1. QUANTITATIVE RISK ASSESSMENT OF TOBACCO-BURNING AND TOBACCO HEATING CIGARETTES. Kristin M. MARANO, Ziad S. Naufal, Michael F. Borgerding and Ryan J. Potts; R. J. Reynolds Tobacco Company, Winston-Salem, NC USA

Two approaches were used to estimate the absolute and relative incremental lifetime cancer risk (ILCR) for mainstream smoke from tobacco-burning and tobacco-heating (i.e., Eclipse) cigarettes. In the first approach, machine-generated mainstream smoke constituent yields from two different smoking regimens were applied to ILCR calculations. Using constituent yields measured with a 55/30/2, 100% vent blocking regimen, mean ILCR estimates for tobacco-burning cigarettes ranged between 4.4 and 5.0E-03, compared to 2.1E-03 for Eclipse. Thus, the Eclipse ILCR was reduced 52-58% compared to tobacco-burning cigarettes. Applying constituent yields measured with a 60/30/2, 0% vent blocking regimen, mean ILCR estimates for tobacco-burning cigarettes ranged between 4.7 and 8.8E-03 (depending on the cigarette comparator) compared to 2.78-03 for Eclipse. Again, the Eclipse ILCR was reduced (42-69%) compared to tobacco-burning cigarettes. In the second approach, changes in carcinogen biomarkers of exposure in smokers who switched from tobacco-burning to Eclipse cigarettes were applied. In order to quantify absolute exposure to mainstream smoke constituents, machine-generated yields were used to represent baseline exposure to tobacco-burning cigarettes. Constituent yield concentrations were then adjusted according to observed differences in representative biomarkers in tobacco-burning cigarette smokers switched to Eclipse for 12 and 24 weeks. Mean ILCR estimates at baseline and after 12 and 24 weeks were B.7E-04, 4.6E-04, and 4.78-04, respectively. Accordingly, after 12 and 24 weeks of Eclipse smoking, mean ILCR for Eclipse was reduced 46-470/o compared to tobacco-burning cigarettes. Using two quantitative risk assessment approaches, a reduction of approximately 50% in the estimated ILCR for Eclipse compared with tobacco-burning cigarettes was consistently observed.

2. TOOLS FOR THE PRIORITISATION OF TOBACCO SMOKE TOXICANTS: AN OVERVIEW. Fiona Cunningham, Stacy Fiebelkorn, Debbie Dillon and Clive MEREDITH; British American Tobacco, Group Research & Development, Southampton, UK

A scientific, evidence-based risk assessment method to assess potential health risks of individual tobacco smoke toxicants, reflecting the range of yields and human characteristics
related to exposure, is a beneficial tool for tobacco product regulation and the establishment of product standards.

In recent years there has been significant interest in characterising these individual toxicants both from the perspective of future regulatory frameworks aimed at monitoring or lowering toxicant levels and from the perspective of tobacco product development focused on selective toxicant reduction, using several different techniques (Fowles and Dybing 2003; Pankow et al. 2007; Burns et al. 2008; Watanabe et al. 2009; Talhout et al. 2011; Cunningham et al. 2011, Xie et al. 2012). A similar approach for smokeless tobacco products toxicants has also been presented (Ayo-Yusuf and Connolly 2011).

A basic criticism of all the techniques employed to date is that they have been applied to individual toxicants rather than toxicants within the complex mixture of tobacco smoke. Progress has been made in the field of risk assessment of simple mixtures of chemicals but a complex mixture such as tobacco smoke presents additional challenges. We have initiated work to investigate the utility of the Margin of Exposure (MOE) segregation tool for use in small scale mixture assessment of three aldehydes (Cunningham et al. 2012) through careful consideration of their mode of action.

We suggest that a data-driven, physiologically relevant risk assessment strategy is a useful tool for the identification and prioritization of tobacco smoke toxicants for risk reduction research prior to construction of a more complex mixture-based risk assessment platform.

10:00 AM Break

10:30 AM MONDAY

3. QUANTITATIVE RISK ASSESSMENT VS. THE WHOLE STORY? Liam SIMMS, Imperial Tobacco Ltd, Bristol, UK

This paper provides an overview of the composition of tobacco and tobacco smoke, provides discussion on the various analyses (chemical and toxicological methods) that have been used to assess different products and some of the challenges and weaknesses that have been highlighted in the literature when assessing the “quantitative risk” of complex mixtures such as whole smoke.

Currently, no Government or regulatory authority has developed a set of predictive tests whereby one tobacco product can be compared to another, however, the FDA (2012a) has published draft guidance on the broad types of studies required for a modified risk tobacco product “MRTP” application. The mechanisms by which diseases associated with cigarette smoking develop are not fully understood, and there are no agreed animal models for many of these diseases. Epidemiology has been unable to identify cigarette smoke components responsible for diseases in smokers. Likewise, laboratory studies have also been unable to identify such components. Smoking has been associated with multiple diseases and multiple postulated mechanisms, involving many organ systems. It is not appropriate to assess the toxicology of individual cigarette smoke components in isolation through Quantitative Risk Assessment (QRA) methodology. Cigarette smoke is a highly complex mixture consisting of thousands of components and the only appropriate way to assess a combustible tobacco product is to test the whole smoke of such a product in biological assays.
4. PAST, PRESENT AND FUTURE APPROACHES FOR REDUCING CADMIUM CONTENT IN TOBACCO LEAVES. Murli Manohar1,2, Toshiro Shigaki3, Lopa Shigaki3 and Kendal D. Hirschi1,2; 1United States Department of Agriculture/Agricultural Research Service Children’s Nutrition Research Center, Baylor College of Medicine, Houston, TX USA, 2Vegetable and Fruit Improvement Center, Texas A&M University, College Station, TX USA and 3Papua New Guinea National Agricultural Research Institute, Papua, New Guinea.

Contamination of soils by heavy metals such as cadmium is of great concern for global agricultural practices and human health. To overcome the toxic effects of heavy metals, plants have evolved various mechanisms such as reduced intake of toxic metals, enhanced detoxification, or increased capacity to store cadmium in metabolically inactive cellular compartments. The emphasis here is on the health hazards, and possible ways to limit the amount of cadmium in the foods and tobacco products we consume. This talk will be divided into six sections: 1) Cadmium exposure and human health; 2) Cadmium hyperaccumulating plants; 3) Limiting cadmium accumulation in plants; 4) Cadmium health hazard caused by cigarette smoking; 5) Strategies to lower cadmium content in tobacco; and 6) Future directions. Our central thesis is that plant biotechnology can be used to significantly reduce cadmium levels in tobacco products.

11:30 AM MONDAY

5. APPLIED PRODUCT RISK ASSESSMENT. Emad A. Choudhury and E. Karl Enters; Celanese International Corporation, Dallas, TX USA

The presentation, using generic terminology, will provide an overview of a supplier’s approach to Applied Product Risk Assessment utilizing an internal proprietary system. The purpose of our Applied Product Risk Assessment process is to ensure that our products meet our corporate safety and legal standards, as well as all applicable safety, regulatory, and legal requirements in each country where the product will be marketed. Our internal global risk management process targets the identification and management of product related risk associated with raw materials, manufacturing, handling and distribution, sale, and the end-use of our products. The system is designed to broadly balance risk from multiple internal and external sources. Targeting increased institutional awareness and organizational decision-making at risk relevant levels, the process incorporates ad hoc risk review committees of technical professionals with backgrounds in toxicology & medical technology, legal, product development, manufacturing, and others as appropriate. Success has been achieved by embedding the product risk management process into our new product commercialization systems, creating a cultural change towards proactive consideration of critical risk management elements such as product risk, increased organization awareness, escalation of decision-making, and risk mitigation.
6. STUDY OF FIFTEEN COMPONENTS IN MAINSTREAM SMOKE OF ONE TYPE OF CHINESE WATER PIPE AND THE FILTRATION EFFICIENCY OF WATER PIPE.
Juxing JIANG, Jian Zhang, Li Jiang, Yanqing Duan, Shijie Li and Wei Zhe; Hongyun Honghe Tobacco (Group) Co., Ltd., Kunming, PR, China

Water pipe smoking has been popular for centuries in China as well as in Arabic countries. Although water pipe tobacco and smoking habits in China are considerably different from those in Arabic regions, there is no detailed research about the chemical components in mainstream smoke of Chinese water pipe. This paper puts forward a method imitating Chinese water pipe smoking, and thereafter 15 components in mainstream smoke are determined with this developed method. The results show that the tar and nicotine yielded by Chinese water pipe smoking are about four times higher than those produced by cigarette smoking with ISO method. The filtration efficiency of Chinese water pipe for 15 components of mainstream smoke is 2.3 ~ 89.7%. For easily water-soluble components, such as formaldehyde, acetaldehyde, acetone, acraldehyde, propylaldehyde, crotonaldehyde, butylaldehyde, hydroquinone and catechol, the filtration efficiency of water pipe is notably higher than for other compounds. Noticeably, only 2.3% of nicotine is absorbed by water pipe. In general, Chinese water pipes do filter varying quantities of toxic elements, but they still deliver dramatically more tar and nicotine than cigarette smoking, especially for switch smokers, that the users should be aware of.

7. COMPLETE ARTIFICIAL SALIVA ALTERS EXPRESSION OF PROINFLAMMATORY CYTOKINES IN HUMAN DERMAL FIBROBLASTS. Gloria E. MALPASS¹, Subhashini Arimilli¹, G. L. Prasad² and Allyn C. Howlett¹; ¹Wake Forest University Health Sciences, Winston-Salem, NC USA and ²R. J. Reynolds Tobacco Company, Winston-Salem, NC USA

Complete artificial saliva (CAS) is a saliva substitute often used as a vehicle for test articles, including, smokeless tobacco. We discovered, using polymerase chain reaction gene expression array analyses, that CAS increased gene expression for certain proinflammatory cytokines including interleukin 8 (IL8) and interleukin 1α (IL1α) within 5 hours of treatment in cultured normal adult human dermal fibroblasts. Expression of the vascular cell adhesion molecule 1 (VCAM1), a gene upregulated by certain proinflammatory cytokines including IL1α, was also increased. Furthermore, cytometric bead array assays indicate that CAS increased the release into the culture media of proinflammatory cytokines including IL8. These results suggest that constituents of CAS may induce a proinflammatory response in cultured human dermal fibroblasts. To elucidate which components of CAS alter the expression and release of proinflammatory cytokines, we investigated the following: α-amylase, lysozyme, acid phosphatase, and urea. Comparison of the effects of CAS and modified preparations of artificial saliva indicate that α-amylase is responsible for the CAS-induced changes in the expression and release of the proinflammatory cytokines in dermal fibroblasts. Further investigation, using colorimetric assays, shows that this response correlates with the enzymatic activity of α-amylase. Therefore, it is important to carefully evaluate the “vehicle effects” of CAS and its constituents in oral/dental research.

8. METHOD DEVELOPMENT FOR ENUMERATION OF ENDOTHELIAL PROGENITOR CELLS (EPCs). G. L. PRASAD¹, Subhashini Arimilli², Bobbette A. Jones¹ and Ian Fearon³; ¹R. J. Reynolds Tobacco Company, Winston-Salem, NC USA, ²Wake Forest
Endothelial progenitor cells (EPCs) are a type of white blood cells present in circulation at low numbers and are hypothesized to play a role in the protection and repair of the cardiovascular system. EPCs, characterized as cells expressing the surface markers CD34, CD133 and CD309 (VEGFR2), were reported to decrease and/or be functionally impaired in smokers. To evaluate whether EPC levels can be utilized as a potential smoking-related biomarker of effect in clinical studies, several technical challenges remain. Therefore, we assessed various experimental conditions and other parameters that potentially impact EPC viability and thus influence the enumeration of EPCs by flow cytometry. Among the parameters evaluated were the effect of a time lag in processing blood samples, the effects of anticoagulants used for blood collection, the conditions used to detect EPCs and the effect of overnight shipping. The method optimization involved collecting fresh blood from ten healthy volunteers who were non-tobacco users. Lysing the red blood cells prior to labeling with specific antibodies against the cell surface markers (CD34, CD133 and CD309) improved the resolution of white blood cells and enumeration of EPCs. While freshly collected blood is ideal for enumeration of EPCs, blood stored up to 24 hours at room temperature could be used. Among the anticoagulants used for blood collection, citrate and CPDA allowed better enumeration of EPCs. Shipping the blood samples overnight at room temperature did not seem to have an impact on EPC counts. We also showed that functional EPC colonies can be grown in culture. In summary, we suggest a simple and reproducible method that can be used for evaluating EPCs in clinical studies.

9. AN IMPROVED METHOD FOR THE DETERMINATION OF SELECTED HUMECTANTS IN TOBACCO PRODUCTS. Carrie T. SODEN, Alexandra Martin, Steven Kalata and Fraser Williamson; Arista Laboratories, Richmond, VA USA

Humectants are an important class of compounds applied to tobacco primarily for the improvement of hydroscopic properties and to act as a carrier for flavor components. For the purposes of this study, we focused on the most prevalent humectants used, glycerol and propylene glycol.

Arista’s original humectants method was based on published Health Canada and CORESTA reference methods and involved a lengthy methanol extraction, GC-FID analysis and was limited in scope to only tobacco from cigarettes.

The objectives of this study were to improve methodology by reducing sample extraction time and expand the scope to include a variety of other tobacco products (e.g. moist snuff and kreteks). During development, it was observed that moist snuff products reached an extraction plateau after 30 minutes, while dry products had not. It was postulated that the elevated moisture content of certain tobacco products proved beneficial to the extraction efficiency of humectants. Water was incorporated into the extraction process resulting in a more efficient extraction of the dry products.

Using the improved method, humectants were extracted from tobacco, first with water to hydrate the tobacco cell structure; then with methanol to allow the extracts to remain amenable to GC-FID analysis. Quantitation was achieved using 1,3-butanediol as an
internal standard. The improved method was fully validated with a reduced extraction time and the scope extended to include various tobacco products.

10. DOSIMETRIC ASSESSMENT OF WHOLE SMOKE PARTICULATE DEPOSITION IN VITRO: A PROPOSED COMMON APPROACH USING QUARTZ CRYSTAL MICROBALANCE TECHNOLOGY. Jason Adamson, David Thorne, Annette Dalrymple, Clive Meredith and Deborah Dillon; British American Tobacco, Southampton, UK

There are a number of different smoking machines and exposure chamber combinations used by our industry to assess the toxicological impact of cigarette smoke in vitro. The amount of smoke delivered to cells within an in vitro exposure system can be presented in many ways: ratios of smoke to air, mixing airflow rate, vacuum rate, percentage or fraction. However, dosimetry (the quantifiable amount of smoke cells are directly exposed to) is more relevant and is becoming increasingly important in the field of cigarette smoke in vitro assessment. Dosimetry techniques will hopefully bridge the gap between different technologies and allow cross-platform comparisons.

Installed into various exposure chambers, quartz crystal microbalance (QCM) technology has allowed us to quantify cigarette smoke particulate dose in vitro. For example, 4 QCMs installed into the Vitrocell 6PT-CF exposure module enabled quantification of whole smoke deposited mass at a range of diluting airflows (0.25 - 4.0 L/min) resulting in 24.00 ± 2.00 µg/cm² - 1.13 ± 0.03 µg/cm² deposited particulate mass (3R4F cigarettes). Moreover, QCM tools have enabled the quality control of smoke runs and highlighted limitations/improvements to established whole smoke methodologies. Furthermore, for the first time we are able to perform direct comparisons of whole smoke particulate dose delivered from different in vitro whole smoke exposure systems: the Borgwaldt RM20S and Vitrocell VC 10. We can demonstrate that QCM technology is a reliable, effective and simple tool that can accurately quantify smoke particulate deposition in real-time, in vitro. Additionally, QCM data can be used to unify in vitro toxicological data irrespective of exposure system.

11. LIMITATION OF STANDARD DEVIATION TO EXPRESS VARIABILITY. Béatrice Teillet¹, Thomas Verren¹, Xavier Cahours¹, Stéphane Colard² and Steve Purkis²; ¹SEITA Imperial Tobacco Group, Fleury-les-Aubrais, France and ²Imperial Tobacco Limited, Bristol, UK

In the framework of growing regulations regarding tobacco products, increased requirements are observed for reporting of analytical figures (e.g. FDA). This paper deals with the reporting of data variability in this context. The objective was to evaluate the consistency of the short term standard deviation to describe the variability of measurements as well as the effect of the number of replicates.

The dataset of the CORESTA 2006 joint experiment included a number of cigarette smoke constituents identified by FDA for reporting. The testing protocol required the analysis of Kentucky reference cigarettes 2R4F and 1R5F performing 5 replicates (run over consecutive days) in 3 independent experiments (run within different time periods).

This data set provided different sources of variability across measurements: short term variability (replicates), medium term variability (periods) and lab-to-lab variability
(laboratories). For each reference cigarette, ANOVA with one factor (laboratories) combined with Newman-Keuls’s multiple range tests was performed to compare data generated across laboratories.

The distribution of variance between laboratories and the residual error (period and/or replicate) was estimated with different numbers of replicates (from 2 to 15). A hierarchical ANOVA on the 3 factors (laboratories, period and replicates) allowed the complete split of variance and evaluates their respective relative contributions to the full variability. Results show that the expression of variability as individual standard deviation (repeatability) gives false differentiations, whatever the number replicates, due to the major contribution of the lab-to-lab variability.

12. DETERMINATION OF AROMATIC AMINES THROUGH THE USE OF TANDEM MASS SPECTROMETRY COUPLED TO GAS PHASE CHROMATOGRAPHY. Sherri S. BROWN and I. Gene Gillman; Enthalpy Analytical, Durham, NC USA

The number of compounds of regulatory interest in cigarette smoke has increased in recent years. The US FDA Tobacco Products Scientific Advisory Committee (TPSAC) proposed list includes six aromatic amines compounds. The established GC-MS methods for the analysis of aromatic amines involve complex sample preparation and encompass only a portion of the six compounds of interest. The objective of this study was to develop a single analytical method capable of determining all HPHC aromatic amines (o Anisidine, o Toluidine, 2,6 Dimethylaniline, 1 Aminonaphthalene, 2 Aminonaphthalen, and 4 Aminobiphenyl). Gas chromatography coupled with tandem mass spectrometry was used to measure these compounds. Cambridge pads were extracted with aqueous acid and the extracts were subsequently processed through two solid phase extraction steps and derivatized prior to analysis. The aromatic amines were separated on an Agilent 7890 using an HP-5MS dual-column system with post-run backflushing through the first column. Detection followed on an Agilent 7000 QQQ tandem mass spectrometer. The mass spectrometer was operated in electron impact mode and all compounds were detected using multiple reaction monitoring (MRM). In general, all compounds gave levels of detection less than 1 ng/mL. Calibration and other analytical details will be presented for all compounds.

13. ACCUMULATION OF NICOTINE AND NORNICOTINE ENANTIOMERS IN LEAF OF NICOTINE DEMETHYLASES MUTANTS DURING GROWTH AND CURING. Bin CAI¹, Anne Jack¹, Ramsey S. Lewis², Ralph E. Dewey², Huihua Ji and Lowell Bush¹; ¹University of Kentucky, Lexington, KY USA and ²North Carolina State University, Raleigh, NC USA

Nicotine metabolism in tobacco plants is mostly through N-demethylation, resulting in nornicotine. There are three functional nicotine demethylases CYP82E4 (E4), CYP82E5v2 (E5) and CYP82E10 (E10) in tobacco. In this study, we investigated the nicotine and nornicotine enantiomer accumulation in nicotine demethylase mutants during growth and air-curing.

Three nicotine demethylase mutants and mutant combinations were grown in the field and leaf samples were analyzed for alkaloid levels plus nicotine and nornicotine enantiomer level. Results demonstrate that there was no significant difference in nicotine
and nornicotine accumulation among plants with/without active E5 and E10. In leaves of plants with active E5 or E10, little (R)-nicotine accumulated throughout all sampling times, however (S)-nicotine accumulated throughout all sampling times. In plants with active E4, both (R)- and (S)-nicotine started to be converted to the corresponding enantiomer of nornicotine at harvest and significant (S)-nornicotine accumulated during curing. Nicotine levels decreased as demethylation occurred. A continuously decreased (R)-enantiomer percentage of total nicotine was measured throughout growth and curing in all mutants. (R)-enantiomer percentage of total nornicotine remained around 75% when only E5 or E10 were present in tobacco and decreased significantly when E4 was present.

Of the three nicotine demethylases, only E4 has significant effects on nicotine and nornicotine accumulation during growth and curing. E5 and E10 selectively use (R)-nicotine as substrate throughout growth and curing. When expressed, mainly after harvest, E4 uses both enantiomers of nicotine.

14. HIGHLY SELECTIVE LC-MS/MS ANALYSIS OF CEMA, 3-HPMA AND HBMA. Veniamin LAPKO, Alan Dzerk, Ridha Nachi, Kirk Newland and Curtis Sheldon; Celerion, Lincoln, NE USA

2-Cyanoethylmercapturic acid (CEMA), 3-hydroxypropylmercapturic acid (3-HPMA), 3-hydroxypropyl-1-methylmercapturic acid (HBMA) are urinary markers of exposure to acrolein, crotonaldehyde and acrylonitrile, respectively. These glutathione conjugates have been shown to be selective markers of tobacco smoke exposure. Due to the variety of similar and isomeric structures, selectivity of the analysis is a critical issue; it has not been adequately addressed in a majority of published methods, potentially resulting in significantly elevated values of some biomarkers. We report here development a selective LC-MS/MS method for simultaneous analysis of CEMA, 3-HPMA and HBMA in human urine using RP UPLC LC-MS/MS. Human urine (0.100 mL) was spiked with internal standards and extracted using 96-well Oasis HLB plate. An improved sample clean-up with recovery of all mercapturic acids over 80% was achieved by elution employing significant decrease in hydrophobicity of the analytes at neutral pH. Extracted samples were injected on a C18 analytical column using a Waters Acquity UPLC. Electrospray ionization (ESI) data was acquired by multiple reaction-monitoring (MRM) in positive mode on an AB|MD Sciex API 4000 tandem mass spectrometer was used for analysis. The total acquisition time was less than 5 minutes. Selectivity of the method was established by quantitation of endogenous levels of 3-HPMA, HBMA and CEMA in more than 30 urine lots. Complete separation of interference peaks on HBMA channel for all lots not achievable using ion-exchange or RP chromatography with different selectivity was demonstrated. Selectivity of the method against 2-hydroxypropylmercapturic acid (3-HPMA isomer), 2-carboxyethylmercapturic acid (HBMA isobar) and several other mercapturic acids was shown. Comparative results of matrix effect tests using different internal standards tested during method qualification will be presented.
15. QUANTIFICATION OF ETHYL CARBAMATE IN TOBACCO AND SMOKELESS TOBACCO PRODUCTS BY GC / MS. Sharad K. MEHTA, T.K. Dinesh and Sathya Gourisankar; ITC Limited, Bengaluru, India

Ethyl carbamate (Urethane) is a genotoxic carcinogen found in fermented foods and beverages. Ethyl carbamate in Tobacco is formed from Maleic hydrazide, which is a plant growth regulator applied to Tobacco. It is also formed from hydrogen cyanide and ethanol or urea and ethanol.

Although there are a number of analytical methods reported in the literature, they all have two chief drawbacks, namely, (1) Require sophisticated instruments like UPLC/MS/MS and (2) Need laborious clean up procedure to remove the matrix effect of Tobacco.

The method developed in our laboratory and presented here is relatively simple, where the Tobacco is extracted with water and the water extract is partitioned with dichloromethane. The dichloromethane extract is concentrated and injected in GC/MS -SIM mode. This method has been validated using standard validation protocols and there is excellent linearity over concentration range from 100 ng / gm to 4000 ng / gm with correlation coefficient of 0.9939. The recoveries are more than 90% for Ethyl Carbamate with limit of quantification -100ng/g and limit of detection- 40ng/g.

16. A METHODOLOGY FOR CALCULATING THE EFFECT OF PUFFING ON DESORPTION OF CONDENSATE IN HCI AND ISO SMOKING. Ian F. TINDALL¹, Linda P. Crumpler² and Timothy J P Mason; Cerulean, ¹Milton Keynes, UK and ²Richmond, VA USA

During routine analytical smoking on a commercial smoking machine air flows through the Cambridge filter pad. It is suspected that this air flow is responsible for desorption of volatile components of TPM which can be a source of variability in measured yields. Furthermore it is known that the HCI (Health Canada Intense) method of smoking has a higher level of desorption / deposition of these volatile components.

Using a synthetic TPM mixture that is of known compensation and can be dosed accurately onto a substrate, an empirical model has been developed for the influence of puff volume and puff period on desorption. This has been further developed to compare how this might vary with semi-constant puffing as occurs in a rotary smoking machine as opposed to intermittent puffing in a linear smoking machine.

It is shown that this has particular relevance when comparing smoking machine types under HCI and ISO smoking and how clearing puffs can introduce unwanted variability if not consistently specified. The applicability of this model is tested by comparing with experimental data gathered from smoking of cigars and cigarettes where high levels of TPM have been generated.
17. EFFECT OF SMOKING LOW “TAR”-TO-NICOTINE RATIO CIGARETTES ON SMOKE EXPOSURE. Michael R. MOYNIHAN and Joseph Pandolfino; 22nd Century Group, Clarence, NY USA

The major conclusions of the 2010 Surgeon General’s report “How Tobacco Smoke Causes Disease” include “Through multiple defined mechanisms, the risk and severity of many adverse health outcomes caused by smoking are directly related to the duration and level of exposure to tobacco smoke.” The complexity of the toxicology of cigarette smoke is underscored by the list of 93 harmful or potentially harmful constituents (HPHCs) released by the Center for Tobacco Products. Individual constituents account for only a limited portion of the risk from smoking, and a large part of the risk cannot be accounted for by the total contribution of known harmful constituents. Harm reduction strategies that reduce exposure to tobacco smoke may have an advantage over reduction of individual smoke constituents or classes of constituents. One strategy is for smokers to switch to cigarettes that deliver a satisfactory level of nicotine while reducing exposure to other smoke constituents. Observations of studies using cigarettes with lower tar-to-nicotine ratios, generally produced by fortifying the cigarette filler with added nicotine, were reviewed by Russell in 2000 (In: Nicotine and Public Health, pp. 265-284, American Public Health Association). 22nd Century Group, Inc. has initiated studies of smokers switching to lower tar-to-nicotine ratio (LTN) cigarettes with higher nicotine content as a potential strategy to reduce smoke exposure. Cigarette consumption and biomarkers of exposure to selected smoke HPHCs of different chemical classes are measured. Values after switching to LTN cigarettes are compared to baseline, and to values for smokers continuing to smoke cigarettes with conventional nicotine content.

18. COMPREHENSIVE ANALYSIS AND CHARACTERIZATION OF TOBACCO SMOKE EXTRACTS WITH GCxGC-TOFMS. Mike RILEY and Elizabeth Humston-Fulmer; LECO Corporation, Saint Joseph, MI USA

The Family Smoking Prevention and Tobacco Control Act (H.R. 1256) has authorized the Food and Drug Administration (FDA) to regulate cigarettes, cigarette tobacco, and smokeless tobacco products. Among the areas of regulation is the monitoring of tobacco smoke constituents. As the pyrolysis of tobacco is known to produce harmful vapors, the FDA has established a list of harmful and potentially harmful constituents (HPHCs), including compounds in smoke, that tobacco companies must measure and report in each brand and subbrand. To comply with these regulations, it has become important to analyze and characterize the compounds in tobacco smoke. Because tobacco smoke is a complex mixture of chemical compounds, this is an analytical challenge. The 93 HPHCs required for measurement includes compounds such as benzene, nicotine, phenols, polyaromatic hydrocarbons (PAHs), inorganic compounds, and tobacco-specific nitrosamines (TSNAs) that span a range of chemical compound classes. The complexity of the smoke matrix has traditionally been dealt with by employing multiple methods along with considerable sample clean-up to target various classes of compounds individually. This poster shows a comprehensive two-dimensional gas chromatography with time of flight mass spectrometry (GCxGC-TOFMS) method for the analysis of cigarette smoke extracts. Quantitative calibration data is also demonstrated for representative analytes. This approach can comprehensively analyze tobacco smoke extracts across several compound classes while minimizing sample clean-up and the need for multiple methods of analysis.
19. **DETERMINATION OF BERYLLIUM, CHROMIUM, COBALT, NICKEL, ARSENIC, SELENIUM, CADMIUM, MERCURY, LEAD IN TOBACCO AND TOBACCO PRODUCTS BY INDUCTIVELY COUPLED MASS SPECTROMETRY.** Donald STOGNER and Danielle Benner; Lancaster Laboratories, Winston-Salem, NC USA

The ICP-MS combines a high temperature heat source with a mass spectrometer to convert atoms of elements to ions, which are separated and detected according to their atomic masses. This presentation describes a method for determining the elemental content of tobacco and tobacco products such as cigarettes as well as smokeless products like SNUS, Orbs, Strips and other smokeless products by ICP-MS. This method is capable of determining most of the Periodic Table for elements with a first ionization potential below argon. The method is used to determine chromium, nickel, arsenic, cadmium, lead, selenium, mercury, cobalt, and beryllium. Validation of the standard test method has been completed resulting in determination of optimal internal standards for each element as well as establishing LOQs, MLOQs, method precision, accuracy and other Figures of Merit for both the NexION and ELAN ICPMS instruments. Results of the validation also demonstrated successful removal of common polyatomic overlaps present in tobacco on the $^{60}$Ni, $^{62}$Ni and $^{75}$As isotopes using the NexION instrument in KED mode. The method ensures accurate results using calibration standards, sample blanks, SRMs, ICV, and bracketing samples with a midrange and calibration blank to monitor carry-over and instrument drift.

20. **VALIDATION OF NEW SMOKING SYSTEM FOR DETECTION OF NITROGEN OXIDES.** Randi FRYE¹, Dennis Urban² and Daniel Allen¹; Lancaster Laboratories, ¹Winston-Salem, NC USA and ²Lancaster, PA USA

The need for increased efficiency and throughput in determination of nitrogen oxides in mainstream smoke brought the Cerulean SM450N smoking machine to the forefront. A validation protocol was designed to evaluate the SM450N 20-port linear smoking machine equipped with dual ThermoElectron 42i-HL NO-NO$_2$-NOx analyzers. Once validation is complete, the Cerulean model will replace the current system of Phipps & Bird 10-port linear smoking machine equipped with ThermoEnvironmental 42C-HL NO-NO$_2$-NOx analyzer. Validation included the determination of precision (method and intermediate), accuracy, and limits of detection and quantitation. Precision data was correspondingly evaluated for comparison of the dual analyzers. Validation was completed utilizing the Kentucky reference cigarette (3R4F), under ISO (35-mL puff every 60 seconds for 2 second duration) and Canadian Intense (55-mL puff every 30 seconds for 2 second duration and 100% vent blocking) smoking regimes. Comparison of the two NOx systems utilized validation data along with evaluation of 10 brandstyles under ISO smoking regimen. Brandstyles were smoked simultaneously to exclude variables such as room temperature or pressure, and ranged from approximately 2 – 28 mg/cig ‘tar’ and 30 – 200 µg/cig NOx.

All parameters specified within the designed validation protocol were met with statistical analysis to support them. Investigation of concurrent use of the dual analyzers showed comparable data within and between repetitions. Evaluation of the two generations of NOx systems revealed a difference in yield. Advanced design and engineering fashioned the SM450N to be more efficient, thus supporting the disparities noted between system
datasets. Further data collection with statistical analysis may yield a correction factor to be used for comparing new with historical data.

21. ESTIMATION OF NICOTINE UPTAKE FROM TOBACCO PRODUCTS IN DUAL-USE VS. ABSTINENCE STUDY DESIGNS. Leanne C. Lee and Elaine K. Round; R. J. Reynolds Tobacco Company, Winston-Salem, NC USA

RJRT has explored two approaches for determining nicotine uptake from use of smokeless tobacco products (STPs) and cigarettes in smokers. In a “dual use” design, subjects smoked ad libitum until 30 minutes before in-clinic assessment. Subject assessment involved use of a tobacco product and periodic blood collections before, during, and after product use for measurement of serum nicotine concentrations. For some STPs, serum nicotine concentrations decreased during use, indicating that the elimination rate of pre-existing nicotine from same-day smoking was greater than uptake from the STP. Therefore, estimation of nicotine uptake from STPs required adjustment for baseline nicotine from same-day smoking. To do so, we assumed nicotine decayed following first-order kinetics and used a published nicotine half-life of 120 minutes. Baseline nicotine remaining at each time point was calculated and subtracted from the observed concentrations. Area-under-the-curve (AUC) was calculated using the adjusted concentrations.

In the abstinence design, smokers abstained from all nicotine-containing products, including cigarette smoking, for 12 hours prior to in-clinic assessment. The longer nicotine abstention minimized baseline nicotine levels, resulting in a smaller impact of nicotine decay than in the dual-use design. The previously described adjustment for incoming nicotine was performed, and baseline-adjusted AUCs were calculated. Results from the two study designs were in good agreement for all STPs evaluated. Cigarette AUC results from some dual use studies were lower than the corresponding AUC results from the abstinence design. Average baseline-adjusted AUCs for products ranked as follows: Cigarettes > Camel Snus > Camel Stick > Camel Strip. The dual use design, with same-day smoking and minimal 30-minute abstinence, provided valid and accurate estimates of nicotine uptake in spite of generally larger variability.

22. A MODIFIED AND IMPROVED ION CHROMATOGRAPHY METHOD FOR THE DETERMINATION OF AMMONIA IN MAINSTREAM CIGARETTE SMOKE. Ninitha Perumalla and Jonathan Mark Wilkins; Lancaster Laboratories, Inc., Winston-Salem, NC USA

Ammonia is listed as one of the Harmful and Potentially Harmful Constituents in tobacco products and tobacco smoke under Section 904(a)(3) of the Federal Food, Drug, and Cosmetic Act. Lancaster Laboratories, Inc has developed an analytical method that can effectively quantitate ammonia in tobacco mainstream smoke. Our modified and improved method is specific, sensitive, simple and robust. The new developed and validated method is an improvement over the historical method as it is run on the new Dionex ICS-2100 ion chromatograph which is equipped with an eluent generator and which utilizes a lower flow rate which in turn saves the amount of chemical waste generated. Also the new method uses a new Cerulean SM450 smoke machine equipped with a Hoffman capture system. Sample preparation is now simple and consistent where as previously the volume and presence of a smoke pad varied by smoke regime. An Ionpac CS12 column is now used to provide better
separation of ammonia from interference. Mainstream cigarette smoke is collected onto a Cambridge filter pad and only one impinger containing 40 mLs of 0.006 M HCl is used for effectively trapping ammonia.

The average recoveries obtained from the validation studies for 3R4F cigarettes were within ±15% for both ISO and the Health Canada smoke regimes. The established Limits of Quantitation and Limits of Detection are 0.055 µg/mL and 0.016 µg/mL, respectively. The results obtained by running the above method for IR5F, 2R4F and 3R4F cigarettes were within the range of results from the CORESTA Special Analytes Task Force.

23. DEVELOPMENT OF BIOMARKERS OF EFFECT FROM CHRONIC TOBACCO USAGE: PART 4. METABOLOMIC PROFILES FROM CIGARETTE SMOKERS AND MOIST SNUFF CONSUMERS. G. L. PRASAD1, Peter Chen1, Bobbette Jones1 and Adam D. Kennedy2; 1R. J. Reynolds Tobacco Company, Winston-Salem, NC USA and 2Metabolon, Inc., Durham, NC USA

The long-term health effects associated with cigarette smoking have been shown to be more harmful compared to those associated with the consumption of non-combustible tobacco products, such as moist snuff. In order to investigate the long-term effects of tobacco exposure, we evaluated the biochemical profiles of 40 smokers, 40 moist snuff consumers (MSC), and 40 non-tobacco consumers (NTC) using UHPLC-mass-spectrometry based global metabolomics. Twenty-four hour urine samples and matching plasma samples were collected from adult male subjects who abstained overnight from both food and tobacco. Metabolomic profiling and data analyses were performed at Metabolon Inc., (Durham NC). In this global profiling study, a total of 511 biochemicals (290 known and 221 unknown metabolites) were detected in the plasma, whereas 972 biochemicals (396 known and 596 unknown) were found in urine. For example, biochemicals from amino acid, carbohydrate, fatty acid, lipid, nucleotide and xenobiotic metabolism were identified. These biochemicals fit into distinct metabolic pathways such as oxidative stress and inflammation, and cholesterol, glucose and amino acid metabolism. In addition, a large number of structurally unknown biochemicals were detected. Cigarette smoking, relative to moist snuff consumption, appears to lead to the most changes in biochemical profiles observed in this study. Biochemical changes which point to a hyperglycemic state and hyperlipidemia are among the key perturbations noted in the smoker cohort, compared to non-smoking cohorts. In summary, these data show changes in global biochemical profiles in generally healthy cigarette smokers and moist snuff consumers. These differences in the metabolite profiles may be useful in understanding the higher risks associated with smoking relative to consumption of smokeless tobacco products.
24. DEVELOPMENT OF BIOMARKERS OF EFFECT FROM CHRONIC TOBACCO USAGE: PART 1. STUDY DESIGN AND BIOMARKERS OF EXPOSURE. Bobbette Jones, Peter Chen, Eckhardt Schmidt and G. L. Prasad; R. J. Reynolds Tobacco Company, Winston-Salem, NC USA

To assess the effects of chronic exposure to combustible and noncombustible tobacco product use, a single site, cross-sectional clinical study was conducted. Three cohorts of healthy males (40/cohort, 35-60 years) were enrolled: long-term smokers and moist snuff consumers (MSC), and non-tobacco consumers (NTC). Select biomarkers of exposure (BioExp) and potential biomarkers of effect (BioEff) indicating oxidative stress, inflammation and metabolomic changes, among others, were investigated (accompanying presentations). Blood biomarkers were measured in subjects abstaining overnight from both food and tobacco. Blood carboxyhemoglobin and thiocyanate were significantly higher in smokers relative to both non-smoking cohorts. Whereas the fasting blood nicotine levels were not significantly different between the tobacco consumers, fasting blood cotinine levels were significantly different among all three cohorts (MSC>smokers>NTC). Urinary BioExp from a 24-hr collection included: total nicotine exposure calculated from nicotine and its 9 metabolites (NicEq-T), tobacco-specific nitrosamines (TSNAs), polycyclic aromatic hydrocarbons (PAHs), aromatic amines (AAs), and thiocyanate. MSC had significantly higher 24-hr urine levels (mass/24-hr) of NicEq-T than smokers; NTC had the lowest. Urinary levels of NNAL (metabolite of NNK) were significantly higher in MSC relative to smokers, indicating potentially increased exposure and/or preferential metabolism in MSC. Total NNN urinary levels also were higher in MSC compared to smokers, and the underlying mechanism requires further research. Smokers had significantly higher levels of PAHs, AAs, and thiocyanate than MSC and NTC; no differences observed between MSC and NTC. Among trace metals evaluated, only urinary cadmium levels were significantly higher in smokers compared to MSC and NTC. In summary, combustion-related BioExp (e.g., carboxyhemoglobin, thiocyanate, PAHs) were markedly reduced in the non-smoking cohorts compared to smokers, with levels in MSC resembling those observed in NTC.

25. DEVELOPMENT OF BIOMARKERS OF EFFECT FROM CHRONIC TOBACCO USAGE: PART 2. INFLAMMATION AND OXIDATIVE STRESS. G. L. Prasad, Peter Chen, Eckhardt Schmidt and Bobbette Jones; R. J. Reynolds Tobacco Company, Winston-Salem, NC USA

In a recent clinical study, we investigated the long-term effects of smoking and moist snuff consumption using a panel of biomarkers of effect (BioEff) indicative of inflammation, oxidative stress and lipid metabolism. In this, cross-sectional study, we enrolled generally healthy adult males into three cohorts: non-tobacco consumers (NTC) and long-term smokers and moist snuff consumers (MSC). Twenty-four hour urine samples and matching
plasma samples were collected from subjects who abstained overnight from both food and tobacco. Compared to NTC and MSC, smokers exhibited elevated levels of biomarkers associated with oxidative stress (urinary isoprostanes and leukotriene E4), inflammation (white blood cell count), and platelet activation (thromboxane metabolites). A trend for elevated levels of several lipids and lipoprotein markers were observed in smokers. Statistically significant higher levels were only observed for apolipoprotein B100 and oxidized low-density lipoprotein in smokers relative to NTC and MSC. Thus, alterations in BioEff suggesting inflammation, oxidative stress and altered lipid metabolism were detected in smokers compared to the non-smoking cohorts. These findings are generally consistent with a previously conducted RJRT study which showed similar BioEff changes in oxidative stress and inflammatory pathways in smokers relative to MSC, with exceptions in some biomarkers. Collectively, our data suggest smokers, relative to the non-smoking cohorts, exhibit perturbations in pathways that could contribute to the development of smoking-related diseases. In summary, our findings are in agreement with existing epidemiological data which show the reduced harm from smokeless tobacco consumption compared to smoking, with no-tobacco-use being the least risky. The BioEff evaluated in this study are likely to be useful in future assessments of the health effects of new tobacco products.

3:00 PM MONDAY


Epidemiological data indicate that consumption of moist snuff is associated with reduced harm relative to cigarette smoking. However, a need exists for interim biomarkers of effect (BioEff) that would be useful to assess the health effects of tobacco. To identify potential BioEff in smokers and moist snuff consumers (MSC), we performed global untargeted metabolomic profiling of plasma and urine collected from smokers and MSC, using mass spectrometry. Analyses revealed a general concordance between the data obtained from the two matrices among biological pathways influenced by tobacco exposure (details presented in an accompanying poster). For example, smokers experienced exacerbated oxidative stress and inflammatory pathways relative to MSC. Based on the differential levels of metabolites detected, random forest analyses separated non-tobacco consumers (NTC), smokers, and MSC with a high (96%) accuracy when all metabolites were included. Overall, smokers showed more pronounced biochemical changes compared to MSC, which facilitated the separation of smokers from non-smokers (MSC and NTC). On the other hand, MSC showed more subtle changes in metabolite profiles, and were more difficult to separate from NTC but could still be separated. The smokers and MSC cohorts could also be segregated with a high (95%) accuracy. In addition, panels of a few metabolites that may be useful for segregating the two tobacco consumer cohorts were identified from these metabolomic profiling data. These metabolites could be used as potential BioEff, pending further validation. In summary, global metabolomic profiles and panels of selected metabolites may be used to assess the effect of tobacco consumption on biological pathways in plasma and urine.
A series of studies were conducted to evaluate dual use (DU) of cigarettes with Camel Strips (Strips), Camel Sticks (Sticks), or Camel SNUS (CS) by adult smokers in their natural environment. Subjects smoked normally for one week (baseline). Over three subsequent weeks, subjects were instructed to gradually reduce cigarettes per day (CPD) by 75% and include use of a smokeless tobacco product. One hundred subjects were enrolled; 88 completed the studies.

At the end of DU, mean CPD reductions of 60%, 60%, and 59% were reported by the Strips, Sticks, and CS groups, respectively. Mean reported smokeless product use on the last day of DU was 8.6 Strips, 4.6 Sticks, and 3.5 CS pouches. Selected biomarkers of tobacco exposure were measured at baseline and the end of DU. No statistically significant increases in biomarker levels were observed. Reductions in some urinary biomarkers confirmed that smoking reductions occurred but were not as great as the reported CPD reductions.

Mean daily nicotine and tar mouth level exposure (MLE) from cigarettes significantly decreased for all DU groups. Mean per cigarette nicotine and tar MLE did not statistically change from baseline in the Strips and CS groups. Small but statistically significant mean increases in nicotine (12%) and tar (13%) MLE per cigarette were observed in the Sticks group. These results suggest subjects did not significantly alter their smoking behavior to compensate for decreased CPD.

Subjective ratings of study products were also measured. Of interest, likeability of cigarettes statistically decreased during DU, while likeability of Strips and Camel SNUS increased. However, likeability of smokeless products was not as great as cigarette likeability at any point during DU.

In the past years, significant progress has been made in the development and use of experimental settings for collection of massive and precise experimental data on tobacco exposure and the diseases induced by it. Despite the growing number of such data, there is a need to facilitate the centralization and integration of tobacco exposure data scattered
throughout a range of disparate sources. Moreover, to fulfill the aim of exposure and
disease impact studies, it is of utmost importance to more reliably and efficiently establish
the causal link to disease. Ontologies are structural frameworks for organizing information,
enabling data integration, analysis and exchange within the community. We demonstrate
our work in developing a specialized ontology with particular focus on the cigarette smoke
exposure and the impact that it poses to the lung and airway system. To ensure global
acceptance and usability, we seek broad contribution of institutions associated to this
domain, provide their domain annotation which will subsequently be represented in a
standardized ontology. We seek to engage key contributors from various fields related to
toxicology, pharmaceutical and biotechnological industries, and academia. We then apply
terminology analysis and concept enrichment followed by a computational evaluation as
well as domain expert review. After several iterations, this ontology will be finalized in web
ontology language (OWL) format and shared with the community.

4:30 PM ADJOURN
29. OVERVIEW OF CORESTA SMOKELESS REFERENCE PRODUCTS: DESIGN, MANUFACTURING, STORAGE AND TESTS. Michael W. OGDEN¹ and John E. Bunch²; ¹R. J. Reynolds Tobacco Company, Winston-Salem, NC USA and ²American Snuff Co., LLC, Memphis, TN USA

The Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) formed a Smokeless Tobacco Sub-Group in 2008 to address, among other things, the need for new smokeless tobacco reference products. Specifications were agreed upon for 4 CORESTA reference products (CRP) in May, 2009 and an approximate 5-year supply was manufactured and made available since January, 2010 from the North Carolina State University Tobacco Analytical Services Lab. The new reference products include: Swedish-style pouched snus (CRP1); US-style moist snuff (CRP2); US-style dry snuff (CRP3); and US-style loose leaf tobacco (CRP4). The composition of these 4 new CRPs will be presented, along with results of analytical tests showing initial characterization and ongoing stability monitoring for nicotine, pH, moisture, and 4 tobacco-specific nitrosamines (NAB, NAT, NNK, NNN). Comparison of initial test results with those obtained after 18 months of storage (at -20°C) shows good stability for all analytes. In addition, more extensive initial chemical characterization has been conducted for a larger number of analytes (29) that includes all 9 Harmful and Potentially Harmful Constituents (HPHC) recommended for testing and reporting by FDA in their April 3, 2012 Draft Guidance for Industry. Examining the maximum percent difference within individual reference products across testing labs using in-house methods shows 50-90% difference for some analytes (e.g., nicotine, NNK, NNN) and 400-2300% difference for others (e.g., acetaldehyde, arsenic, B(a)P, formaldehyde). Such results are likely to be typical of the range in smokeless tobacco product constituent values that will be reported to FDA under the HPHC Draft Guidance as a result of employing multiple non-standardized test methods in multiple laboratories with unknown method variability and/or bias.

30. COMBUSTED, BUT NOT SMOKELESS TOBACCO PRODUCT PREPARATIONS, CAUSE DNA DAMAGE IN HUMAN ORAL CAVITY CELLS. Wolfgang ZACHARIAS¹, Hong Gao¹ and Gaddamanugu L Prasad²; ¹Brown Cancer Center, Univ. of Louisville, Louisville, KY USA and ²R. J. Reynolds Tobacco Company, Winston-Salem, NC USA

We examined the effects of reference tobacco preparations on DNA damage in human oral cavity cells. The oral squamous cell carcinoma cell line (101A), normal human gingival epithelial cells (HGEc), and human gingival fibroblasts (HGF) were treated with total particulate matter from 3R4F cigarettes (TPM), 2S3 smokeless tobacco extracted with complete artificial saliva (ST/CAS), or nicotine alone (NIC). Cells were treated for 24 hours with TPM at respective EC50 doses (13.7, 8.6 or 17.2 µg/ml of equi-nicotine units, as determined in previous experiments), or the doses with equi-nicotine units for ST/CAS.
Also, cells were exposed to a high dose of ST/CAS (565.3 μg/ml of equi-nicotine units). DNA damage in exposed cells was assessed by alkaline Comet assays and immunofluorescence staining for the damage-specific protein γ-H2AX.

Both assays showed that only TPM caused readily detectable DNA breaks in exposed cells whereas ST/CAS or NIC did not; only the high dose of ST/CAS caused some weakly measurable DNA damage. Intriguingly, the malignant 101A cells were more susceptible to DNA damage than the normal HGEC and HGF cells.

These studies demonstrate that combusted tobacco products can cause substantial DNA damage in normal and malignant oral cavity cells, whereas non-combusted ST/CAS, or NIC alone, exert no detectable or only minimal DNA damage after 24 hour of exposure. The data will assist in evaluating relative genotoxic and other harmful effects of different categories of tobacco products on oral cavity cells. Such knowledge may help to further understand the involvement of combusted versus non-combusted tobacco products in the etiology of oral cancers.

3:00 PM   MONDAY

31. MENTHOL DESORPTION PROPERTY FROM THE MENTHOLATED FILTER DURING SMOKING. Masato MIYAUCHI and Ayako Chiku; Japan Tobacco, Inc., Yokohama, Kanagawa, Japan

An empirical study has been carried out on the aspects of menthol distribution within a pack of cigarettes and menthol mainstream yield from the tobacco column and the cellulose acetate filter during smoking. As reported in our previous study, with the Micro-Raman Spectroscopy the menthol has diffused deeply into cellulose acetate fiber during aging. Therefore, the influence of the menthol diffusion on the mainstream yield from the filter should be investigated.

In this work, a GERSTEL thermal desorption–GC/MS method is implemented to determine the menthol amount desorbed from the fiber at the several conditions of temperature. Two series of tests were performed to evaluate the effect of plasticizer levels (0, 2, 6, 9%) and aging conditions (room or high temperature). Firstly, nearly all menthol was thermally desorbed below 200°C and the desorbed efficiency below 40°C showed no marked difference between any plasticizer levels. Secondly, after aging for 2 months at the high temperature (55°C), the desorbed efficiency below 80°C fell to an extremely low level.

A relationship between the thermal desorption efficiency and the mainstream yield was examined when the filter was aged with a mentholated tobacco column but smoked with an unmentholated tobacco column. The results show that the desorption efficiency below 40°C shows a linear correlation with the mainstream yield for all kinds of filters; 1) 4 plasticizer levels x 2 aging conditions, 2) 18 kinds of commercial cigarette brands. As a result, it was concluded that the thermal desorption property was an important factor affecting the mainstream yield and it was significantly affected by the aging temperature.

3:20 PM   Break
3:50 PM        MONDAY

32. EFFECT OF CELLULOSE ACETATE FILTER ON REMOVAL FOR SEMI-VOLATILE COMPOUNDS. Tatsuya MASUI and Masato Miyauchi; Japan Tobacco, Inc., Yokohama, Kanagawa, Japan

The filtration of semi-volatile compounds by cellulose acetate filter is very complex; semi-volatile compounds are removed not only by particle filtration but also by vapor adsorption, and their removal efficiency is higher than that for tar. It’s well known that the removal efficiency for tar is mainly determined by pressure drop. Although it has already been known that the removal of semi-volatile compounds is affected by many factors such as plasticizer additive value, the removal efficiency for semi-volatile compounds has not yet been summarized systematically by filter properties. The objective of this study was to specify filter properties affecting the removal efficiency for semi-volatile compounds and elucidate dominant removal process of them.

In experiments, cigarettes with various filters were smoked under ISO smoking condition with filter ventilation blocked and the smoke was analyzed by GC-MS. The filters ranged in length from 10 to 40mm, in denier value from 2.2Y35000 to 5.9Y35000, in plasticizer additive value from 0% to 6%.

The results showed the removal efficiency for semi-volatile compounds was expressed as a function of the surface area of cellulose acetate filters, which was considered as a factor affecting diffusion of vapors from a smoke stream to a filter and calculated using outer perimeters of the fibers measured by a microscope. It was also confirmed that the plasticizer additive value affected semi-volatile compound removal. Consequently, these results indicated that vapor adsorption was a dominant removal process of semi-volatile compounds.

4:10 PM        MONDAY

33. THE EFFECT OF SMOKING PARAMETERS ON THE YIELDS OF E-CIGARETTES. Tony MCCORMACK, Mike Taylor and Bill Guthery; Filtrona Technology Centre, Jarrow, UK

In recent years there has been a large increase in the number of e-cigarettes on the market, the majority of which are designed to resemble the appearance of a standard cigarette. These devices are generally claimed to deliver nicotine and tobacco flavour to the smoker without producing the combustion and pyrolysis products associated with lit cigarettes. Most e-cigarettes use a heating element to vaporize liquids contained within a separate attached cartridge and thereby enable the delivery of these vapours to the smoker. As a result, the way in which an e-cigarette produces and delivers substances to the consumer is totally different to conventional cigarettes and it may be anticipated that smoking regimes that have been developed for standard cigarettes may not provide the most suitable basis comparing yields from e-cigarettes. Owing to the very recent introduction of these products, little information is currently available into the effects of puff volume, puff duration and puff number on the yields of e-cigarettes. This present work is part of a programme intended to provide more data into the effects of smoking parameters on the yields of e-cigarettes.
In this paper, results will be presented into the effect of puff number on the yields of total particulate matter, nicotine and water from a range of different commercially available e-cigarettes and these results will be compared with data from standard cigarettes.

4:30 PM    ADJOURN
TUESDAY MORNING, SEPTEMBER 11, 2012
SESSION A  Session Chair: David Zaitlin

8:50 AM TUESDAY

34. EFFECTS OF VARIETY AND HARVEST MANAGEMENT ON CURED LEAF QUALITY AND TSNA CONTENT OF BURLEY TOBACCO. Robert D. MILLER, Lowell P. Bush and Anne M. Jack; University of Kentucky, Lexington, KY USA

A study was conducted to determine the relative effects of variety, transplant/harvest date, and harvest management on leaf quality and TSNA content of burley tobacco. Six varieties were evaluated at two transplant/harvest dates at three locations. For each date, all plots were cut on the same day. Harvest management treatments included pick up from the field on the same day as cutting, three days after cutting, six days after cutting, and ten days after cutting. When data were combined across locations, transplant/harvest dates, and harvest management practices, varieties had a minimal effect on cured leaf quality. However, much larger effects on quality were observed between transplant/harvest dates and among harvest management treatments, particularly when rainfall occurred while the tobacco was in the field. In the absence of rainfall, the grade index for the earlier transplant/harvest date was 19 points higher than for the later date. Leaving the tobacco in the field for up to six days before pick-up resulted in a relatively small decrease in the grade index if no rainfall occurred; four tobacco companies that evaluated the cured leaf preferred tobacco that had been left in the field six days rather than three days in terms of usability and price per pound. However, waiting ten days before pick-up resulted in a noticeable decrease in quality. Rainfall occurring shortly after cutting resulted in a 30-50 point drop in the grade index, regardless of how long the tobacco remained in the field. A subset of the varieties is being analyzed for TSNA content, with results to be presented at the TSRC meeting.

9:10 AM TUESDAY

35. THE EFFECT OF HARVEST MATURITY ON TSNA ACCUMULATION IN BURLEY TOBACCO – DIFFERENT APPROACHES. Anne JACK, Colin R. Fisher, Angela Schoergendorfer, Huihua Ji, Neil F. Fannin and Lowell P. Bush; University of Kentucky, Lexington, KY, USA

The accumulation of tobacco specific nitrosamines (TSNAs) in burley tobacco is affected by a number of interacting factors. Previous work by several researchers has indicated an initial increase in TSNA accumulation with increasing maturity at harvest, but no increase or a decrease in the later harvests. In all these studies, the treatments were transplanted at the same time, and harvested at different times, so that different treatments were cured under different conditions. It is likely that the later harvests had lower TSNAs than expected because curing conditions later in the season are less favorable for TSNA accumulation. In this two-year study, our objective was to harvest at 21, 28 and 35 days after topping, but cure all treatments under the same conditions. To achieve this, the tobacco was transplanted and topped at seven day intervals, then harvested and cured together. Varieties were TN 90LC and TN 90H, a high converter selection. There were no significant differences between treatments for total TSNAs in TN 90LC, but in TN 90H, the 35-day harvest had higher
TSNAs than the 28- and 21-day harvests (7.8, 5.7 and 5.3 ppm, respectively). Total alkaloids would be expected to increase with maturity, but while the 28-day harvest was higher than the 21-day harvest (5.9 and 4.1 % DM, respectively), the 35-day harvest was similar to the 21-day harvest (4.4 % DM). We attribute this to the fact that the basal fertilizer was all applied at the time of the first transplanting, and the sidedressing was insufficient to correct for subsequent leaching. Without controlled curing, there are problems with both approaches to this type of study.

9:30 AM TUESDAY

36. IDENTIFICATION OF FRUCTOOLIGOSACCHARIDES IN CURED TOBACCO LEAVES. Atsushi NAGAI¹, Takeshi Yamamoto¹ and Hiroyuki Wariishi²; ¹Japan Tobacco Inc., Yokohama, Kanagawa, Japan and ²Graduate School of Bioresource and Bioenvironmental Sciences, Kyusyu University, Japan

Sugar is one of the main components of tobacco leaves, and is related to the taste and aroma of cigarette smoke. It is important to investigate the sugar composition in order to understand the quality of tobacco products. In this study, several oligosaccharides in cured tobacco leaf were examined. Fructooligosaccharides (FOSs) and maltooligosaccharides (MOSs) in cured tobacco leaf (Nicotiana tabacum) were detected and quantified using high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry.

Although these oligosaccharides were not detected in air-cured tobacco leaf, they were present in flue-cured Virginia and sun/air-cured tobacco leaf. However, no FOSs such as kestose, nystose and fructosyl-nystose have been reported in green tobacco leaves yet, and are therefore considered to form after harvesting.

In order to clarify the formation mechanism in detail, the effect of storage on cured tobacco leaves was subsequently estimated using the following samples: a burley type leaf with sucrose, without sucrose and a heat-treated burley leaf prior to the addition of sucrose. The results revealed that the formation of FOSs in cured tobacco leaves occurred in the samples with sucrose and that the increase of FOSs was interrupted by the heat-treatment of tobacco leaves. The changes in the amounts of FOSs were thought to be caused by enzymatic reactions using sucrose as a substrate.

9:50 AM TUESDAY

37. A NUMERICAL STUDY ON FLOW FIELDS OF CONDITIONING ROOM IN TOASTER. Yongjoon JANG and Hanjoo Chung; KT&G Research Institute, Daejeon, Korea

The purpose of the toasting process is to adjust for better taste quality of Burley tobacco intended for American-blend products. The Burley tobacco toaster is normally tunnel-shaped, with the tobacco passing through it on an apron conveyor belt. In the first chamber, the tobacco is heated to 120-160°C, in the second it is cooled and in the third it is remoistened to a limited extent either by means of steam or by water sprayed through a very fine nozzle. In a conditioning room, the air flow at the apron is not well distributed because this space has structural limitations.
In this study, the air flow and the droplet motion in the conditioning room were predicted using computational fluid dynamics. Numerical simulations of the fluid flow, discrete phase models were done in Fluent 6.03. Steady state was calculated with varying operations and boundary conditions. The flow resistance of the apron was formulated as a porous jump model. The predicted flow fields at the apron are shown to be very sensitive to the guide vane position. The guide vane location optimization in the conditioning room was interpreted to mean that the humidity effect could be further improved.

10:10 AM  Break

10:40 AM TUESDAY

38. SMALL-SCALE PRODUCTION OF PROCESSED CIGARETTE LEAF (“PCL”). Michael F. FORBES and John H. Lauterbach; Lauterbach & Associates, LLC, Macon, GA USA

Processed cigarette leaf (“PCL”) refers to several types of band-cast reconstituted tobaccos developed by Brown and Williamson Tobacco Corporation (“B&W”) and other British-American Tobacco (“BAT”) units during the 1950s and 1960s (http://legacy.library.ucsf.edu/tid/wsa51f00/pdf). In the B&W implementation of PCL, there were no chemical additives used in the PCL process except for addition of glycerin as a humectant. PCL was used until the late 1970s, when it was replaced by a paper-type reconstituted tobacco that had much reduced costs associated with its use. However, the PCL process may offer advantages today if it could be used to produce a truly additive-free reconstituted tobacco. Consequently, we have produced PCL on a laboratory-scale using a recipe and process conditions from a 1967 B&W report (http://legacy.library.ucsf.edu/tid/hao00f00/pdf), and we have profiled the starting furnish and finished product with classical chemical analyses and two GC-MS techniques. Aqueous slurries of ground burley stem and ground flue-cured stem were cooked under pressure for about 30 minutes at 143°C and 132°C, respectively. The cooked slurries were mixed and processed in a high-speed blender. After additional dilution with water, mixed 50/50 flue-cured/burley lamina fines were added (lamina/stem 1.4/1), and the mixture was cooked for 4 h at about 90°C with further water added to dilute the slurry to about 9% solids. The slurry was applied to drying sheets and dried at 100°C until most of the water had evaporated. At ~ 10% oven volatiles, the sheets were flexible and routine analyses showed alkaloids 2.1%, total sugars 8.8%, reducing sugars 9.5%, nitrate 2.3%, and chloride 1.5%.

11:00 AM TUESDAY

39. THE DETAILED CHEMISTRY OF PCL AND ITS IMPLICATIONS ON PRODUCT ACCEPTABILITY. John H. LAUTERBACH and Michael F. Forbes; Lauterbach & Associates, LLC, Macon, GA USA

Processed cigarette leaf (“PCL”) refers to several types of band-cast reconstituted tobaccos developed by Brown and Williamson Tobacco Corporation (“B&W”) and other British-American Tobacco (“BAT”) units during the 1950s and 1960s (http://legacy.library.ucsf.edu/tid/wsa51f00/pdf). In the B&W implementation of PCL, there were no chemical additives used in the PCL process except for addition of glycerin as a humectant. PCL was
used until the late 1970s, when it was replaced by a paper-type reconstituted tobacco ("PJS") that had much reduced costs associated with its use. However, when PCL was replaced by PJS in 1979, the market share of B&W’s flagship brand, Kool, began a steady decline. Did the change from PCL to PJS precipitate the decline in Kool or were other factors at work? We have attempted to answer this question by producing PCL on a small scale and having routine and detailed chemical analyses performed on the PCL we produced as well as on the tobaccos that were used to make PCL. Our GC-MS analyses and routine analytical data indicated that the PCL process resulted in hydrolysis of carbohydrates during the cooking of the mixture of autoclaved stems and lamina fines. These reductions point to the generation of new species that improve both the physical quality of the sheet and its smoking properties. These physical and chemical changes would likely bring improved acceptability in a cigarette blend over a similar blend with a PJS-type reconstituted tobacco substituted for the PCL.

11:20 AM    TUESDAY

40. DETERMINATION OF GLUCOSAMINE AND MANNOSAMINE IN TOBACCO BY LC-MS/MS. Eun-Jung HAN, Hye-Joeng Min and Hye-Gun Kim; KT&G Research Institute, Daejeon, Korea

Nitrogenous compounds such as amino acids and proteins are frequently analyzed in tobacco since they are considered to be main precursors of biologically active substances in cigarette smoke. However, relatively less attention has been paid to other class of nitrogenous compounds such as amino sugars and deoxyfructosazines, although their levels in tobacco are known to be equal to or even higher than those of most free amino acids. These nitrogenous compounds seem to contribute to the formation of biologically active substances in smoke, or characteristic sensory properties of cigarette smoke. Therefore, to set up an analytical method for those compounds became important.

This study describes a procedure for the analysis of mannosamine and glucosamine in tobacco and employed a liquid chromatography-tandem mass spectrometry (LC-MS/MS) technique to quantify amino sugars. Sample preparation steps consist of the extraction of the tobacco with a mixture of water and methanol (9:1), followed by filtration through syringe filter (0.2 µm). Analytes were separated on a HILIC column. Recovery was ranged from 90% to 100%, and RSD was lower than 5%. Contents of glucosamine and mannosamine in some tobacco were determined by this method. This analytical method seems to be applicable to tobacco research relevant to tobacco aging or tobacco toasting.

11:40 AM    LUNCH
TUESDAY MORNING, SEPTEMBER 11, 2012

SESSION B  Session Chair: Paul Busby

8:50 AM  TUESDAY

41. FIT FOR PURPOSE BIOANALYTICAL VALIDATION AND SAMPLE PROCESSING.
Raymond H. Farmen and Kirk E. Newland; Celerion, Lincoln, NE USA

Since the tobacco industry is moving into the area of regulated bioanalysis we have noted that there is a lot of confusion surrounding fit-for-purpose bioanalytical methods. Additionally, there are many bioanalytical guidances from different regulatory agencies that sometimes seem to contradict each other. Celerion is a CRO that has a long history of regulated bioanalytical expertise. This presentation will focus on explaining the following bioanalytical topics with a focus on compliance:

1. GLP – when is a study truly GLP, and when does a study follow GLP guidelines?
2. The importance of assuring sample integrity.
3. An analytical batch; this is the cornerstone of bioanalytical chemistry and its principles are very different from clinical chemistry and GMP assays.
   a. The value of standards
   b. The relationship between standards and quality control samples
   c. The importance and timing of proper chromatographic integration
   d. The various types of regression parameters used to define a batch
   e. Batch acceptance criteria
   f. Dilution integrity
4. The importance of incurred sample reproducibility will be explored.
5. A proper way to report sample concentrations will be discussed

Depending upon the type of study and how the results will be used determines which criteria and which tests need to be performed to make the method “fit-for-purpose.” How a “fit-for-purpose” method will be used must be defined a priori.

9:10 AM  TUESDAY

42. DETERMINATION OF COUMARIN AND AFLATOXIN IN TOBACCO AND SMOKELESS TOBACCO PRODUCTS BY LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY.
Jingcun Wu, Bill Rickert, Andrew Masters and Peter Joza; Labstat International ULC, Kitchener, Ontario, Canada

A simple and selective method for the determination of coumarin has been developed using isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS). In this study, a simple ethanol;water (1:1 v/v) extraction with coumarin-5,6,7,8-d4 as the internal standard was used. The mass detection conditions were optimized utilizing three mass transition (MS/MS) pairs. The method exhibits good linearity (R² ≥ 0.999) and a wide concentration range (0.1-1000ng/mL) with a lower limit of quantification (LLOQ) near 67 ng/g. The method was validated with three reference products; Kentucky Reference KY3R4F cigarette filler, 1S2 dry snuff and 2S3 moist snuff. While no coumarin was detected in KY3R4F, coumarin was found in both smokeless tobacco products 1S2 and 2S3.
(approximately 1100 and 800 ng/g respectively) with relative standard deviations less than 10% for both samples (n=7).

An independent LC-MS/MS method was developed for the determination of aflatoxins (B1, B2, G1 and G2). Tobacco product was extracted with methanol:water (80:20 v/v) using a commercially available internal standard (U-[13C17]-AFB1). After dilution with a phosphate buffer solution (PBS), sample clean up was achieved using an immunoaffinity cartridge. This procedure was simplified when only aflatoxin B1 was required using only water. Three mass transitions for each analyte were evaluated. The method exhibits good linearity (R² ≥ 0.999) over a concentration range of 0.1 to 100ng/mL. In this study, no detectable levels of aflatoxins were found in the reference products (1S2 dry snuff and 2S3 moist snuff). Method accuracy and precision was assessed using laboratory fortified matrix samples with recoveries ranging from 89.7 to 109% across all aflatoxins tested.

9:30 AM TUESDAY

43. A SENSITIVE METHOD FOR QUANTIFICATION OF HYDRAZINE IN MAINSTREAM TOBACCO SMOKE. Mehran SHARIFI, Carmen Donisa, Peter Joza, Andrew Masters and William Rickert; Labstat International ULC, Kitchener, Ontario, Canada

The recent publication of the FDA's HPHC list suggests that reporting of the hydrazine content of mainstream smoke will eventually be required. This paper describes method development challenges, such as potential loss of hydrazine through interactions with other smoke constituents (e.g. volatile carbonyls).

The mainstream smoke of cigarettes was passed through a glass fiber filter pad followed by a trap containing 40mL of an aqueous buffer:methanol (55:45, v/v) solution, with 2-nitrobenzaldehyde (10 g/L) used as a derivatizing agent and 15N₂-hydrazine used as internal standard. After smoking, the filter pad was extracted with the trapping solution then incubated for 30 minutes at 35°C. An aliquot of the extract was centrifuged and the resultant hydrazone was quantified by liquid chromatography tandem mass spectrometry (LC-MS/MS). Two transitions were monitored for both the hydrazone and the internal standard, with the more abundant transitions used for quantification.

This LC-MS/MS method demonstrated good linearity (R² > 0.999) from 0.079 to 248 ng/mL, with a limit of quantification in mainstream smoke of 0.2 and 0.4 ng/cig for ISO and Canadian Intense smoking regimens respectively. The method recovery was assessed using samples spiked with known amounts of hydrazine. The results showed good accuracy with recovery percentage values ranging from 98 to 111%. Although there were no detectable levels of hydrazine in the reference cigarettes used in the validation (KR3R4F), the method precision was estimated to be approximately 10% based on the variability of spiked samples. Trapping efficiencies were assessed using a hydrazine permeation tube which provided a known amount of hydrazine.
44. PUFF-BY-PUFF ANALYSIS OF MAINSTREAM SMOKE CONSTITUENTS OF NON-LIP/FSC AND LIP/FSC CIGARETTEs. Stefan BACHMAN, Maria Gleinser, Dieter Möhring, Irene Rohregger and Dietmar Volgger; Papierfabrik Wattens GmbH & Co KG, Wattens, Austria

Cigarette base paper parameters like permeability and burn additives have a large impact on mainstream smoke yields of cigarettes. Especially CO and potentially also CO$_2$ and O$_2$ yields could change by applying LIP/FSC-bands on cigarette papers. Other analytes will show similar trends. Physical band parameters like permeability, diffusivity as well as the band design have an influence on those smoke yields.

The sample set involved cigarettes with permeabilities in a range of 50-130 CU and burn additive levels between 1-2%. Cigarettes have been produced with base papers containing bands with different diffusivities and band designs. The sample cigarettes were smoked according to ISO 3308, with fully blocked filter ventilation. Changes in mainstream smoke yields of CO, CO$_2$ and O$_2$ and other analytes will be presented.

A single channel smoking machine (Borgwaldt RM1) and a mass analyzer (Airsense Compact) were used for the determination of mainstream smoke yields. A specially designed interface was applied to split a fraction of the mainstream smoke. The smoking machine triggered the interface in such a way that smoke can only enter the mass analyzer during a puff. Electron impact ionization was used for detecting CO$_2$ and O$_2$ whereas CO and other analytes were determined by ion molecule reaction ionization.

This study compares smoke yields of non-LIP/FSC and LIP/FSC cigarettes with different base paper parameters (e.g. permeability, burn additives), different band diffusivities and different band designs. To determine further differences between non-LIP/FSC and LIP/FSC cigarettes puff-by-puff analysis have been carried out.

Further smoke constituents have been measured by smoking cigarettes through desorption tubes and were analyzed by GC-MS afterwards. A puff-by-puff smoking regime served to determine the differences between a puff smoked on a band area and a puff taken on the band spacing.

10:10 AM        Break

10:40 AM        TUESDAY

45. COMPARISON OF ANALYTICAL DATA PROVIDED BY DIFFERENT LABORATORIES. Xavier CAHOURS$^1$, Thomas Verron$^1$, Steve Purkis$^2$ and Stéphane Colard$^2$; $^1$SEITA, Imperial Tobacco Group, Fleury-les-Aubrais, France and $^2$Imperial Tobacco Limited, Bristol, UK

Regulatory authorities are currently discussing the measurement and imposition of ceilings on certain smoke constituents. However, if routine measurement of these constituents is to be required, the laboratories need to use the same validated methods and the precision
of the methods has to be known. To assess method precision, the widely used method is to perform a collaborative study in order to determine the repeatability and reproducibility. Therefore, comparing data coming from different laboratories are relevant only if all the uncertainties are taken into account. For doing that, the ISO 5725 part 6 [1] recommends using the critical difference (described in the standard) that is based on the precision of the method.

In this paper, using the CORESTA 2006 joint experiment data [2], we show, on a number of smoke constituents from reference cigarettes, the importance i) to use the appropriate statistical methods to compare results from different laboratories in order to avoid misleading conclusions; ii) to have validated and standardised methods with known precision. Moreover, using the critical difference computed from repeatability and reproducibility of the methods, we demonstrate that the number of replicates have a small effect on product comparison.


11:00 AM TUESDAY

46. ANALYTICAL METHOD VALIDATION – APPLICATION OF THE ACCURACY PROFILE TO THE METHOD “DETERMINATION OF TOTAL ALKALOIDS IN TOBACCO. Béatrice TEILLET¹, Thomas Verron¹, Xavier Cahours¹ and Stéphane Colard²; ¹SEITA Imperial Tobacco Group, Fleury-les-Aubrais, France and ²Imperial Tobacco Limited, Bristol, UK

In the context of methodology for cigarette product characterization, it is important to subject the method to its intended-use (e.g. regulatory requirement) in order to confirm its “fitness for purpose” as described in ISO17025 and ASTM E2857-11. Method validation by the accuracy profile approach determines if the method can provides future results with the required accuracy (precision and trueness). As an example, validation of the determination of total alkaloids in tobacco by continuous flow analysis illustrates how this approach can be carried out. The end-user defines first the limit of acceptability (e.g. ±15%) on the difference between the measurement and the true value, and the confidence level of the tolerance interval (e.g. 95%). As no reference products with assigned values currently exist, 5 different samples with different alkaloid levels from 0.27 to 4.10% (dry weight basis) have been selected and values were assigned by spike calibration. The experimental design consisted of preparing 2 independent replicates of the calibration standards and tobacco samples. This was repeated for 5 days involving 3 different operators preparing new reagents each day. From this experimental design, Intermediate precision standard deviation, bias, acceptability interval and expected tolerance interval are calculated. The accuracy profile representation allowed the conclusion that the total alkaloid method is valid from 0.73 to 4%, with 0.73% as the limit of quantification. Unlike classical validation methods that do
not manage simultaneously trueness and precision, the Accuracy Profile approach allows a clear and easy comparison between method performance and the intended use.

11:20 AM TUESDAY

47. CIGARETTE BURNING AND ALTERNATIVE SMOKING REGIME. Stéphane COLARD¹, Thomas Verron², Steve W. Purkis¹ and Xavier Cahours²; ¹Imperial Tobacco Limited, Bristol, UK and ²SEITA, Imperial Tobacco Group, Fleury-les-Aubrais, France

Context and objective: Some regulations require the use of two smoking regimes. The objective of this study is to better understand the impact of the smoking regime on the cigarette burning process.

The combustion of a cigarette during smoking can be described as successive steps of active (during the puff) and passive (smoldering) burning from which a simple modeling approach can be derived. A sequential cigarette burning model has been developed in order to better understand the impact of the smoking regime on the cigarette burning process. This model takes into account a limited number of cigarette characteristics (smoldering rate, paper and filter ventilations), and three smoking parameters (puff volume, duration and frequency).

The calculated number of puffs has been compared to the measured values from a ventilated cigarette smoked under 32 different smoking regimes, with filter ventilation holes blocked or opened. The close values of the measured and calculated number of puffs have validated the proposed model. Several parameters can be deduced from this approach such as the length and weight burnt during the puffs or the burning time. The results compare well with other published models.

The approach has then been used to understand the tar, nicotine and carbon monoxide yields as a function of the smoking regime, and in particular to investigate the effect of the filter ventilation blocking. It is concluded that when using the ISO smoking regime as the basic smoke collection method, any other regime is of poor added value in terms of product characterization.

11:40 AM LUNCH
TUESDAY AFTERNOON, SEPTEMBER 11, 2012

SESSION A  Session Chair: Karen Williams

1:30 PM  TUESDAY

48. SMOKING MACHINE DESIGN AND YIELD ERRORS UNDER INTENSE SMOKE REGIMES. PART 1: THE INFLUENCE OF DEAD VOLUME ON YIELD. Ian F. TINDALL\textsuperscript{1}, Linda P. Crumpler\textsuperscript{2} and Timothy J P Mason\textsuperscript{1}; Cerulean, \textsuperscript{1}Milton Keynes, UK and \textsuperscript{2}Richmond, VA USA

The difference in key analyte yields between smoking machine employing the rotary and linear methods has long been tolerated on the basis that these are small under the ISO regime and within the limits of expected experimental variance. These differences are magnified under Canadian Intense conditions and give rise to concerns that machines using the rotary principle for smoking have low NFDPM, water and TPM yields.

The influence of the essential difference in design of a remote capture pad has been investigated by eliminating other machine differences and “mimicking” the behavior of the two different machine types on a single machine. Careful deconstruction of the smoke path has shown that the “dead volume” in a rotary system is both greater than a linear system and significant in determining the apparent yield. Moreover this effect is exacerbated by using the CI regime through a change in the smoke matrix formed. The relationship between regime conditions and dead volume has been investigated and an empirical relationship derived.

The relationship between puff volume and vapor desorption is identified as a secondary mechanism for lowering yields and is the subject of a separate paper. The hazards inherent in using a remote capture pad and the consequences for CI smoking are made clear. Recommendations for design changes that minimize the apparent lowering of yields are presented.

1:50 PM  TUESDAY

49. SMOKING MACHINE DESIGN AND YIELD ERRORS UNDER INTENSE SMOKE REGIMES. PART 2: THE INFLUENCE OF PUFF VOLUME ON DESORPTION OF VOLATILE SMOKE COMPONENTS. Ian F. TINDALL\textsuperscript{1}, Linda P. Crumpler\textsuperscript{2} and Timothy J P Mason\textsuperscript{1}; Cerulean, \textsuperscript{1}Milton Keynes, UK and \textsuperscript{2}Richmond, VA USA

The difference in key analyte yields between smoking machine employing the rotary and linear methods has long been tolerated on the basis that these are small under the ISO regime and within the limits of expected experimental variance. These differences are magnified under Canadian Intense conditions and give rise to concerns that machines using the rotary principle for smoking have low NFDPM, water and TPM yields.

The hypothesis that these differences are in part due to the greater volume of air passing through the capture pad in CI smoking was explored by a series of experiments capturing the desorption products from smoking in both ISO and CI modes after the pad capture
stage. These were found to be more significant in the CI system and constitute a major loss of condensate to the system.

Analysis of the desorption products allowed an estimate of the proportion of water present in this desorbed fraction and how this influences the apparent water content of the smoke matrix. Furthermore the higher volume of air passing through the capture pad in the rotary system was found to be significant in the vapour losses observed.

A model was developed using a synthetic TPM mixture that allowed the relationship between measured yield, puff intensity and machine type to be defined empirically. The problems inherent in using a capture pad with high total air flow and the consequences for CI smoking are made clear. Predictions are made on the basis of an empirical model developed using the synthetic TPM mixture.

2:10 PM TUESDAY

50. AN APPROACH TO THE DEVELOPMENT OF TEST METHODS IN A REGULATED ENVIRONMENT. Carol H. A. GOSS and Christopher G Wright; British American Tobacco, Southampton, UK

Incoming regulation of tobacco and tobacco products requires scientifically robust methods to ensure that regulatory standards can be clearly defined and to enable manufacturers and regulators to generate comparable testing data. Although many laboratories have developed and validated test methods to determine the constituents of tobacco and tobacco smoke, few methods have been cross-validated between laboratories and for many of the chemical constituents of tobacco and smoke the between-laboratory comparability of data may be insufficient to meet future needs.

Key to the development of test methods are a clear definition of the intended purpose, appropriate performance/technical specifications and a rigorous process to evaluate, optimise and validate candidate methods. In order to deliver appropriately selective, sensitive and rugged methods, best technical practice must be applied which includes the removal of matrix effects (for example by use of orthogonal clean-up), the optimisation of chromatographic resolution, the use of stable-isotope dilution approaches where practicable and the provision of structural information (e.g. mass spectrometric or spectroscopic) to assure unambiguous measurement. Cost efficiency and the ability to generate large volumes of data quickly must also be considered.

The presentation will review analytical method development and explore the potential to improve the robustness of this process.

2:30 PM TUESDAY

51. THE SCIENCE REQUIRED FOR SUCCESSFUL BIOANALYTICAL METHODS. Ridha NACHI; Celerion, Lincoln, NE USA

The selectivity advantage of MS/MS technology alone is not always sufficient to develop the most suitable method to successfully analyze biological samples. The need for extremely low
limits of quantification in bioanalysis requires strong coordination of sample preparation, chromatographic separation and MS/MS conditions. The use of a novel derivatization, two-dimensional chromatography and specialized internal standard are three techniques used to provide robust bioanalytical data. Several different approaches used in the development of methods for tobacco related compounds will be presented. The low limit of quantification of 50 fg/mL for 3-hydroxybenzo[a]pyrene (3-OHBAP) in human urine was achieved by combining an aggressive sample cleanup utilizing the hydrophobic nature of the analyte with derivatization to compensate for the poor efficiency of ionization and mass fragmentation. The combination resulted in a 20-fold increase in sensitivity. The RP UHPLC separation provided additional selectivity to the method as well as increasing signal to noise due to narrow peaks. An elegant two-dimensional chromatography strategy was combining similar column chemistries under different pH conditions was used to provide the needed selectivity to achieve a sub pg/mL limit of quantification for N’-nitrosonornicotine (NNN) at 0.75 pg/mL in human urine.

A more traditional two-dimensional LC-MS/MS approach using different types of columns was applied to develop a robust and selective method for monohydroxy-3-butenyl mercapturic acid (MHBMA) in human urine. The fast separation and narrow peak obtained with UHPLC condition may require re-evaluation of the stable internal standard. Carbon-13 labeled internal standards were significantly superior to deuterated internal standards for mercapturic acids, including hydroxybutyl mercapturic acid (HBMA) and 2-cyanoethyl mercapturic acid (CEMA) with respect to compensation for matrix effects and maximizing reproducibility.

2:50 PM Break

3:20 PM TUESDAY

52. EXTRACTION EFFICIENCY OF SELECTED ALKALOIDS FROM TOBACCO PRODUCTS. Alexandra MARTIN and Fraser Williamson; Arista Laboratories, Richmond, VA USA

Nicotine and four minor alkaloids (nornicotine, anabasine, myosmine and anatabine) are compounds synthesized within the tobacco plant and are precursors of the tobacco-specific N-nitrosamines (TSNAs). The accurate quantitation of these compounds is important in evaluating tobacco leaf quality and final product composition. Products on the market today consist of a wide variety of tobacco blends and widely differing levels of these compounds. Also, many new smokeless tobacco products (STPs) contain flavors that have been shown to compromise quantitation in currently published methods (e.g. CDC method for nicotine).

The objective of this work was to develop a simple, robust and efficient method to analyze nicotine and the minor alkaloids in a wide range of tobacco products/matrices without requiring standard addition. A variety of published methods were evaluated with the focus on extraction efficiency and quantitation. It was observed that the most difficult alkaloid to extract was nornicotine. The methods examined required up to 3 hours to reach an extraction plateau, which is often (erroneously) equated with complete extraction.
Due to the poor extraction efficiency of the methods evaluated, a new method was developed that extracts the alkaloids within 30 minutes using 5N NaOH (aqueous) to pretreat the matrix and diluting with methanol to make the extract suitable for GC/MS. By using GC/MS as the analytical system, the alkaloid levels in Quest 3 tobacco and a variety of flavored STPs were determined with excellent precision and no observed interferences. The levels of nornicotine from this method ranged from 15 to 80% higher than the other methods tested, while the nicotine was not significantly different.

3:40 PM TUESDAY

53. COMPARISON OF THE LEVELS OF SEVERAL ORGANIC ACIDS IN DIFFERENT TOBACCO TYPES. Serban MOLDOVEANU, R. J. Reynolds Tobacco Company, Winston-Salem, NC USA

The content in tobacco leaf of several organic acids, including citric, malic, trihydroxybutanoic (two isomers), and quinic is relatively high. The levels of these acids are different in burley, flue-cured and oriental, and they contribute to distinction between these tobacco types. Tobacco specific acids are important contributors to the taste of cigarette smoke as well as that of alternative tobacco products (e.g., snus). Quantitative determination of these acids poses several analytical problems, caused by the lack of chromophors in the molecules of the acids and their high hydrophilic character. An analytical method using LC/MS/MS with separation on a special column that can be used with 100% aqueous mobile phase has been developed and applied for the quantitation of tobacco specific acids. The MS instrument used for the analysis was an API-5000 from AB Sciex connected to an HPLC system, from Agilent. The column used for the separation was a Synergy 4u-Hydro RP, 4.6 x 250 mm with 4 mm particles, from Phenomenex. The separation was done isocratically with a mobile phase consisting of an aqueous solution of 0.04 M HCOOH brought at pH 2.85 with a diluted solution of ammonia. The flow rate of the mobile phase was 0.8 mL/min. To the post column flow was continuously added acetonitrile at a flow rate of 0.3 mL/min, and the total flow was split 50/50 to waste and MS for reducing the amount of liquid introduced in the ion source. The detection was done by electrospray (ESI) working in negative ionization mode and using multiple reaction monitoring (MRM) with specific parent/daughter ions for each analyte. Citric acid-d$_4$ was used as an internal standard. For the analysis, 50 mg tobacco was treated with 1 mL extracting solution for 30 min at 78°C. The solution was filtered through 0.45 mm PVDF membrane and diluted 50 times with the mobile phase, for analysis. The extracting solution consisted of 0.04 M HCOOH in water.

4:00 PM TUESDAY

54. N-NITROSODIETHANOLAMINE IN TOBACCO: METHOD VALIDATION AND LEVELS PRESENT IN US TOBACCO PRODUCTS. Fraser WILLIAMSON and Alexandra Martin; Arista Laboratories, Richmond, VA USA

N-Nitrosodiethanolamine (NDELA) is a non-volatile nitrosamine classified by the International Agency for Research on Cancer (IARC) as a group 2B carcinogen. It has been identified in both tobacco and tobacco smoke and is currently included on the United States Food and Drug Administration (FDA) list of Harmful and Potentially Harmful Constituents (HPHCs). NDELA is not a natural component of tobacco or tobacco smoke.
and its presence is due entirely to the pesticide MH-30 (Trademark of Chemtura Corp.), which consists of the diethanolamine salt of maleic hydrazide. The use of MH-30 was suspended in the early 1980s eliminating the pathway for the introduction of NDELA in tobacco.

The objective of this study was to validate an analytical method for NDELA in tobacco and evaluate levels present in various research products manufactured in the United States since the late 1960s together with commercially available products. This study is not meant to be an exhaustive analysis of all tobacco products on the US market, but rather a selective analysis of the various product types available.

NDELA was extracted from tobacco with water and partitioned into ethyl acetate with excess sodium sulfate. An aliquot of the ethyl acetate fraction was dried with sodium sulfate, derivatized with BSTFA and analyzed by gas chromatography using nitrogen chemiluminescence detection. Quantitation was performed using N-Butyl-N-(4-hydroxybutyl)nitrosamine as an internal standard.

Levels observed in the research products tested ranged from not detected to 3400 ng/g. NDELA was not detected in any of the commercial samples analyzed and this study suggests that this compound should be removed from the HPHCs list.

4:20 PM TUESDAY

55. THIS BLUNT IS FOR YOU – THE ROUTINE AND DETAILED CHEMISTRIES OF BLUNTS AND BLUNT WRAPS. John H. LAUTERBACH and Deborah A. Grimm; 1Lauterbach & Associates, LLC, Macon, GA USA and 2Tulane University, New Orleans, LA USA

At the FDA’s February 29, 2012, meeting, Expanding the Research Base for Tobacco Product Regulation, a member of the audience asked FDA scientists about what they were going to do about blunts. Unfortunately the FDA scientists did not know much about blunts and a related product, blunt wraps. The purpose of this presentation is to provide routine and detailed chemical data on blunts and blunt wraps. Blunts are inexpensive machine-made cigarillos typically about 110 mm long and about 10 mm in diameter. They often have both a wrapper and a binder and generally do not have a filter. Many are flavored with fruit flavors. The tobacco fillers in blunts were lower in alkaloids and sugars than were the fillers in filtered cigars. GC-MS analyses indicated presence of humectants such as propylene glycol. Blunt wraps (aka wraps) are pieces of very flexible reconstituted tobacco about 50 mm in width and 110 mm in length. They are sold in sealed pouches with each wrap and a plastic sheet rolled around a plastic tube. The wraps are quite moist (> 20% oven volatiles) and contain humectants such as propylene glycol. Samples of wraps must be dried over a desiccant before DS scan analyses are attempted. Alkaloid and nitrate levels are typically less than 1%. GC-MS of methanol extracts showed that products contain benzyl alcohol and compounds associated with the flavors such as grape, strawberry, and cherry.

4:40 PM ADJOURN
56. CIGARETTE SMOKE INDUCES INFLAMMATION AND OXIDATIVE STRESS IN THE BRAINS. Ashwani KHANNA and Walter Royal, III; University of Maryland, Baltimore, MD USA

Chronic tobacco exposure is known to affect central nervous system (CNS) resulting in the high risk for the development of diseases such as multiple sclerosis, dementia, Parkinson, Alzheimer’s and others. Despite this knowledge, direct effect of cigarette smoke (CS) exposure on the brain has not been systematically investigated. Cigarette smoke exposure is known to induce systemic inflammation and oxidative stress, two major mediators of its detrimental effects in habitual smokers. This is, in particular, due to the inability of scientists to perform studies among the human population, since it is difficult to control and monitor smoke exposure. Therefore, we have developed a system to simulate human smoking behavior and performed investigations in a rat model. Rats were exposed to cigarette smoke (4 cigarettes/day for 30 days) and the efficacy of smoke exposure was confirmed by quantization of cotinine, the metabolite of nicotine, the key component of cigarette smoke. Rats were exposed to CS from 4 cigarettes/day for 30 days using a system developed to simulate human smoking behavior. After 30 days, the animals were sacrificed, brain and spleen tissues were collected and serum was separated from blood. Immunocytochemical analysis of brains from the rats showed, compared to rats not exposed to CS, there was increased staining for CD4, TNFalpaha-α, IFNgamma-γ, class II MHC, ED, Nrf2 and GFAP. Using quantitative PCR, there was also found to be increased relative gene expression for the cytokines IFN-γ, TNF-α, IL-6, IL-17, IL-18 and IL-23 and for the chemokines MCP-1/CCL2, MIP-1α/CXCR3 and SDF-1α/CXCL12. Also increased was gene expression for p22phox, superoxide dismutase and Nfr2, whereas thioredoxin gene expression was decreased. Immunofluorescence staining demonstrated that cytoplasmic localization of Nrf2 in astrocytes in brain tissues from rats exposed to CS compared to nuclear localization in rats not exposed to CS. These studies, therefore, demonstrate that in this model CS exposure can result in increased brain immune cell trafficking, pro-inflammatory responses and oxidative stress. These novel findings suggest that further studies of this model could be useful for elucidating mechanisms and developing strategies for understanding and treating inflammatory CNS disorders that may result from exposure to CS.

57. ALTERATIONS IN HUMAN BRONCHIAL EPITHELIAL CELL TRANSCRIPTOMES FOLLOWING EXPOSURE TO MAINSTREAM SMOKE FROM TOBACCO AND NON-TOBACCO CIGARETTES. Michael P. TIMKO, Michael J. Wolkowicz and Tatyana Kotova; University of Virginia, Charlottesville, VA USA

Increased public awareness of the health risks associated with smoking has led to the development of numerous products aimed at promoting smoking cessation by purportedly
reducing nicotine addiction and the harmful effects of tobacco smoke exposure. Using differentiated human bronchial epithelial (HBE) cells, we established an in vitro liquid-air interface model for smoke exposure and examined the transcriptional changes associated with exposure to mainstream smoke (MSS) generated from three tobacco-based cigarettes (1R5F University of Kentucky Reference, Marlboro Red, and Marlboro Gold) and SmokeFree®, a non-tobacco based smoking product fabricated from cocoa husks. Cell toxicity and cell-proliferation assays showed that MSS from SmokeFree® was significantly more toxic to HBE cells than smoke from any of the three tobacco-based cigarettes. Exposure of HBE cells to MSS from SmokeFree® and traditional tobacco containing cigarettes elicited both general (i.e., smoke exposure regardless of source) and product-specific (tobacco versus non-tobacco) alterations in gene expression associated with signal transduction and stress and toxicity response pathways. The implication of these studies with respect to the need for greater analysis of potential harm reduced smoking products will be discussed.

2:10 PM TUESDAY

58. RNA-SEQ ANALYSIS OF ALTERATIONS IN HUMAN BRONCHIAL EPITHELIAL CELL TRANSCRIPTOMES FOLLOWING EXPOSURE TO ELECTRONIC (E)-CIGARETTE VAPORS. Michael P. TIMKO, Michael J. Wolkowicz, Tatyana Kotova and Stephen N. Holby; University of Virginia, Charlottesville, VA USA

Increased public awareness and governmental concern of the health risks associated with smoking has led to the development of products aimed at reducing the harmful effects of tobacco smoke exposure and potentially promoting smoking cessation. Electronic cigarettes (also known as e-cigarettes) are battery-operated devices designed to simulate smoking and deliver nicotine, flavor and other chemicals when inhaled by the user. The safety and efficacy of these products have not been critically evaluated and the direct effects of e-cigarette vapors on human cellular function have not been studied in detail. Using differentiated human bronchial epithelial (HBE) cells we have developed an in vitro liquid-air interface model for exposure and examined the cellular effects and transcriptional changes associated with exposure to e-cigarette vapor (ECV) delivered from a commercially available product “MAGMA brand” marketed by Volcanoecigs.com [http://www.volcanoecigs.com/]. Cell toxicity and Trans-Epithelial Electrical Resistance (TEER) assays were carried out on HBE cells exposed for various times to ECV generated from charging media containing 0 mg and 16 mg (Full Flavor) of nicotine. In general ECV was significantly less toxic than exposure to mainstream smoke from traditional tobacco containing 1R5F reference cigarettes. Next generation (RNA-seq) transcriptome analysis and qRT-PCR based analysis were used to profile changes in gene expression following exposure of HBE cells to ECV. Our results indicate that ECV exposure elicits specific alteration in transcriptomic activity that contrast with those observed during exposure to mainstream smoke from traditional tobacco containing cigarettes. The implication of these studies with respect to the need for greater analysis of potential harm reduced smoking products will be discussed.
59. TOBACCO SMOKE EXPOSURE ACCELERATES CARDIAC ALLOGRAFT REJECTION, VASCULAR INFLAMMATION, AND GRAFT LOSS. Ashwani KHANNA¹ and Mandeep R. Mehra²; ¹University of Maryland, Baltimore, MD USA and ²Harvard Medical School, Boston, MA USA

Tobacco exposure in cardiac transplant recipients, before and after transplantation, may increase the risk of cardiac allograft vasculopathy and allograft loss, but no direct evidence for this phenomenon is forthcoming. In this experimental study, we investigated early consequences of tobacco smoke exposure in cardiac transplant donors and recipients with an emphasis on allo-inflammatory mediators of graft outcome. Using heterotopic rat cardiac transplantation, we tested the effects of donor or recipient tobacco smoke exposure in 6 groups of animals (rat heterotopic cardiac transplantation) as follows: tobacco-naive allogeneic rejecting controls (n=6), tobacco-naive nonrejecting controls (n=3; killed on day 5 to simulate survival times of tobacco-treated animals), isografts (n=3), both donor and recipient rats exposed to tobacco smoke (n=4), only donor rats exposed to tobacco smoke (n=7), and only recipient rats exposed to tobacco smoke (n=6). Polymerase chain reaction studies of tissue and peripheral (systemic) protein expression were performed to evaluate inflammatory (tumor necrosis factor-alpha, interferon-gamma, interleukin-6) and alloimmune (interleukin-1 receptor 2, programmed cell death-1, and stromal cell-derived factor-1) pathways, as was histological analysis of the cardiac allografts. Our experiments reveal that pretransplantation tobacco exposure in donors and/or recipients results in heightened systemic inflammation and increased oxidative stress, reduces posttransplantation cardiac allograft survival by 33% to 57%, and increases intragraft inflammation (tumor necrosis factor-alpha, interferon-gamma, interleukin-6) and alloimmune activation (CD3, interleukin-1 receptor 2, programmed cell death-1, and stromal cell-derived factor-1) with consequent myocardial and vascular destruction. These sentinel findings confirm that tobacco smoke exposure in either donors or recipients leads to accelerated allograft rejection, vascular inflammation, and graft loss. Molecular pathways that intersect as arbiters in this phenomenon include instigation of alloimmune activation associated with tobacco smoke-induced inflammationn.

2:50 PM Break

3:20 PM TUESDAY

60. DIETARY SUPPLEMENT ANTIOXIDANT N-ACETYL CYSTEINE MITIGATES CIGARETTE SMOKE-INDUCED MYOCARDIAL INFARCTION IN A RAT MODEL. Ashwani KHANNA¹ and Mandeep R. Mehra²; ¹University of Maryland, Baltimore, MD USA and ²Harvard Medical School, Boston, MA USA

The contribution of chronic tobacco exposure in determining post-myocardial infarction (MI) left ventricular (LV) remodeling and possible therapeutic strategies has not been investigated systematically. In this small animal investigation, we demonstrate that chronic tobacco smoke exposure leading up to acute MI in rats is associated with greater histological extent of myocardial necrosis and consequent worse LV function. These findings are associated with increased transcriptomic expression of pro-inflammatory cytokines,
tissue repair molecules and markers of oxidative stress in the myocardium. The results demonstrate that an N-acetyl cysteine (NAC) treatment significantly reduced tobacco-exposed induced infarct size and percent fractional shortening. A significantly increased IV end-systolic diameter was observed in tobacco-exposed sham compared to tobacco-naïve sham (4.92±0.41 vs 3.45±0.33; P<0.05), and tobacco-exposed MI compared to tobacco-naïve MI (8.24±0.3 vs 6.1±0.49; P<0.01) rats. Decreased intracardiac mRNA expression of the markers of inflammation, tissue repair and oxidative stress and circulating levels of pro-inflammatory cytokines accompanied these positive effects of NAC. The treatment of tobacco-exposed MI rats with NAC resulted in significantly increased levels of intracardiac mRNA expression of antioxidants, including superoxide dismutase, thioredoxin and nuclear factor-E2-related factor 2, as well as circulating levels of glutathione (7±0.12 vs 10±0.18; P≤0.001), where the levels were almost identical to the tobacco-naïve sham rats. These findings identify a novel post-infarction therapy for amelioration of the adverse effects of tobacco exposure on the infarcted myocardium and advocate the use of dietary supplement antioxidants for habitual smokers to prevent and reverse cardiovascular adverse effects in the absence of successful achievement of cessation of smoking.

3:40 PM TUESDAY

61. BRIDGING CARDIOVASCULAR IN VITRO AND IN VIVO MODELS. Brian K. NORDSKOG, Joya E. Brown, Geoffrey M. Curtin and Betsy R. Bombick; R. J. Reynolds Tobacco Company, Winston-Salem, NC USA

To better understand the impact cigarette smoking has on cardiovascular disease, in vivo and in vitro models have been explored. First, atherosclerosis was assessed in the aorta of apoE knockout mice. ApoE is a gene involved in lipoprotein clearance. Inactivation of the gene results in arterial cholesterol accumulation and the development of atherosclerosis. ApoE -/- mice (+/- atherogenic diet) were exposed nose-only to mainstream cigarette smoke (MCS) (0-0.48 µg/mL wet total particulate matter [WTPM]) and evaluated for CVD using histopathology, virtual histology, lipid chemistry and global genomic approaches. Second, primary cultures of human aortic endothelial cells (HAEC) were exposed acutely or chronically to aqueous extracts of MCS. Global genomic profiling was used to assess the effects of cigarette smoke extract on molecular changes in HEAC.

Atherosclerosis development in the thoracic aorta was enhanced in apoE knockout mice in the presence of both an atherogenic diet and MCS. Gene expression analysis identified several novel and “traditional” cardiovascular gene alterations (GREM1, CXCL1, THBS1, and VCAM1). Acute exposures of HAEC to aqueous MCS extracts resulted in limited numbers of gene expression changes. However, in the HAEC chronic exposure model, several gene expression changes were observed, many of which, were similarly observed in the in vivo model.

When using simple in vitro models to simulate complex disease processes, it is imperative that the endpoints are linked back to human data or a relevant biological model. The HAEC chronic in vitro exposure model shows promising linkages to our in vivo model of atherosclerosis and may further our understanding of the biological relevance of responses in in vitro models.
4:00 PM TUESDAY

62. THE QUANTITATIVE ESTIMATION OF TOXICANT DOSE TO CIGARETTE SMOKERS. F. Kelley ST. CHARLES¹, John McAughey² and Jim Shepperd³; ¹St. Charles Consultancy, Winston Salem, NC USA and ²British American Tobacco (Investments) Limited, Southampton, UK

For the risk assessment of cigarette smoke, a reliable estimate of the mass of chemical compounds retained in the body would be beneficial. Filter studies provide estimates of mouth exposure to compounds in cigarette smoke, but do not account for mouth spill and respiratory retention. Except for nicotine, biomarkers provide the relative uptake of certain compounds when comparing products, but generally do not provide quantitative uptake values. In addition, many compounds of interest do not have reliable biomarkers. Estimates of the respiratory retention and mouth spill allow mouth exposure to be converted to a quantitative estimate of dose. A method to estimate respiratory retention of compounds from cigarettes smoke was presented at the 64th TSRC. A method of estimating mouth spill is shown using data from two clinical studies. Mean values of mouth spill from both studies were slightly greater than 30% with a mid-quartile range of 20 to 45%. Mouth exposure, respiratory retention and mouth spill combine to allow a quantitative estimation of smoker uptake for almost any chemical compound that can be measured analytically. The samples needed from subjects are spent filters and a 24-h urine sample. These could be obtained from ambulatory studies which would have a minimal influence on smoker behavior.

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63. EXAMINATION OF AN ANALYTICAL METHOD OF BENZO(A)PYRENE IN TOBACCO AND SMOKELESS TOBACCO USING GC/MS. Hiroyuki YOSHIDA, Japan Tobacco Inc., Tsuchi, Japan

Currently an analytical method for the determination of Benzo[a]pyrene (B[a]P) in tobacco is prescribed by Health Canada (T-307, 1999). It involves tedious multi-step sample cleaning to remove matrix interference. This method uses an HPLC – fluorescence detector system for the qualitative and quantitative determination of B[a]P in conjunction with an internal standard. On the other hand, a Gas Chromatography-Mass Spectrometry Method is used in Determination of Benzo[a]pyrene in Cigarette Mainstream Smoke with an external standard(CORESTA Recommended Method N° 58).

A simple extraction and clean-up method has been developed and validated for the qualitative and quantitative determination of B[a]P in tobacco and smokeless tobacco. Analysis was performed by GC/MS in selected-ion-monitoring-mode with the almost same operating condition as CRM N° 58. B[a]P was extracted from tobacco by liquid – liquid partition with a mixture of Hexane and Methanol using an ultrasonic bath for 10 min., and a SPE(silica) was used for the clean-up. The method has been validated by standard validation protocols using Certified Reference Material of B[a]P (NMIJ CRM 4213-a, No.7) emphasizing limit of detection, limit of quantification, recovery, repeatability and reproducibility. Minimum recovery of 97% was obtained with a linear regression coefficient of 0.9999 for the range of 0.27-200 ppb of B[a]P. This method was applied to four CORESTA reference products.
(CRP1, CRP2, CRP3, CRP4). The data of CRP1, CRP2, CRP3, and CRP4 were 0.73ng/g, 40.00ng/g, 34.40ng/g, and 1.11ng/g respectively, those uncertainty estimates were 0.21ng/g, 4.24ng/g, 5.54ng/g and 0.18ng/g respectively with relative standard deviations under 8% on a “wet basis”.

4:40 PM   ADJOURN
64. INVESTIGATION AND COMPARISON OF HARDNESS METERS. Clarissa E. Tatum and Larry Renfro; Eastman Chemical Company, Kingsport, TN USA

Cigarette filter hardness is an important factor in consumer perception of cigarette quality. Many different hardness testers are produced and used within the industry, each using a unique design and testing protocol. The variations among the testers inevitably lead to differing hardness values among the instruments. Understanding both the operation of the individual testers and also the variances between hardness testers is essential in comparing the values obtained on different instruments.

The Filtrona Digital Hardness Tester (DHT, no longer produced) has been used in our lab for hardness testing and is the basis for current models in our database. For this study, a Quality Test Module 7 (QTM 7), a hardness meter produced by Cerulean (formerly Filtrona Instruments), was compared to the DHT. Rods representing a span of hardness values were tested with both instruments and a correlation between the DHT and the QTM 7 was developed. The penetration data from the two instruments exhibit a strong linear correlation ($R^2=0.9925$). Additionally, various adjustable parameters on the QTM 7 were investigated to observe the effects on hardness readings. For filter rod penetration, an increase in both load amount and load duration led to an increase in rod penetration. The speed of compression of the filter rods was also studied. Results showed that compression speed had a negligible effect on rod penetration and the resulting hardness of the filter rod. Understanding the relationship between hardness testers and the parameters that influence hardness data is important in comparing hardness values among various instruments and our database model.

9:10 AM WEDNESDAY

65. INFLUENCE OF BAND WIDTH AND BAND MATERIAL COVERAGE RATE (TOTAL BAND AREA / TOTAL BAND AREA) ON SMOKE YIELDS, SE TEST AND FREE BURN. Mario Mayr and Dietmar Volgger; Papierfabrik Wattens, Wattens, Austria

The objective of this analysis was to evaluate the difference in smoke yields, self-extinguishment performance and free burn rate depending on the different amount of band coverage rate on the FSC cigarette papers. The cigarette base sheet parameters were kept constant, for substance at a level of 24gsm, chalk content at a level of 29% and an amount of burning additive (tri-potassium-citrate) of 2,0%. The aim was to vary the coverage rate of the band material with a constant diffusivity level (RT) of 0,05cm/s on the bands. The defined levels are:
- 5/19mm with 20,8% coverage rate
- 6/21mm with 22,2% coverage rate
- 6/18mm with 25,0% coverage rate
- 7/20mm with 25,9% coverage rate
All cigarettes were produced maintaining one specification and the same tobacco blend (American Blend). At least 2 identical bands are present on each of the cigarettes. All cigarettes were smoked according ISO 4387 with the ISO Regime (35/2/60) and the Canadian Regime (55/2/30). The results show a clear increase of T-N-C with increasing the band coverage rate on the FSC cigarette paper. All cigarettes were tested on self-extinguishment performance according ASTM E.2187-09. They have been also tested on three layers of Whatman #2 and LIPCan filter papers to achieve a better characterization and discrimination of the self-extinguishment performance.

9:30 AM WEDNESDAY

66. TOBACCO COLUMN INFLUENCE ON CIGARETTE PAPER. Joseph WANNA\textsuperscript{1}, C. Le Moigne\textsuperscript{2} and L. Le Bec\textsuperscript{2}; SWM Intl., \textsuperscript{1}Alpharetta, GA USA and \textsuperscript{2}Allonnes, France

Cigarette paper has a dynamic interaction with the cigarette tobacco column. Ion exchanges occur between the tobacco column and cigarette paper during cigarette ageing. Previous reports have discussed ion transfer from the tobacco column to conventional cigarette paper. This presentation will review and report on ion transfer from the tobacco column to cigarette paper with bands applied to the paper to provide $>75\%$SE on the ASTM test method E2187-09. Cigarettes with American type and special blends will be investigated to identify if there are any differences and levels of ion transfer. In addition the influence of these ions on base paper and band diffusion under lab conditions (23°C and 50%RH) and after heating to 230°C for 30 minutes will be discussed.

Preliminary investigations showed band diffusion after heating at high temperature for papers removed from the cigarette after ageing is different than control papers (not taken from cigarette).

9:50 AM Break

10:20 AM WEDNESDAY

67. Applications of GC tandem mass spectrometry to the analysis of chemical constituents in mainstream cigarette smoke. I. Gene GILLMAN, Sherri S. Brown and Katherine E. Humphries; Enthalpy Analytical, Durham, NC USA

The US FDA Center for Tobacco Products is currently collecting data on mainstream cigarette smoke constituents, but it is not clear how this will impact future product testing requirements. To allow for maximum flexibility in our laboratory we have tried to develop analytical methods to analyze a wide range of compounds using only a few instrument types. The US FDA Tobacco Products Scientific Advisory Committee (TPSAC) proposed HPHC list includes at least 93 compounds in mainstream smoke; over 50% can be analyzed by gas chromatography combined with mass spectrometry (GC-MS). In our laboratory we use GC-MS to measure volatile, semi-volatile and polycyclic compounds. We will present separate methods for the application of a tandem GC-MS/MS system for the analysis of nitrosamines, N-nitrosodiethanolamine (NDELA), aromatic amines, and large polycyclic aromatic hydrocarbons including dibenzopyrenes in mainstream...
smoke. These methods have several advantages including simple sample cleanup and/or greatly reduced limits of detection. All compounds were separated on an Agilent 7890 GC system using an HP-5MS column followed by detection on an Agilent 7000 QQQ tandem mass spectrometer. For the determination of nitrosamines, the mass spectrometer was operated in positive chemical ionization mode using ammonia as the reagent gas. NDELA, 1-Aminonaphthalene, 2-Aminonaphthalene, 4-Aminobiphenyl, o-Anisidine, o-Toluidine, and 2,6-Dimethylaniline were derivatized prior to analysis. Detection for all compounds was performed using multiple reaction monitoring (MRM). All compounds gave levels of detection of less than 1 ng/mL. Method validation information will be presented for all compounds including calibration range, method reproducibility, and other analytical details.

10:40 AM WEDNESDAY

68. FAST IDENTIFICATION OF CARBON-CENTERED RADICALS BY ELECTROSPRAY ION-TRAP MASS SPECTROMETRY WITH 3-AMINO-2,2,5,5-TETRAMETHYL-1-PYRROLIDINYL OX. SUN Shihao, Peng Shuhai, Liu Hailin, Li Peng, Zong Yongli and Xie Jianping; Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou, Henan, China

A method of electrospray ion-trap mass spectrometry was developed to investigate carbon-centered radicals (·R). ·R generated by thermal decomposition of model compounds (2,2'-azobisisobutyronitrile and 2,2'-azobis (2,4-dimethyl) valeronitrile) was trapped by 3-amino-2,2,5,5-tetramethyl-1-pyrrolidinyl ox (3AP) to form a radical adduct (3APR) and then analyzed by electrospray ion-trap mass spectrometry. MS<sup>n</sup> data of 3APR showed a similar fragmentation character in the procession of mass spectrum analysis. The ions of [3APRH], [3APRH-NH<sub>3</sub>], and [3APRH-NH<sub>3</sub>-CH<sub>4</sub>] can be used as the confirmative ions to identify the structure of 3APR, and then molecular weight of radicals can be obtained from a mass difference of 3APR and 3AP. Good linearity (Y = (1.08X + 0.0311)E+8, R<sup>2</sup>=0.9989) relationship of ·R and 3APR can be achieved in the range from 0.12 to 6 µmol/mL. Moreover, the reaction condition of 3AP trapping carbon-centered radicals is mild, without isolating of oxygen or light. This indicated that the method could be used to quantitatively analyze carbon-center radical. The developed method with simple, fast and effective provide an alternative way to identify carbon-center radical.

11:00 AM WEDNESDAY

69. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC-TANDEM MASS SPECTROMETRY METHOD FOR THE DETERMINATION OF THREE HYDROXYLATED METABOLITES OF POLYCYCLIC AROMATIC HYDROCARBONS IN THE URINE OF CHINESE SMOKERS. HOU Hongwei, Xiong Wei, Zhang Xiaotao, Tang Gangling and Hu Qingyuan; China National Tobacco Quality Supervision & Test Center, Zhengzhou, Henan, China

A liquid chromatography-tandem mass spectrometry method (LC-MS/MS) for the simultaneous determination of 1-hydroxypyrene (1-OHP), 3-hydroxybenzo[a]pyrene (3-OHBaP) and 3-hydroxybenz[a]anthracene (3-OHBaA) in human urine has been developed using D<sub>9</sub>-1-OHP as internal standard. LC–MS/MS analysis was carried out on
an Applied Biosystems API5500 LC–TSQ Quantum mass spectrometer using positive ion atmospheric pressure chemical ionization (APCI+) and multiple reaction mode (MRM). The linear ranges of 1-OHP, 3-OHBaP and 3-OHBaA were 0.50 to 25.00 ng/mL, 0.25 to 12.50 ng/mL and 0.10-5.00 ng/mL, respectively. The average recoveries were better than 80%, except for 3-OHBaP at low spiked level. The intra- and inter-day precision values were 1.8%-11.4%. The developed method was successfully used to measure urinary PAH metabolites from smokers and nonsmokers.

11:20 AM ADJOURN