

3:50 PM MONDAY

34. ANALYSIS OF MINOR ALKALOIDS IN TOBACCO I: PREPARATION OF STANDARD SOLUTION. Shun YAMAUCHI and Hiroyuki Yoshida; Japan Tobacco Inc., Leaf Tobacco Research Center, Tochigi, Japan

A collaborative study regarding analysis of minor alkaloids in tobacco was initiated by TSRC, and the results were reported in 2005. In this study, a mixed standard solution of 4 minor alkaloids (nornicotine, anabasine, anatabine, and myosmine) was used. When we verified the collaborative study, it was clear that the level of recovery of myosmine (Laboratory Fortified Blanks: LFB) was extremely low (around 30%). The preparation of the standard solution should be modified for the accurate quantification of myosmine.

When we performed individual analyses of the minor alkaloid standard reagents, we found that myosmine was also contained in the nornicotine standard reagent we used. We tested nornicotine standard reagents from several different manufacturers and found that they all contained myosmine.

When preparing the standard solutions above, using the improved TSRC collaborative method (ITCM), the recovery rate of nornicotine, anabasine, anatabine and myosmine in LFB were 100.3%, 100.2%, 96.8% and 103.4%, respectively; 84.8%, 99.3%, 95.7% and 105.3%, respectively, in the Laboratory Fortified Matrix (LFM) of burley; and 82.7%, 99.2%, 95.0% and 106.5%, respectively, in the LFM of flue-cured.

4:10 PM MONDAY

35. ANALYSIS OF MINOR ALKALOIDS IN TOBACCO II: RAPID ANALYTICAL METHOD BY GC/FID. Hiroyuki YOSHIDA and Shun Yamauchi; Japan Tobacco Inc., Leaf Tobacco Research Center, Tochigi, Japan

A collaborative study regarding the analysis of minor alkaloids in tobacco was initiated by TSRC and the results were reported in 2005. Under the TSRC collaborative method, it takes about 50 minutes per injection to analyse by GC/FID. As shown in the former report, the improved TSRC collaborative method (ITCM) requires two sets of standard solutions, mixed nornicotine, anabasine, anatabine standard solution and myosmine standard solution. Therefore, to analyze such a large number of samples, a substantial amount of time is required. We investigated whether a new rapid method of analyzing minor alkaloids using GC/FID was able to shorten the runtime while maintaining an equal level of accuracy and reproducibility compared to the ITCM.

The accuracy and reproducibility of the new rapid analysis method were equal to that of the ITCM in the quantification of nornicotine, anabasine, anatabine and myosmine and the equipment analysis time was shortened by about 50%, thereby doubling analysis capacity. Furthermore, as the regression analysis confirmed that the quantitative values from the new rapid analysis method were consistent with those of the ITCM, the new rapid analysis method showed a good robustness.

4:30 PM MONDAY

36. THE X200AH – A NEW GENERATION FULLY AUTOMATED SMOKING MACHINE. Chris CRAWLEY[1], Terry J. Chai[2] anjd Ming Hou[3]; [1]Axiom Select LLC, Richmond, VA, [2]Columbus, Ohio, USA and [3]Shanghai, China

The X200AH is a fully automated rotary smoking machine which has been designed to incorporate some features not found in existing smoking platforms. The 20-channel rotary smoking platform has been improved to reduce losses and make cleaning and access easier. The user interface has been substantially upgraded and improved. Hoffmann analysis is possible. A CO / CO2 analyzer is available. The flexible design permits research and routine analytical smoking.

Platform is designed to incorporate future upgrades combining linear and rotary advantages. Smoking results, using CM6 monitor cigarettes, are well within statistical limits for rotary or linear designs.

4:50 PM ADJOURN

MONDAY AFTERNOON, SEPTEMBER 19, 2011

SESSION B ***Session Chair: Anthony Gerardi***

2:20 PM MONDAY

37. A STRATEGY FOR MEASURING THE *IN VITRO* CYTOTOXICITY AND GENOTOXICITY OF TOBACCO SMOKE AEROSOL. Ken SCOTT, Debbie Dillon, Annette Dalrymple, Ian Crooks and Clive Meredith; British American Tobacco, Group Research and Development, Southampton, UK

The *in vitro* genotoxicity of tobacco smoke has traditionally been measured using its particulate phase. This is a useful surrogate for tobacco smoke, because its preparation has been standardised, *in vitro* exposure can be controlled and measured and it provides clear dose responses, which are sensitive to product changes. The limitations of particulate matter are that it does not include gas phase constituents, it is not an aerosol and it is not fresh. Recent developments have addressed these limitations. One approach has been to collect the gas phase as an aqueous extract and combine it with the particulate phase to test a reconstituted smoke extract. Another approach is to expose the cells directly to fresh whole smoke at an air-liquid-interface. The feasibility of air-liquid-interface exposures has also been demonstrated in other sectors (nano-particles, volatile organic compounds and automotive emissions). Its relevance for tobacco smoke will be studied with *in vitro* data from the Neutral Red Uptake, Ames and micronucleus tests as well as the TK mammalian mutation assay. The strategy behind further development of this approach for tobacco smoke includes the following steps.

1. Compliance with relevant *in vitro* testing guidelines, *e.g.* OECD. This relates to the choice of assay, cell line, metabolic activation, duration of exposure; and interpretation of results.
2. Validation of the *in vitro* methods, including repeatability, reproducibility and minimum significant difference.
3. Dosimetry for both particulate and gas phases, to demonstrate and quantify exposures.
4. GLP accredited data.

2:40 PM MONDAY

38. CYTOTOXIC EFFECTS OF DIFFERENT TOBACCO PREPARATIONS ON HUMAN PERIPHERAL BLOOD MONONEUCLEOCYTES. Subhashini ARIMILLI[1], Brad E. Damratoski[1] and G. L. Prasad[2]; [1]Department of Microbiology & Immunology, Wake Forest University School of Medicine, Winston Salem, NC, USA and [2]R.J. Reynolds Tobacco Company, Winston Salem, NC, USA

Acute exposure to cigarette smoke or its components has been known to trigger diverse cellular effects, such as oxidative stress, DNA damage and cell death in a number of cell culture models. However, available data regarding the potential cytotoxic effects of Smokeless Tobacco (ST) lacks consensus. This research was initiated to investigate the

relative biological effects of ST, and whether ST elicits differential biological responses compared to the combustible tobacco preparations.

Total Particulate Matter (TPM) and whole smoke conditioned medium (WS-CM) were prepared from 3R4F reference cigarettes, while 10% 2S3 reference moist tobacco extract was prepared in artificial saliva with enzymes (ST/CAS). Human peripheral blood mononuclear cells (PBMCs), isolated from non-tobacco consumer donors, were used as a model to examine the systemic effects of exposure to the tobacco preparations and nicotine, in short-term cell culture.

Corresponding EC_{50} values, normalized for nicotine content of the tobacco preparations, suggest that combustible tobacco preparations induced markedly higher cytotoxicity as follows: WS-CM ≥ TPM >> ST/CAS > nicotine. WS-CM and TPM similarly induced time-dependent cytotoxicity in PBMCs. While all three tobacco preparations induced detectable levels of DNA damage in a dose-dependent manner, the combustible tobacco preparations were significantly more potent than ST/CAS. Further, the leukocyte subsets exhibited differential cytotoxicity when exposed to combustible tobacco preparations in the following order: T helper cells > cytotoxic T cells> monocytes > NK cells. In contrast, the treatment with nicotine and ST/CAS was less cytotoxic and did not exhibit cell type selectivity.

Thus, the data presented herein suggest that in terms of nicotine content/unit, the combustible tobacco preparations are markedly more cytotoxic than ST/CAS or nicotine to human PBMCs.

3:00 PM MONDAY

39. ASSESSMENT OF NICOTINE METABOLITES AND SPECIFIC CIGARETTE SMOKE CONSTITUENTS IN HUMAN URINE. Mi JANG, Han-Jae Shin, Hyung-Seok Lee, Hak-Chul Hyun, Hyung-Ok Sohn and Yong-Ok Kim; KT&G Research Institute, Daejeon, Korea

Recently, US FDA has issued a regulation regarding MRTP (modified risk tobacco products) applications and a demand for the study on the assessment of risk reduction is increased. The measurement of changes in biomarkers for cigarette smoke is reliable and relevant approach for assessment of MRTP in human. Biomarkers of exposure are constituents or metabolites that are measured in a biological fluid such as urine.

Among them, nicotine and five major metabolites in urine are highly specific markers and routinely used for assessing tobacco smoke exposure. 3-HPMA(3-hydroxypropylmercapturic acid) and MHBMA(monohydroxybutenyl-mercapturic acids) are major metabolites of acrolein and 1,3-butadiene, respectively. Also mutagenicity of smoker's urine has been reported higher than that of nonsmoker's urine. These metabolites used as biomarkers of exposure to specific cigarette smoke constituents.

In this study, we assessed the nicotine metabolites level and specific cigarette smoke constituents biomarker. Using urinary sample of volunteers, biomarkers such as nicotine

metabolites, 3-HPMA, MHBMA and urine mutagenicity were evaluated by LC-MS/MS and urine mutagenicity assay, respectively. As a result, selected biomarkers such as acrolein and 1,3-butadiene have a correlation with nicotine and nicotine metabolites.

3:20 PM ***Break***

3:50 PM **MONDAY**

40. AN EVALUATION OF CEMA, AND ADDUCTS OF CYSTEINYLGLYCINE AS POTENTIAL BIOMARKERS OF EXPOSURE TO ACRYLONITRILE, FORMALDEHYDE, AND ACETALDEHYDE FROM CIGARETTE SMOKE. Emmanuel MINET[1], Francis Cheung[1], Graham Errington[1], Katharina Sterz[2] and Gerhard Scherer[2]; [1]British American Tobacco, Group R&D, Southampton, UK and [2]Analytisch-Biologisches Forschungslabor GmbH, Munich, Germany

Introduction: The WHO Study Group on Tobacco Product Regulation recommended a list of toxicants in mainstream smoke of cigarettes for mandated and recommended lowering. This list comprises 18 tobacco smoke constituents, including acrylonitrile, acetaldehyde, and formaldehyde. A reduction in exposure to those toxicants, through the use of new filters and tobacco technologies, can be assessed with suitable biomarkers of exposure measured in biofluids such as urine. However, no biomarkers for the smoking-related exposure to formaldehyde and acetaldehyde are available, whilst the dose response of cyanoethylvaline (CEMA), a urinary acrylonitrile biomarker has not been thoroughly characterized.

Objective: The objective of this project was to develop analytical methods for the determination of urinary CEMA, 2-methyl-thiazolidine-4-carbonyl-glycine (MTCG) (potential acetaldehyde biomarker), and thiazolidine-4-carbonylglycine (TCG) (potential formaldehyde biomarker), and evaluate the dose-response correlation with urinary nicotine.

Method: LC-MS/MS methods were developed, validated, and applied to a urine sample series obtained from non-smokers and smokers of 1 mg, 6 mg, and 10mg ISO-tar yield cigarettes. Nicotine and five of its metabolites were quantified to establish correlations with CEMA, TCG, and MTCG.

Results: The analytical method used for the determination of CEMA is sufficiently sensitive and specific to detect differences between smokers and non-smokers. Furthermore, urinary CEMA show a clear dose-response relationship to urinary nicotine. MTCG (0.72±0.45 ng/ml) and TCG (13.76±9.58 ng/ml) were detectable in human urine, but no correlation could be established with nicotine measured in the urine of smokers. The high TCG and MTCG background observed in non-smoker urine indicates the interference of possible confounding factors.

4:10 PM MONDAY

41. THE USE OF ENDPOINTS IN *IN VITRO* MODELS OF CARDIOVASCULAR DISEASE EXPOSED TO HUMAN SMOKERS' SERUM AS POTENTIAL BIOMARKERS OF SMOKING-RELATED CARDIOVASCULAR DISEASE. Ian M. FEARON, Karina McQuillan, Mark Taylor, Tony Carr, Olivia Mayland, Katherine Hewitt, Karsta Luettich and Frazer Lowe; British American Tobacco, Group R&D, Southampton, UK

Introduction: Cigarette smoking is a recognised cardiovascular disease (CVD) risk factor. *In vitro* models, in which cells are exposed to particulate or aqueous cigarette smoke extracts, are used to study CVD mechanisms and to examine the role of smoking in disease development. Here we explore the use of human sera as a more biologically-relevant exposure model in studies monitoring a number of CVD endpoints.

Methods: Human umbilical vein endothelial cells were grown to confluency and a scratch wound created with a pipette tip. Cells were exposed for 24 hours to 50% serum taken from 10 healthy smokers or non-smokers. Migration was monitored using IncuCyte imaging apparatus. After 24 hours, media and cells were harvested for gene and protein expression analysis using the TaqMan and MesoScale Discovery (MSD®) platforms, respectively.

Results: 8 hours after wounding, endothelial migration rate in cells exposed to smokers' sera was 20.4±18.6μm/hour. This was significantly increased ($P<0.05$, unpaired Student's t test) in cells exposed to non-smokers' sera (38.2±10.2μm/hour). Exposure to smokers' sera caused a significantly greater secretion of both MCP-1 (6105±849pg/ml) and IL-6 (446±121pg/ml), compared to cells exposed to non-smokers' sera (MCP 1, 4195±380pg/ml and IL 6, 210±21pg/ml). MCP 1 and IL 6 gene expression levels were also greater in cells exposed to smokers' sera. In addition, smokers' sera caused significantly greater expression of genes for IL1-R1, TGFβ1 and VEGFA.

Conclusions: Smokers' sera impaired endothelial migration and increased expression of a number of genes/proteins associated with CVD, when compared with non-smokers' sera. We propose that serum is a relevant *in vitro* exposure agent to examine the effects of cigarette smoke on CVD processes and that this approach may provide additional biomarkers of cardiovascular disease risk in smokers.

4:30 PM MONDAY

42. CLINICAL STUDIES TO INVESTIGATE THE INFLUENCE OF REDUCED TOXICANT PROTOTYPE CIGARETTES ON LEVELS OF BIOMARKERS OF EXPOSURE AND BIOLOGICAL EFFECT IN HEALTHY SMOKERS. Jim SHEPPERD, A.C. Eldridge, K. McAdam and C.J. Proctor; British American Tobacco, GR&D, Southampton, UK

We have conducted a study that addresses the first part of the IOM's definition of a PREP by analyzing biomarkers of exposure (BoE) in smokers switched to prototype cigarettes which produced lower levels of some smoke toxicants compared to conventional cigarettes.

The 6-week study involved short periods of clinical confinement. One hundred smokers of 6mg ISO tar cigarettes were recruited. After 2 weeks smoking a commercial 6mg ISO

tar yield product, 50 were switched to a 6mg ISO tar Reduced Toxicant Prototype (RTP) cigarette containing diluent sheet in the blend, and a high activity charcoal filter. In addition, 150 smokers of 1mg ISO tar commercial cigarettes were also recruited. 50 of these subjects were switched to a 1mg ISO tar yield RTP containing the diluent sheet, and a three stage filter containing high activity carbon and an amine functionalized resin material; 50 to an RTP containing tobacco treated to reduce polyphenols and proteins, and the same three stage filter.

BoE, mouth level exposure to tar and nicotine were measured, and the sensory response to the product scored. The study found that switching to RTPs at both tar yields reduced levels of BoE for several smoke toxicants compared to conventional cigarettes. The reductions in BoE correlated well with machine measured reductions in smoke toxicant levels.

The next stage of RTP prototype testing is in planning and will include biomarkers of biological effect (BoBE) and an extended switching period of 6 months, to allow time for changes in these biomarkers to occur. The primary objective will be to determine whether longer term use of an RTP results in continued exposure reduction and a reduction in BoBE.

4:50 PM ADJOURN

TUESDAY MORNING, SEPTEMBER 20, 2011

SESSION A ***Session Chair: Colin Fisher***

8:50 AM TUESDAY

43. ADOPTION OF STRIP-TILLAGE SYSTEMS FOR DARK-FIRED TOBACCO PRODUCTION IN WESTERN KENTUCKY. Andy BAILEY; Univ. of Kentucky/Univ. of Tennessee, UKREC, Princeton, KY, USA

Strip tillage production has increased each year since 2007 and currently accounts for at least 15% (1325 Ha) of the total dark tobacco production in Kentucky and Tennessee. Strip tillage is somewhat of a hybrid between traditional conventional tillage and no-tillage where tobacco is transplanted into cultivated strips 30 to 40 cm wide while the remainder of soil surface is uncultivated. Strip tillage should provide many of the benefits of no tillage production, such as soil and water conservation, fuel and labor savings due to reduced field preparation, reduced delays in post-transplant field operations following rains, and cleaner tobacco at harvest. Phosphorus and potassium fertilizer can also be applied as band applications as strips are being made, and research with burley tobacco has shown that recommended application rates can be reduced by one-third if these nutrients are applied as band applications. On most soils in western Kentucky, the use of a powered tiller implement designed to match the strips is also recommended just prior to transplanting. Field research trials with grower cooperators has been conducted since 2008 with the following objectives: 1) evaluate potential costs and labor savings in land preparation between strip-tillage and conventional tillage systems; 2) compare crop performance in different tillage systems; 3) evaluate potential for reduced phosphorus and potassium use with banded applications compared to broadcast; and 4) evaluate any other potential benefits to strip tillage compared to traditional, conventional tillage systems. Results of this field research suggest a 30 to 50% reduction in land preparation costs, as well as possible yield benefits in dry seasons. Additional results of this research will also be discussed.

9:10 AM TUESDAY

44. INVESTIGATION OF TOBACCO BLACK SHANK RESISTANCE CONFERRED BY AN INTROGRESSED N. RUSTICA GENOMIC REGION. Katherine E. DRAKE and Ramsey S. Lewis; Crop Science Department, N.C. State University, Raleigh, NC, USA

Black shank, caused by *Phytophthora nicotianae*, is typically the most important pathogen affecting tobacco production in the United States. Genetic resistance offers the most attractive means of reducing economic loss due to this pathogen. Several recently commercialized flue-cured tobacco hybrids possess a gene designated as *Wz* that was derived from a Zimbabwean breeding line called 'WZ'. The genomic region carrying *Wz* was reportedly introgressed from *N. rustica*. The effect of this genomic region against multiple races of the black shank pathogen has not previously been investigated in an organized way. A doubled haploid mapping population was generated from a cross between WZ and black-shank susceptible cultivar, 'NC 55.' The population was evaluated for resistance in multiple field environments and after race-specific inoculations in growth chambers. The population was also genotyped with a large number of AFLP markers. Results suggest that

the introgressed *N. rustica* region has a large effect on resistance to race 0 and race 1 of *P. nicotianae*. A large number of AFLP markers of *N. rustica* origin were found to be linked to the black shank resistance factor. This genetic variation and associated markers may be of value for breeding for black shank resistance in U.S. tobacco.

9:30 AM TUESDAY

45. A DRAFT GENOME SEQUENCE OF PERONOSPORA TABACINARTHE TOBACCO BLUE MOLD PATHOGEN. David ZAITLIN; KTRDC, University of Kentucky, Lexington, KY, USA

Peronospora tabacina D.B. Adam (syn. *P. hyoscyami* de Bary) causes blue mold disease, a major foliar disease of cultivated tobacco in many parts of the world. *P. tabacina* is classified as a downy mildew, a group of biotrophic oomycetes that are closely related to photosynthetic brown algae and diatoms. Many downy mildews are destructive plant pathogens with the potential to cause severe crop disease and economic losses. Research conducted over the past decade has identified a diverse class of proteins, called effectors, secreted by plant pathogens from three different Kingdoms (bacteria, fungi, oomycetes) that act to suppress the host defense response. Oomycete effectors are secreted into the apoplast or from specialized structures called haustoria, and are then transported into the plant cell cytoplasm. The sequenced *Phytophthora* genomes are predicted to contain between 350 (*Ph. sojae, Ph. ramorum*) and >550 (*Ph. infestans*) cytoplasmic effector genes. The genome of *Hyaloperonospora arabidopsidis*, a close relative of *P. tabacina*, contains <150 predicted effector genes, however. The *P. tabacina* genome (~60 Mbp) has been sequenced at the University of Kentucky. The latest assembly consists of 35.9 Mbp of sequence in 2198 scaffolds (N50=51,722 bp). Bioinformatic searches (*ab initio* gene prediction and effector-specific searches) and comparative genomics against the *H. arabidopsidis* and *Pseudoperonospora cubensis* genomes will give an estimate of the number of *P. tabacina* effector genes and enable their isolation. *P. tabacina* is only the second downy mildew genome to be completely sequenced, and will add to our understanding of biotrophy and pathogenesis in these important pathogens. The final P. *tabacina* genome sequence will be made available to the research community via a BLAST server and a genome browser.

9:50 AM TUESDAY

46. SELECTION OF RESISTANCE TO MULTIPLE PATHOGENS IN TOBACCO ASSISTED BY MARKERS AND GREENHOUSE SCREENINGS. Natalia MARTINEZ, Robert D. Miller, Dandan Li and Glen Weinberger; Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY, USA

The use of molecular markers in combination with greenhouse screens provides an efficient method for identification of flue-cured and burley tobacco genotypes resistant to several diseases. The objective of this study was to compare the consistency of each method for testing resistance to black shank and black root rot, diseases caused by two distinct soil-borne fungal pathogens. The roots of four week-old seedlings were inoculated with each pathogen and at least two resistant and susceptible control lines were included for each pathogen trial. For black shank disease evaluations, plants were inoculated with either zoospores or chlamydospores and there were no observable differences between the disease development

within the genotypes tested. The majority of the lines were selected as being resistant or susceptible for each disease by marker-assisted or greenhouse screening alike. However, in the case of black shank, inoculation with the pathogen (*Phytophthora nicotianae* race 0) gave insight into the presence of genotypes that were likely to be segregating recombinants by showing signs of partial resistance, due to the polygenic nature of existing resistance to black shank. It was useful to include the pathogen screenings in order to confirm the marker selection and to recognize lower resistant gene frequency in some of the lines prior to final testing in field disease nurseries.

10:10 AM ***Break***

10:40 AM TUESDAY

47. EVALUATION OF BURLEY CULTIVARS FOR LEAF QUALITY. Robert D. MILLER; University of Kentucky, Lexington, KY, USA

Adverse weather conditions during recent curing seasons have often resulted in undesirable leaf color of burley tobacco at marketing. During the same period, consolidation of burley production into larger acreages has resulted in a decrease in the number of burley cultivars being utilized, with a large percentage of the crop comprised of varieties released within the last eight years. This has raised concern that these newer varieties may produce lower quality leaf than older varieties such as TN 90 and ms KY 14 X L8. To investigate this possibility, the grade indices (an indicator quality based on USDA grades historically used for price supports) of ten burley cultivars that were included in 18 variety trials were evaluated. The trials were conducted at Versailles and Lexington, Kentucky, and Greeneville and Springfield, Tennessee, from 2006 through 2010; randomized complete blocks with three replications were used for each test. After curing, all plots were separated into four farm grades and evaluated by USDA graders. A wide range in grade indices occurred between years and locations, with relatively small differences observed among varieties. The highest average grade index of 79, indicative of high quality for all varieties, was recorded in Lexington in 2008 while the lowest average index of 32, with all grades of all varieties deemed by the graders to be variegated, was observed in Greeneville in 2010. However, when averaged across all trials there was little difference among locations, with a range from 59 to 62. The average grade index among the ten varieties ranged from 57 to 61, with both TN 90 and ms KY 14 X L8 having an average index of 58.

11:00 AM TUESDAY

48. PRELIMINARY STUDY OF A METHOD TO VERIFY THE COUNTRY OF ORIGIN FOR RAW AND PROCESSED SAMPLES OF TOBACCO LEAF. Bob PEARCE, Arny Stromburg, Jason Unrine, Limin Feng and Colin Fisher; University of Kentucky, Lexington, KY, USA

There is a need for a method to independently verify the country of origin for leaf tobacco traded on the world market. The objective of this study was to determine the feasibility of using trace element content and linear discriminant analysis to correctly classify leaf tobacco samples according to their country of origin. Samples of re-dried burley tobacco leaf were collected from the United States (58), Malawi(20), and Brazil (19). The samples

were processed using a non-metallic grinding method and analyzed for 26+ elements including trace elements. Linear discriminant analysis was applied to the data and resulted in plots with three distinct groupings of points. Two additional samples were obtained for each of the three countries. Each of these six samples were prepared by two methods: 1) the nonmetallic method as above and 2) a typical metallic knife grinding mill. These additional samples were treated as unknowns to see if they could be correctly classified. Using the non-metallic grinding method all six unknowns were correctly classified as to their country of origin. When the typical grinding mill was used one of the six unknowns was incorrectly classified. Mixtures of samples were also tested to determine how blended tobaccos might influence the method. Two way mixtures generally plotted along a line between the two endpoints, while a three way mixture (1/3 from each country) plotted along a line between the Malawi and United States endpoints. This study has demonstrated that verification of origin is feasible by this method and further investigation is warranted.

11:20 AM TUESDAY

49. USING TRANSCRIPTION FACTORS TO REGULATE TOBACCO METABOLIC PATHWAYS. Ling YUAN; Kentucky Tobacco Research and Development Center and Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY, USA

Coordination of transcriptional control of biosynthetic genes has emerged as an effective approach for plant metabolic engineering. Such regulation can be achieved by specific transcription factors (TFs). We have isolated and characterized a set of tobacco TFs that are involved in flavonoid biosynthesis and seed-coat development. We have demonstrated that tobacco (*Nicotiana tabacum*) comprises two functional basic helix-loop-helix TFs (bHLH TFs) that have originated from the two ancestors, *N. sylvestris* and *N. tomentosiformis*, respectively. These bHLH TFs interact with a MYB-type TF to form a protein complex and regulate a subset of flavonoid biosynthetic pathway genes. Ectopic expression of these TF genes in transgenic tobacco induce whole-plant anthocyanin production and changes in seed-coat characteristics. Suppression of the TF genes have resulted in white-flower phenotypes. We have also demonstrated that the gene transactivation activity and DNA-binding specificity of the bHLH TFs can be modified and improved through protein engineering. Our results validate an effective approach to metabolic engineering of tobacco using TFs.

11:40 AM ***Lunch***

TUESDAY MORNING, SEPTEMBER 20, 2011

SESSION B ***Session Chair: Buddy Brown***

8:50 AM TUESDAY

50. EVALUATION OF THE PRECURSORS GENERATING HYDROGEN PEROXIDE IN AN AQUEOUS EXTRACT OF PARTICULATE PHASE CIGARETTE SMOKE. Yuichiro TAKANAMI; Japan Tobacco Inc., Yokohama, Kanagawa, Japan

It is well known that an aqueous extract of cigarette smoke generates reactive oxygen species. Our previous studies suggested that the hydrogen peroxide generated in such an extract was a possible source of hydroxyl radicals. In our fractionation study reported at TSRC 2010, fractions containing not only hydroquinone and catechol but also other smoke constituents, possibly phenolic compounds, generated hydrogen peroxide. In this study, an extract of smoke was subjected to triple-stage fractionation, and the selected fractions were analyzed using UPLC-TOF MS. In this analysis, the two catechols were found to be precursors for the generation of hydrogen peroxide. Then, the concentrations of seven phenolic compounds, including the two constituents, were analyzed in the extract of smoke. A model solution was prepared by mixing the seven phenolic compounds in their corresponding concentrations. When the hydrogen peroxide generated in the model solution was analyzed, the solution generated a higher amount of hydrogen peroxide than the solution containing only hydroquinone and catechol. This indicated that the five phenolic compounds other than hydroquinone and catechol contributed to the generation of hydrogen peroxide. However, the amount of hydrogen peroxide generated in the model solution was significantly smaller than that generated in the extract of the smoke, which indicated that many other smoke constituents contribute to the generation of hydrogen peroxide.

9:10 AM TUESDAY

51. DIFFERENTIATION IN CYTOTOXICITY AND INFLAMMATORY EFFECTS OF TOBACCO FREE FATTY ACID-IRON COMPLEXES. Florian R. PERINI, Edward A. Robinson and Manoj Misra; Lorillard Tobacco Company, Greensboro, NC, USA

Free fatty acids (FFA) comprise 1% of a standard tobacco blend dry weight and transfer to smoke up to 5.9% of the smoke condensate. FFA combined with Fe may play a role in the biological damage produced by cigarette smoke. Our objective was to synthesize, isolate, characterize FFA-Fe complexes, and evaluate their individual biological activity. To assess the potential of these species to induce biological damage, we synthesized complexes from saturated FFA (palmitic, stearic) and unsaturated (oleic, linoleic), and determined their cytotoxicity and inflammatory cytokine levels. The complexes were prepared from dichloromethane-water mixtures and isolated from the organic layer prior to bioassays. Cyclic voltammetry and ultraviolet spectroscopy confirmed the formation of uncharged Fe(III) complexes, establishing a 3:1 FFA:Fe ratio. Further characterization was carried out. FFA and their complexes were tested in a 2.5-20 μM range for biological evaluations. Neutral red dye uptake in cells, interleukin-8 (IL-8), and 8-iso-prostaglandin measurements in a cell supernatant were determined over 7 days in immortalized normal human lung cells (BEAS-2B). Iron as iron ascorbate did not induce cytotoxicity, but effected inflammatory

endpoints. Both oleic and linoleic acids were toxic in our system at ≥ 10 μM, but palmitic and stearic acids did not produce significant toxicity. All FFA induced IL-8 and 8-iso-prostaglandin release in a dose-dependent manner. However, the levels released from the FFA-Fe complexes were significantly greater than from either of the pure components alone. The stearic acid-iron complex, the least cytotoxic FFA tested, produced the highest inflammatory response. Our study demonstrates that FFA-Fe complexes display significant toxicological effects. The differential response between saturated FFA and unsaturated FFA complexes indicates that their biological effects may be modulated by the FFA ligand structure.

9:30 AM TUESDAY

52. PUFF-RESOLVED ON-LINE REAL-TIME ANALYSIS AND QUANTIFICATION OF TOBACCO SMOKE COMPONENTS BY A COMMERCIAL SMOKE PROFILER (SMOKING MACHINE – PHOTO-IONIZATION TOF MASS SPECTROMETER SYSTEM). Ralf ZIMMERMANN[1], Nils Rose[2], Andreas Walte[3], Mohammad Saraji-Bozorgzad[3], Matthias Bente[3], Thomas Gröger[4] and Markus Eschner[4]; [1]University of Rostock, Germany, [2]Borgwaldt KC, Hamburg, Germany, [3]Photonion GmbH, Schwerin, Germany and [4]Helmholtz Zentrum, München, Germany

Recently, laser based photo-ionization mass spectrometry has been successfully coupled to smoking machines, allowing the puff-resolved on-line analysis of volatile and semi-volatile tobacco smoke constituents [1]. The introduction of special incoherent VUV-light sources for photo ionization mas spectrometry then allowed the construction of more reliable and easy to use systems [2] and finally led to commercial photo ionization mass spectrometers (Photonion, Schwerin, Germany) as well as to a commercial smoking machine - photo ionization TOF mass spectrometer system (Borgwaldt KC, Hamburg, Germany).

In order to get quantitative puff-resolved results, the photo ionization cross-sections were measured using a gas chromatography (GC) - photo ionization TOF mass spectrometer (MS) system. In addition isobaric mass spectrometric interferences were evaluated using GC/MS data. Based on this data, the results obtained of the commercial smoking machine - photo ionization TOF mass spectrometer system can be quantified. This is demonstrated by comparing the on-line measurement results of different cigarettes, including reference cigarettes, with literature/reference data.

[1] T.Adam, R.R.Baker and R.Zimmermann, Characterization of puff-by-puff resolved cigarette mainstream smoke by single photon ionization-time-of-flight mass spectrometry and principle component analysis, *J. Agr. Food Chem.* 55 (2007) 2055-2061

[2] L. Hanley, R. Zimmermann, Light and Molecular Ions: The Emergence of Vacuum UV Single-Photon Ionization in MS, *Anal. Chem.* 81 (2009) 4174–4182

9:50 AM TUESDAY

53. DETECTION OF THE MOLECULAR COMPOSITION OF PYROLYSIS GASES IN THERMAL ANALYSIS (TA) USING PHOTO IONIZATION TOF MASS SPECTROMETRY FOR EVOLVED GAS ANALYSIS (EGA): INSTRUMENTAL SET-UP AND FIRST RESULTS ON TOBACCO AND CIGARETTES MATERIALS. Ralf ZIMMERMANN[1], Mohammad Saraji-Bozorgzad[2], Matthias Bente[2], Andreas Walte[2] and Markus Eschner[3]; [1]University of Rostock, Germany, [2]Photonion GmbH, Schwerin, Germany and [3]Helmholtz Zentrum München, Germany

Evolved Gas Analysis (EGA) in Thermogravimetry (TG) by mass spectrometry (MS) is a powerful technology for analysis of thermal degradation products from organic materials such as polymers, bio mass, paper or tobacco. TG-MS with electron ionization (EI) gives predominately information on the evolved small molecules such as CO, CO_2, or H_2O. Complex organic molecules, however, often are not detectable due to the fragmentation associated with EI. Mass spectrometry using single photon ionization (SPI-MS) now allows the analysis of the evolved pattern of intact organic molecules in conjunction with thermal analysis (TA) data. In this work the coupling of thermal analysis (TG and differential calorimetry, DSC) with SPI-TOFMS is described [1]. The SPI approach used for the TG-SPI-MS coupling is based on the innovative incoherent EBEL VUV-light source (**E**lectron **B**eam pumped rare gas **E**xcimer **L**ight source). The VUV-light emitted by the EBEL is focused into the ion source of an ultra-compact orthogonal-acceleration-TOFMS (oaTOFMS). The new EBEL-SPI-oaTOFMS instrument achieves detection limits for organics in the low ppb region. In addition to the technology, applications on different materials are presented. This includes polymers, tobacco, cigarette filter material as well as cigarette paper. Further applications in the field of tobacco science are motivated and discussed

[1] R. Geißler, M. R. Saraji-Bozorgzad, T. Gröger, A. Fendt, T. Streibel, M. Sklorz, B. M. Krooß, K. Fuhrer, M. Gonin, E. Kaisersberger, T. Denner, R. Zimmermann, *Anal. Chem.* 81 (2009) 6038-6048

10:10 AM ***Break***

10:40 AM TUESDAY

54. EFFECT OF SUGAR CONTENT ON ACETALDEHYDE YIELD IN CIGARETTE SMOKE. Xavier CAHOURS[1], Thomas Verron[1] and Stephen W. Purkis[2]; [1]SEITA, Imperial Tobacco Group, Fleury-les-Aubrais, France and [2]Imperial Tobacco Limited, Bristol, UK

The relationship between cigarette blend sugar and acetaldehyde formed in its smoke is a matter of current regulatory interest. Some tobacco scientists have used data by Phillpotts [1] to illustrate that cigarette smoke yields of acetaldehyde are independent of inherent or added sugar levels in the parent tobacco blends. Some tobacco control advocates (O'Connor and Hurley [2]) have recently interpreted the data differently. Using the Phillpotts's data, O'Connor and Hurley recently applied a multivariate analysis to determine the relationship between acetaldehyde in smoke and sugar in tobacco taking into account the NFDPM yields. Indeed, a multivariate analysis enables the partial effect of different factors, especially product design, to be taken into account. With this analysis normalized for NFDPM, the

authors concluded that the sugar-acetaldehyde relationship had a coefficient of correlation of 34%. However, the multivariate analysis used by the authors is incomplete. Therefore, in order to avoid any misleading conclusions, we decided to carry out a multivariate analysis (General Linear Model (GLM)) taking into account all the factors (Sugar, NFDPM, FP and Country). Contrary to the O'Connor conclusion, our results have shown the sugar content does not have a significant impact on aldehyde yield.

In a second step, we have examined some data sets obtained in our laboratory between 2001 and 2010 on 99 commercial brands from the EU market. In our studies, a GLM analysis with the sugar factor nested in the country factor has shown no effect of sugar content on acetaldehyde yields whatever the country. Moreover, an analysis taking into account the blend style has also highlighted no relationship.

[1] D. F. Phillpotts, D. Spincer, D. T. Westcott. The effect of the natural sugar content of tobacco upon the acetaldehyde concentration found in cigarette smoke. *Beitraege zur Tabakforschung*, 8 (1975) 7
[2] R.J. O'Connor, P.J. Hurley. Existing technologies to reduce specific toxicant emissions in cigarette smoke. *Tobacco Control*, 18 (2008) 139.

11:00 AM TUESDAY

55. PYROLYZATE COMPOSITION FOR SEVERAL POLYMERIC CARBOHYDRATES.
Serban MOLDOVEANU; R.J. Reynolds Tobacco Co., Winston-Salem, NC, USA

A considerable number of natural organic polymers are carbohydrates. Many of these polymers are present in tobacco leaf (*e.g.* cellulose, pectin, and starch) and other are occasionally used in cigarette manufacturing as additives to reconstituted tobaccos or as various types of glue. Present study describes the results of pyrolysis of several polymeric carbohydrates and compares the pyrolyzate composition from one polymer to another and with the pyrolysis products of the monomeric units that form the polymer. The list of carbohydrate polymers evaluated in the study includes cellulose, starch, maltodextran, pectin, carrageenan, xanthan gum, locust gum, guar gum, gum Arabic, and sodium alginate. The momomeric compounds that were pyrolyzed include glucose, mannose, and galacturonic acid. Pyrolysis of each compound was performed in helium at 900°C in flash pyrolysis mode. Pyrolysis of cellulose in helium with 3% oxygen did not show any significant difference from the pyrolyzate in pure helium. The composition of the pyrolyzates was established using mass spectrometric detection, using a GC/MS system coupled with the pyrolyzer. The identification of the compounds in the pyrolyzates was performed using mass spectral library searches on NIST08 and Wiley275. The number of pyrolyzate components in each pyrolyzate is very high. However, only the largest 100 – 150 components of the pyrolyzates were identified, the rest of compounds being at very low or trace level. For the cases where the monomeric compound (*e.g.* glucose) was compared to the polymer, only small qualitative differences were seen between the pyrolyzates. Also, polymers such as cellulose, starch, and maltodextran showed small differences in their pyrograms. The pyrolyzates of carbohydrate polymers with different monomeric units showed significant differences among their compositions.

11:20 AM TUESDAY

56. SEPARATION OF VOLATILE AND SEMI-VOLATILE COMPOUNDS IN TOBACCO USING ON-LINE COMBINATION OF LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY. Shaofeng LIU, Junchao Bai, Junwei Guo and Jizhao Guo; Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou, China

A method of on-line combination of liquid chromatography and gas chromatography-mass spectrometry (LC-GC/MS) was established for analysis of volatile and semi-volatile compounds in tobacco. The separation mechanism of volatile and semi-volatile compounds was studied. An LC column of 250mm×1.0mm packed with 5μm amino-bonded silica was used as stationary phase for the separation. N-Hexane/dichloromethane/acetonitrile was used as mobile phase, using gradient elution, fifteen fractions were collected by twice separation, and then were analyzed by GC/MS. Comparing with direct GC/MS analysis of the same sample, the on-line combination of LC-GC/MS system was suitable for a complex tobacco sample, and has a higher reliability of qualitative analysis.

11:40 AM ***Lunch***

TUESDAY AFTERNOON, SEPTEMBER 20, 2011

SESSION A ***Session Chair: Lowell Bush***

1:30 PM TUESDAY

57. INFLUENCE OF NITROGEN FERTILIZATION RATE AND ALLELES AT THE YELLOW BURLEY LOCI ON TSNA FORMATION IN AIR-CURED TOBACCO. Ramsey S. LEWIS[1], Robbie Parker[1], Anne Jack[2], David Danehower[1], Scott Whitley[1] and Lowell Bush[2]; [1]Crop Science Department, N.C. State University, Raleigh, NC, USA and [2]University of Kentucky, Lexington, KY, USA

TSNA formation is influenced by alkaloid levels and the availability of nitrosating agents. Tobacco types differ in their potential for TSNA accumulation due to genetic and agronomic factors. Highest TSNA concentrations are typically exhibited by burley tobaccos. One of the main genetic differences between burley tobacco and all other tobacco types is that this tobacco type is homozygous for recessive mutant alleles at the *Yellow Burley 1 (Yb_1)* and *Yellow Burley 2 (Yb_2)* loci. In addition, this tobacco type is typically fertilized at higher N rates than most other tobacco types. In this study, we utilized nearly isogenic lines (NILs) differing for the presence of dominant or recessive alleles at the Yb_1 and Yb_2 loci to investigate the potential influence of genes at these loci on TSNA accumulation. Three pairs of NILs were evaluated at three different nitrogen fertilization rates for alkaloid levels, nitrogen physiology measures, and TSNA accumulation after air-curing. As previously observed by others, strong positive relationships were observed between nitrogen application rates and TSNA accumulation. Recessive alleles at *Yb1* and *Yb2* were associated with increased alkaloid levels, reduced nitrogen use efficiency, reduced nitrogen utilization efficiency, and increased leaf nitrate. Acting together, these factors contributed to significantly greater TSNA levels in genotypes possessing the recessive alleles at these two loci relative to those carrying the dominant alleles. The chlorophyll-deficient phenotype conferred by the recessive yb_1 and yb_2 alleles probably contributes in a substantial way to increased available nitrate during curing and, consequently, increased potential for TSNA formation.

1:50 PM TUESDAY

58. THE EFFECT OF ARTIFICIAL CASING METHODS ON TSNA ACCUMULATION. Colin FISHER, Anne Jack, Lowell P. Bush and Robert Pearce; Plant & Soil Sciences Department, University of Kentucky, Lexington, KY, USA

The accumulation of tobacco specific nitrosamines (TSNAs) increases as the moisture content of stored leaf increases. Take-down of air-cured tobacco prior to stripping is done when the leaf is in case after a period of high humidity, most often following rain. During extended periods of low humidity, when the leaf is too dry to handle, growers use artificial methods of casing, such as steam, misting or spraying. An experiment was done to compare the effect of these three artificial casing treatments with natural casing on TSNA accumulation.

Four treatments were assigned to plots of a high converter selection of TN 90 before harvest in a randomized complete block design with four replications. At the end of curing

when the cured leaf was naturally in case after a period of high humidity, leaf samples were taken for moisture content determination and TSNA accumulation. The naturally cased treatments were immediately packed in the centre of a bundle of border plants in an airtight polyethylene bag. The tobacco for the artificial casing treatments were placed in a low humidity environment overnight before the artificial casing treatments were applied. Samples of each treatment were taken immediately prior to bulking and after fourteen days. Data loggers recorded temperature and humidity data in each bulk. The moisture content at bulking and after 14 days varied considerably between casing methods, and this was reflected by the equilibrium relative humidity.

TSNA data showed overall low levels of accumulation due to unfavorable curing conditions, difference between treatments were minimal.

2:10 PM TUESDAY

59. CONTRIBUTIONS OF THREE NICOTINE DEMETHYLASES TO NICOTINE AND NORNICOTINE COMPOSITION IN THE PLANT. Bin CAI[1], F. Fannin[1], R. Lewis[2], R. Dewey[2] and L. Bush[1]; [1]Dept of Plant and Soil Sciences, University of Kentucky, Lexington, KY, USA and [2]Dept of Crop Science, North Carolina State University, Raleigh, NC, USA

Nicotine is synthesized in the tobacco root and translocated to the leaf where it may be demethylated to nornicotine. Three functional nicotine demethylases (CYP82E4, CYP82E5 and CYP82E10) have been identified in tobacco to date. CYP82E4 (E4) is the major demethylase and mainly functions in senescent leaf. Expression of *CYP82E10* (E10) is reported to be in the roots and CYP82E5 (E5) functions in both root and leaf.

In tobacco leaf, nicotine is comprised of 0.2 % of (R)-nicotine, but the nicotine metabolite, nornicotine, consists of 4-75 % of (R)-nornicotine. The reasons for this discrepancy between nicotine and nornicotine enantiomeric composition is not known. Previously, we have confirmed the preference of E4 for (R)-nicotine *in vitro* which can explain why a high (R)-nornicotine content could result from a low (R)-nicotine percentage. In this study, our goal was to evaluate the role of each demethylase in nicotine and nornicotine composition in root and leaf tissue.

Two sets of grafts were generated from nicotine demethylase tobacco mutants and tomato. First set of grafts were generated from nicotine demethylase tobacco mutants and tomato. These grafts were with a tomato (scion) on a tobacco mutant root stock. The second set was grafts with nicotine demethylase tobacco mutants (scion) on the triple mutant root stock. After recovery, the grafts were topped to stimulate nicotine production and then analyzed for nicotine and nornicotine composition. The results indicate that (1) in the root, originally synthesized nicotine may consist of 3% (R)-nicotine; (2) E5 and E10 selectively demethylate (R)-nicotine to (R)-nornicotine in root, before translocation of nicotine to leaf and (3) in leaf, E4 demethylates (S)-nicotine into (S)-nornicotine during senescence.

2:30 PM TUESDAY

60. DEVELOPMENT OF DNA MARKERS FOR NIC1 AND NIC2 GENES REGULATING NICOTINE BIOSYNTHESIS IN TOBACCO. Dandan LI and Robert D. Miller; Dept. of Plant and Soil Sciences, Univ. of Kentucky, Lexington, KY, USA

Tobacco (*Nicotiana tabacum*) synthesizes nicotine and related pyridine alkaloids in the root, and their synthesis increases on the leaf. Two regulatory NIC loci that positively regulate nicotine biosynthesis have been genetically identified, and their mutant alleles have been used to breed low-nicotine tobacco varieties. The objective of this study was to develop efficient, user-friendly DNA markers to identify the NIC1 and NIC2 mutations and quickly introgress the desired NIC1 and NIC2 mutations into commercial varieties by using marker-assisted selection to reduce nicotine levels in tobacco leaves. DNA markers NIC2-179 were found for the NIC2 locus, originally called locus B. Polymorphic AFLP (Amplified Fragment Length Polymorphism) markers were identified for the NIC1 locus, originally called locus A. These markers were tested on the F2 populations from the cross BA21 X LA21, HI X BA21 and LI X BA21, respectively. The F2 plants were harvested and air-cured. Samples were ground and analyzed for alkaloid. These markers can be used in marker-assisted selection programs to quickly introgress the desired NIC1 and NIC2 mutations into commercial varieties to reduce nicotine levels in tobacco leaves.

2:50 PM ***Break***

3:20 PM TUESDAY

61. PERSISTENCE OF TOBACCO SEED IN THE ENVIRONMENT. Richard MUNDELL, James O'Daniel and Orlando Chambers; University of Kentucky, Lexington, KY, USA

The future field-based production of transgenic tobacco for traditional or new applications will rely on robust containment measures based on sound risk assessment. Information concerning the persistence of tobacco seeds in the environment is an essential contributor to that risk analysis. Using three different commercial tobacco cultivars we examined the viability of seed which had been buried at four different soil depths in the field, sampling annually over a 6-year period. Our findings indicate that tobacco seed can persist in the natural environment over this time frame, and suggest that monitoring for volunteer plants should extend beyond 1 year following the production of transgenic tobacco outdoors.

3:40 PM TUESDAY

62. FIELD EVALUATION OF AN INTERSPECIFIC HYBRID AS A CONTAINMENT STRATEGY FOR PLANT-MADE PHARMACEUTICAL APPLICATIONS IN TOBACCO. Orlando CHAMBERS[1], J. Hollis Rice[2], Richard Mundell[1], Reginald J. Millwood[2], C. Neal Stewart[2] and H. Maelor Davies[1]; [1]Kentucky Tobacco Research & Development Center, Lexington, KY, USA and [2]University of Tennessee, Knoxville, TN, USA

Biotechnology allows plants to be utilized for the production of non-native pharmaceutical and industrial materials with tobacco being the most commonly exploited plant for these applications. Genetic containment is a concern for these plant expression technologies

because of strict USDA regulations requiring no outcrossing with commercial tobacco and no unauthorized release of transgenic seed. An interspecific cross between *N. tabacum* and *N. glauca* was tested to assess the level of sterility for contained field production.

Parental lines were engineered for expression of green fluorescent protein (GFP) and homozygous lines were crossed to produce the hybrid. A modified Nelder-wheel with curved, radial arms was utilized for the field test design. Fifty hybrid plants and fifty fertile *N. tabacum* plants were grown in the center of the plot to evaluate the sterility of the hybrids. Groups of five male-sterile plants (cv. MSTN 90) were placed at specified distances from the center plot to measure the outcrossing potential of the hybrid.

Mature seed pods were collected from both the hybrids and the MSTN 90 plants. A small percentage of the hybrid flowers produced mature seed pods and most of them did not contain any seeds. The few seeds that were produced either did not germinate, or if they did germinate, the seedlings lacked vigor and often perished. Pods collected on the MSTN 90 plants produced more viable seed but less than .0006% of the germinated seedlings expressed GFP.

The results suggest that although the hybrids are not fully sterile, the probability of seed set from selfing, or cross pollination from fertile pollen sources, as well as the incidence of outcrossing to compatible species is extremely low.

4:00 PM TUESDAY

63. EFFECT OF BALE WEIGHT ON TSNA CONTENT OF CURED BURLEY TOBACCO IN LARGE BALES. Paul DENTON[1,2], Margarita Velandia[1] and Vickie Witcher[1]; [1]University of Tennessee, Knoxville, TN USA and [2]University of Kentucky, Lexington, KY USA

Cured burley tobacco was placed in "large bales" (106 cm x 106 cm x 103 cm) at varying weights to investigate the effect of density on TSNA formation during storage. Bale weights were 246, 309 and 340 kg in 2006-7, and 250 and 362 kg in 2008. Tobacco was sourced from a University farm in all three years, and from three private farms in 2008. Tobacco was from LC varieties that were air cured on the stalk in typical curing barns. Leaf samples were taken at baling and a data recorder was placed in each bale to record temperature. In 2006 and 2007 bales were stored for four to eight weeks, typical of the time between baling and delivery for sale. In 2008, bales were stored for 12 weeks to more closely simulate a typical time in the bale from baling until processing. Bales were opened and resampled at the end of the storage period. All samples were stemmed and TSNA analysis was conducted on the lamina only. The changes in total TSNA content were generally less than 1.0 ppm. In many cases the measured TSNA content actually showed slight decreases over the storage period, which is probably a result of sampling variability. There was no significant effect of bale weight on change in total TSNA or on final TSNA content. Average temperature within the bale and the change in temperature within the bale after baling did not differ by bale density. These results indicate that use of heavier bale weights will not result in increased TSNA when the tobacco is baled at acceptable moisture levels.

4:20 PM ADJOURN

TUESDAY AFTERNOON, SEPTEMBER 20, 2011

SESSION B ***Session Chair: Ray Robertson***

1:30 PM TUESDAY

64. IMPROVED PRESSURE DROP & VENTILATION INSTRUMENT WITH VARIABLE FLOW RATES. Steven A. WILSON; Eastman Chemical Company, Kingsport, TN, USA

Filter ventilation is a key cigarette design parameter for cigarette manufacturers to achieve low tar deliveries. Historically smoke testing has been conducted at a single set of smoking conditions, but the new aggressive testing conditions mandated by certain governments require higher puff volumes. Designing cigarettes for these aggressive smoking conditions will require an understanding of the influence of higher air flow rates on cigarette ventilation levels.

This paper describes the design and construction of a new instrument for measuring filter properties. The instrument was built by Custom Electronic Systems, Inc. and is a Model CES 508 Cigarette Pressure Drop & Ventilation Tester. It uses two NIST traceable laminar flow elements to measure the total and ventilation air flows. The design provides good precision and accuracy for flow rates up to 100 ml/s. Digital micro processors are used to provide flow control and automatic compensation for environmental conditions and for moderate changes in house vacuum. Filter tip pressure drops can be measured up to 1000 mm of water. A specially designed test head allows the measurement of ventilation level over the range of flow rates without a bias due to high air flows.

To demonstrate the instrument's unique capabilities, the ventilation levels of various cigarette designs were measured at a range of flow rates. The results show that cigarette ventilation levels change with the higher puff volumes seen with aggressive smoking conditions.

1:50 PM TUESDAY

65. APPLICABILITY OF CURRENT TOBACCO RELATED METHODOLOGIES TO NEW OR NOVEL TOBACCO PRODUCTS. Peter JOZA, Andrew Masters, Bill Rickert and Xinyu Liu; Labstat International ULC, Kitchener, Ontario, Canada

In this study, methods validated for use on tobacco and traditional tobacco products, were investigated to determine their adequacy when applied to some additional matrix types.

The determination of nicotine was performed on a series of "dissolvable" tobacco products using Health Canada T-301. This methanolic potassium hydroxide (0.05N KOH) extraction method yielded lower than expected results. When repeated using the extraction defined by the Centers for Disease Control and Prevention (CDC), higher results were observed. Dependent on product type (matrix), results obtained using the T-301 method ranged from 29% to 100% of those obtained using the CDC methodology. This suggests either the composition of the product and/or the form of nicotine in the product can influence the extraction efficiency of the method and the results reported.

Advances in instrumentation utilizing the same 'basic' technology can influence results. Tobacco specific nitrosamines (TSNA) were determined in mainstream smoke and tobacco filler using three different LC-MS/MS systems (AB API3000, AB API3200, Waters Xevo UPLC). Each system was optimized using the same transitions and four deuterated analogues for the quantification of TSNA. Analysis of the same solutions from mainstream smoke and tobacco filler on each instrument, yielded comparable results for most TSNA. However, further investigation identified a consistent bias where the NAT results for tobacco filler averaged 12.6% lower than those determined on either API LC-MS/MS system. This bias was eliminated by performing a standard additions assay on each system suggesting specific instrument parameters can be matrix dependent.

2:10 PM TUESDAY

66. THE IMPACT OF DIFFERENT PHYSICAL AND CHEMICAL CIGARETTE PAPER BASE SHEET PARAMETERS ON SMOKE YIELDS. Mario MAYR and Dietmar Volgger; delfortgroup / Wattenspapier, Austria

Three cigarette paper parameters have been varied and their impacts on smoke yields have been measured. The basis weight has been kept constant at a level of 25gsm, the chalk content at a level of 26%. All the samples are wood pulp based. The following parameters were modified:

- the content of Tri-sodium and Tri-potassium citrate (Mix) in the range of 0.75, 1.5, 2.25 %
- air permeability in the range of 50, 100, 120 CU (ml/min/cm²)
- Diffusivity in the range of 0.100, 0.200, 0.300 cm/s

All cigarettes have been produced maintaining one specification with the same tobacco blend (American blend) to avoid interaction of other parameters. All cigarettes were also tested for self-extinguishing rates according to ASTM E2187-09.

ISO 12863 the international equivalent to the ASTM standard allows for the use of alternative substrates like the LIP CAN paper a grade produced by the Finnish paper mill Tervakoski. All cigarettes have been tested on both substrate papers, Whatman #2 and LIP CAN and on three, ten and fifteen layers to achieve a better characterisation and discrimination of the self-extinguishing performance. The differences in the self-extinguishing results were mainly caused by the changes in diffusivity.

2:30 PM TUESDAY

67. BAND DIFFUSION AT HIGH TEMPERATURE. Joseph WANNA[1] and Jean Marie Loureau[2]; [1]SWM Intl, Alpharetta, GA, USA and [2]SWM Intl, Quimperle, France

Cigarettes designed to meet ASTM standard E2187: "Standard Test Method for Measuring the Ignition Strength of Cigarettes" use mainly cigarette paper with bands around the tobacco column. These bands are defined by their width, frequency, and diffusion characteristics. However, cigarette performance on the test method depends on both band and tobacco column properties. Band diffusion is an important parameter and in a commercial or industrial lab setting is measured at 23°C. Band diffusion certainly will

increase at high temperature as the band and base paper start to decompose. There is no defined test method for testing band diffusion at high temperature. High temperature band diffusion will depend on the temperature used, temperature stability, exposure time, sample placement, sample handling after heating, conditioning before testing again, and other factor readily obvious at the current time. Paper strips are placed in an oven where the temperature is set at 230°C for 30 minutes, taken out and set in the lab to condition at 23°C and 50%RH for 30 minutes and band diffusion is tested again.

Preliminary investigations showed certain factors influence band diffusion after heating at high temperature, such as band material and base paper properties. This study revealed that as citrate and chalk levels in the base increase, the band diffusion at high temperature will increase. However, this did not impact ASTM performance which is largely controlled by the target band diffusion initially selected for the cigarette design.

2:50 PM ***Break***

3:20 PM TUESDAY

68. FILTER ADDITIVES FOR THE SELECTIVE FILTRATION OF PHENOLS FROM CIGARETTE SMOKE. Tony McCORMACK and Mike Taylor; Filtrona Technology Centre, Jarrow, Tyne & Wear, UK

Phenols are known toxic substances and accordingly it is recognized that it would be desirable to reduce the levels of phenol and other phenolic compounds found in the semi-volatile fraction of mainstream cigarette smoke. Whilst it is also well-known that cellulose acetate filters plasticized with triacetin exhibit enhanced selective filtration of phenol, there is an ongoing need for filter materials that provide still further enhancement of the removal of phenols.

During this work, a range of liquid additives that may be expected to improve the selective filtration of phenols was identified. A laboratory technique for screening the performance of these additives was developed, in particular to overcome uncertainties arising from interactions between the additive and the choice of filter substrate material (*e.g.* paper or cellulose acetate).

This paper describes the laboratory techniques developed, together with the results from smoking tests carried out on the identified additives over a range of application levels. It was concluded that the greatest levels of enhanced selective filtration of phenols were obtained from glycerol tripropionate. The results from further trials conducted on this material to assess whether the enhanced performance was maintained on machine-made cellulose acetate filters over a three month ageing period are also reported.

3:40 PM TUESDAY

69. STUDY ON MENTHOL DIFFUSION PROCESS INTO ACETATE FIBER USING LASER MICRO RAMAN SPECTROSCOPY. Masato MIYAUCHI, Ayako Chiku and Takashi Hasegawa; Japan Tobacco, Inc., Tokyo, Japan

For previous reports, during aging of mentholated tobacco products, the elution efficiency for menthol from the filter decreases with time. Therefore, it seems that the menthol has diffused deeply into acetate fibers during aging. However, any information concerning the menthol diffusion into the fiber has not appeared to date in the literature. This study focuses on the diffusion into the fiber.

A laser micro-Raman spectroscopic technology was applied for the direct analysis of the menthol distribution into the fiber. Firstly, the Raman spectra of cellulose acetate, menthol, and triacetin were measured. The peak in the Raman spectra of menthol at 771cm^{-1}, which is supposed to be an in-phase ring stretching vibration, does not appear in the Raman spectra of the other compounds. On the other hand, the peak in the Raman spectra of acetate at 912cm^{-1}, which is probably attributed to one of the modes involving COC stretch, does not appear in the Raman spectra of the other compounds. Thus, the ratio of peak area centered at 771cm^{-1} to that centered at 912cm^{-1} was identified as the menthol concentration into the fiber. Secondly, micro-Raman scattering measurements were carried out on the "Y" cross-sectional fiber of the mentholated product. The obtained cross-sectional ratio (771cm^{-1}/912cm^{-1}) demonstrates the following menthol distribution. Before aging, the menthol was located in the neighbourhood of the fiber's surface. After aging for 65 days at room temperature (295K), the menthol began to be distributed deeply into the fiber. After aging for 65 days at high temperature (328K), the menthol was distributed with uniformity into the fiber. This study shows the feasibility of observing the menthol diffusion into the fiber by the micro-Raman spectroscopy.

4:00 PM TUESDAY

70. DETERMINATION OF NITROALKANES IN CIGARETTE MAINSTREAM SMOKE WITH HEART-CUT TWO-DIMENSIONAL GC/MS METHOD. Jizhao GUO, Jingjing Shang, Fuwei Xie and Xiaobing Zhang; Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou, China

A heart-cut two-dimensional GC/MS instrument consisting of a GC and a GC/MS was fabricated based on pneumatic pressure controlled switch (*i.e.* Deans switch). The instrument included dual ovens for two columns and a cold trap cooled by liquid nitrogen between two columns. It was easy to construct and operate. A method for analysis of nitroalkanes in cigarette mainstream smoke was developed with the instrument. The gas phase compounds of cigarette mainstream smoke were directly analysis without cleanup after trapping by ethyl acetate under -70°C. Multiple cuts were executed in one injection, and the cuts were trapped on the head of the 2nd column through transfer line between two GCs. Nitromethane, nitroethane, 2-nitropropane and 1-nitro-n-pentane was detected and quantified from the cigarette mainstream smoke. The limits of detection for nitroalkanes ranged from 1.30 to 9.80ng/cig. The recoveries ranged from 87.1 to 110%, and the RSD was between 7.17~9.43%.

4:20 PM ADJOURN

WEDNESDAY MORNING, SEPTEMBER 21, 2011

COMBINED SESSION ***Session Chair: Serban Moldoveanu***

8:50 AM WEDNESDAY

71. CONSUMPTION PATTERNS AND BIOMARKERS OF EXPOSURE IN CIGARETTE SMOKERS SWITCHED TO DISSOLVABLE TOBACCO (CAMEL ORBS), DUAL USE, OR TOBACCO ABSTINENCE. George R. KRAUTTER and Michael F. Borgerding; R. J. Reynolds Tobacco Company, Winston-Salem, NC, USA

Camel Orbs (Orbs) are comprised of finely-milled tobaccos and food-grade ingredients compressed into a small oval form intended to be ingested. This trial investigated short-term changes in product usage and biomarkers of exposure when adult smokers either continued smoking, switched to consuming Orbs exclusively or partially, or were tobacco abstinent. 114 Participants were randomized into 4 groups (n=27-29/group) and confined in clinic 6 days: usual brand cigarette (UB) continued to smoke *ad lib*; exclusive Orbs (EO) and dual-use (DU) self-regulated product(s) consumption (DU followed a work-place 'non-smoking' schedule); and tobacco abstinent (TA). Biomarkers quantified: (24-hr urine) total nicotine equivalents and select metabolites of TSNAs, PAHs, aromatic amines, acrylamide, acrolein, benzene and urine mutagenicity; (blood) carboxyhemoglobin; (plasma) nicotine, cotinine and thiocyanate; (expired-air) carbon-monoxide (ECO). UB daily cigarette use and nicotine intake did not change from baseline (BL). DU reduced daily cigarette use from 15 to 13/day and consumed 3-4 Orbs/day, resulting in a minimal reduction in nicotine intake from BL. DU diurnal reductions in blood/plasma/ECO biomarkers were observed due to schedule. EO consumed 10-11 Orbs/day, and both EO and TA had significantly reduced nicotine intake from BL. Substantial reductions of all biomarkers from BL were observed in EO and TA, whereas minimal reductions were generally observed in UB and DU. Results demonstrated that when smokers switch to Orbs their exposure to toxicants associated with cigarette smoke are greatly reduced, similar in magnitude to being tobacco abstinent. Dual use resulted in minimal toxicant reductions in the absence of any consistent increase in nicotine or toxicant exposure.

9:10 AM WEDNESDAY

72. NICOTINE UPTAKE, URGE TO SMOKE, AND CARBOXYHEMOGLOBIN CHANGES IN SMOKERS AFTER CONSUMPTION OF SMOKE-FREE TOBACCO PRODUCTS. Elaine K. ROUND, Leanne C. Lee, Sheri A. Bowman, Kelly M. Harger, Tracy M. Hefner, Angela M. Slater and Mitchell F. Stiles; R. J. Reynolds Tobacco Company, Winston Salem, NC, USA

As a harm reduction approach, some public health officials support the use of smoke-free tobacco products in place of cigarettes for smokers who continue to use tobacco. This study was conducted to evaluate endpoints of nicotine uptake, urge to smoke, and carboxyhemoglobin changes in smokers after consumption of modern smoke-free tobacco (MSFT) products. Fifteen subjects completed a randomized, crossover, open-label study of Camel Orbs, Camel Strips, Camel Sticks, Camel Snus, and subjects' usual brand (UB) cigarettes. Subjects consumed a single unit of one product at each of five test visits after a

12-hour nicotine abstention. Study endpoints were assessed for up to three hours following product use.

Nicotine uptake measured as $AUC_{0\text{-}180}$ was highest for UB cigarette. Mean nicotine uptake following use of one snus pouch, ½ stick, one orb, and one strip was 81%, 49%, 46%, and 25% of the mean UB value, respectively. Urge to smoke ratings significantly decreased after use of all products, but the decrease was greatest and remained statistically significant for the longest period of time after smoking UB. Carboxyhemoglobin levels significantly increased after smoking one UB cigarette but either did not change or decreased after use of any MSFT product.

Results from this study were compared to results of previous studies in which nicotine uptake from a single MSFT product was estimated after a 30-minute tobacco abstention. Limitations of using a shorter tobacco abstinence period will be discussed.

9:30 AM WEDNESDAY

73. A NOVEL MODEL MOUTH SYSTEM FOR EVALUATION OF *IN VITRO* RELEASE OF NICOTINE AND TOBACCO-SPECIFIC NITROSAMINES FROM MOIST SNUFF. Jie ZHANG, Peng Li, Yongli Zong, Shihao Sun, Yubing Song and Jianping Xie; Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou, China and Beijing Cigarette Factory of Shanghai Tobacco (Group) Corporation, Beijing, China

It is critical to characterize the release of nicotine and tobacco-specific nitrosamines (TSNA) from smokeless tobacco products for safety evaluation and product quality control. A novel model mouth system for evaluating the *in vitro* nicotine and TSNA release behavior of a smokeless tobacco product (moist snuff) has been developed. The system consists of the release medium reservoir module, the flow speed control module, the temperature control module, nicotine release module, and release solution collection module, and simulates buccal condition in terms of temperature, saliva compositions, and the rate of saliva production, etc. Nicotine concentration was determined with a reversed-phase high-performance liquid chromatographic (RP-HPLC) method. TSNA concentration was determined using a new Liquid chromatography-electrospray tandem mass spectrometry (LC-ESI MS/MS) method. The influences of flow rate and temperature of the release medium on the nicotine release rate were studied. The performance of the model mouth system was compared with *in vivo* data of nicotine and TSNA release in human volunteers. The model mouth system was applied to evaluate nicotine and TSNA release in 21 brands of commercially available moist snuff, and the effects of product weights, nicotine concentration, and product pH values on nicotine and TSNA release were evaluated. The model mouth system and the analytical method were shown to be a useful tool in assessing the bioavailability of nicotine and TSNA to moist snuff users.

9:50 AM ***Break***

10:20 AM WEDNESDAY

74. DIFFERENCES IN FREE AMINO ACID CONTENTS BETWEEN FLUE-CURED TOBACCO AND BURLEY TOBACCO BOTH CURED WITH FLUE-CURING AND AIR-CURING METHOD. Hongzhi SHI[1], Tian Zhao[2], Jinhu Yang[3], Rui Chu[3], Wenbi Li[4] and Junwei Yang[4]; [1]Henan Agricultural University, Zhengzhou, Henan, China, [2]Guoshun Liu, Haiyan Zhou, Henan Agricultural University, Zhengzhou, Henan, China, [3]Binchuan Burley Tobacco Company, Binchuan, Yunnan, China and [4]Dali Tobacco Company, Dali, Yunnan, China

Amino acids are the important precursors of Maillard reaction compounds, and the significant differences in individual amino acid content were measured between different tobaccos. The experiments were conducted in 2010 with a flue-cured tobacco (Hongda) and a burley tobacco (TN86) to investigate the amino acid contents these two tobaccos under two N treatments and two curing method. The results demonstrated that by using the same curing method the contents of proline (Pro) and methionine (Met) were not different in the two types, while the contents of Pro and Met in tobacco cured by the flue-cuing method increased more than 112% and 91%, respectively, over tobacco cured by the air-curing method. Pro and Met were most influenced by curing method and not tobacco type. Contents of free amino acids in burley tobacco were higher than that in flue-cured tobacco and the free amino acids most influenced by genotype were aspartic acid (Asp) and glutamic acid (Glu). Content of Asp in burley tobacco was more than 5 times higher than that in flue-cured tobacco under the same applied nitrogen Contents of Asp and Glu were not altered by curing method. All of the free amino acids increased with increased N application. Conclusions are that flue-curing is the main cause of higher amounts of Pro and Met in flue-cured tobacco, while genetic factors contribute more to the high levels of Asp and Glu in burley tobacco.

10:40 AM WEDNESDAY

75. THE PROTEIN EXPRESSION PROFILES OF MATURE TOBACCO LEAVES ON LOW NITROGEN NUTRITION EXPOSURE. Huijuan YANG, Guoshun Liu, Hongzhi Shi, Hong Cui and Li Xu; Henan Agricultural University, Zhengzhou, Henan, China

To research the mechanisms of the development regulation of tobacco under low nitrogen application, leaf length and width were measured at 50 days, 60 days and 70 days after transplanting. Results showed that both length and width of leaves were smaller under low nitrogen nutrition than under normal nitrogen nutrition. The differences between them were significant even at 70 days after transplanting which is in the mature stage of tobacco leaves. These results suggested that the low nitrogen mostly affected the development of mature leaves. The protein expression profiles of leaves at 70 days grown under the two treatments were analyzed by 2-D electrophoresis and 5 differentially expressed proteins were identified by MALDI-TOF/TOF. Two of these are annotated proteins and the other three are unknown proteins and expression transcripts. One of the identified proteins, a cyclophilin-like protein from Nicotiana tabacum which is a stress-response signal protein, was shown to be more highly expressed in leaves under low nitrogen. This protein may play pivotal biological roles in the development regulation under stress environments. Real-Time Quantitative PCR analysis also confirmed that the high expression of cyclophilin-like

protein gene at mRNA level under low nitrogen which is in line with the protein contents in the 2-D experimental identified result. The expressional level of protein and mRNA between low nitrogen and normal nitrogen differ by more than 1.6 fold. Our results revealed that cyclophilin-like protein was highly expressed in the mature leaves under low nitrogen conditions compared with normal conditions and indicated that cyclophilin-like protein may participate the molecular regulation and signal transduction of the leaf development under the low nutrition exposure. Shorter and narrower leaves developed on tobacco could be an adaptive physiological exhibition under low nitrogen stress. As a stress responsive protein cyclophilin-like protein may conduct the proceeding of this process.

11:00 AM WEDNESDAY

76. SIMULTANEOUS DETERMINATION OF TOBACCO-SPECIFIC NITROSAMINES IN RABBIT'S URINE AND BLOOD BY LC-MS-MS. Jianxun ZHANG, Zhaoyu Wang, Binbin Lu, Sheng Wang and Juan Wang; Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou, China

An improved method has been developed for the determination of the five major tobacco-specific nitrosamines (TSNAs) in rabbit's urine and blood. This method uses isotope dilution liquid chromatography coupled to a tandem mass spectrometer with electrospray ionization and is significantly more sensitive than traditional methods. Sample concentrations were determined for five TSNAs in urine and blood using five isotopically labeled TSNAs analogues as internal standards. The results show that: 1) The limits of detection of each TSNA varied from 2.5 to 10 pg/mL. Recoveries for the five major tobacco-specific nitrosamines (TSNAs) ranged from 92.1 to 104%. Precisions (RSD) were between 2.31 and 6.98%. 2) The new method offers decreased sample preparation as compared to traditional methodologies. 3) The pharmacokinetic parameters of TSNAs have been counted out.

11:20 AM WEDNESDAY

77. THE EVOLUTION OF MOISTURE, TEMPERATURE AND DENSITY OF CUT TOBACCO AND SHRINKAGE CHARACTERISTICS DURING CYLINDER DRYING. Wenkui ZHU[1], Chuanfang Yu[1], Liangyuan Chen[1], Bin Li[1], Qiang Zen[2] and Hongtao Li[3]; [1]Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou, China, [2]Fujian Tobacco Co., Xiamen, Fujian, China and [3]Shandong Tobacco Co., Qingdao, Shandong, China

Cylinder drying is the widely used dehydration method in tobacco primary process. During cylinder drying, the temperature and moisture evolution of cut tobacco could modify physical structure properties of the materials, which would influence the final quality of cigarettes. In the present work, the evolution of moisture, temperature and density of cut tobacco during cylinder drying was investigated, and the drying shrinkage characteristics of cut tobacco particles were further analyzed. The result showed: 1) The effective diffusion coefficient (De) based on Fick's Second Law could be used to reflect the drying characteristics of cut tobacco. De increases significantly with the increase of cylinder wall temperature (130-160°C), which strengthens the transfer and diffusion ability of moisture in cut tobacco. 2) The temperature evolution of cut tobacco during cylinder drying includes the pre heating and constant temperature stage, rapid heating stage and balancing stage, which respectively

corresponded to the constant rate drying stage, falling rate drying stage and stagnation drying stage in moisture evolution curves. 3) Apparent density of cut tobacco on dry basis increased gradually during drying and then tended to be constant finally. The change of apparent density indicated the shrinkage of cut tobacco particles during cylinder drying. 4) The shrinkage coefficient of cut tobacco particles at different drying temperatures showed a almost linear relationship with cut tobacco moisture content.

11:40 AM ADJOURN