63rd Tobacco Science Research Conference

MONDAY MORNING, SEPTEMBER 29, 2009

Symposium

9:00 AM **WELCOME: Rob Stevens, 63rd TSRC Chair**

9:10 AM **SYMPOSIUM: "Changing Times: The Tobacco Industry and Regulations" Chair: Joseph Wanna**

9:15 AM **MONDAY**

1. CHANGING TIMES: REGULATION OF THE US TOBACCO INDUSTRY. Clausen ELY, Jr.; Covington and Burling, LLP, Washington D. C.

The U.S. tobacco industry is currently regulated by a variety of different agencies, under a patchwork of federal and state laws, and pursuant to the 1998 Master Settlement Agreement between the U.S. tobacco companies and the states. FDA's ambitious effort to regulate the tobacco industry a decade ago was narrowly overturned by the U.S. Supreme Court. After a decade of debate, however, Congress is now poised to grant FDA comprehensive regulatory authority over the tobacco industry. The legislation would centralize and transform regulation of the tobacco industry, including resurrection of most elements the 1996 FDA regulation restricting the sale and distribution of tobacco products. FDA would have regulatory authority over virtually every aspect of tobacco product production and marketing. FDA approval would be necessary to market new or modified risk tobacco products, and the agency would have broad power to issue product standards, impose good manufacturing practice requirements, and require product testing. This new regulatory regime would impose huge costs on the industry, restrict competition and limit the development of new products. In large measure, the future of the tobacco industry would be in FDA's hands.

9:45 AM **MONDAY**

2. LOW IGNITION PROPENSITY REGULATION: HISTORY AND IMPLICATIONS. Joseph WANNA; Schweitzer Mauduit International, Alpharetta, GA

The expansion of world wide regulation aimed at reducing the ignition propensity of cigarettes is compelling major changes in cigarette designs that impacts deliveries, burn properties, construction, and consumer perception. The process started when Congress passed the Cigarette Safety Act of 1984, which established the Technical Study Group (TSG). The TSG's mandate was to evaluate the technical and economic feasibility of creating a cigarette with a reduced propensity to ignite upholstered furniture. The TSG issued a report in 1987 stating that it is technically and maybe commercially feasible to produce a cigarette with low ignition propensity and recommended the formation of a group to develop test methods. Congress passed the Fire Safe Cigarette ACT of 1990, which created the Technical Advisory Group (TAG) to develop a test method. The TAG issued a report in 1993 proposing two test methods. During the same time period many studies were performed and results published independently by the tobacco industry and CORESTA. ASTM published test

method E2187 "Standard Test Method for Measuring the Ignition Strength of Cigarettes", which based on testing cigarettes on 15, 10, and 3 sheets of Whatman #2 filter paper. New York State was the first to implement low ignition propensity (LIP) cigarette regulation based on this test method in 2000. The regulation mandated that cigarettes sold in the state to have at least 75% Self Extinguishments on 10 sheets. This presentation will review the history and current status of LIP regulation, methods of complying, and new scientific findings.

10:15 AM ***Break***

10:45 AM **MONDAY**

3. LOW IGNITION PROPENSITY REGULATION: THE ROLE OF CIGARETTE PAPERS, TOBACCO AND CIRCUMFERENCE. Paul CASE, Steven Coburn, Virginie Cotte, Leonardo Nappi, and Matthew Hesford; British American Tobacco, Group R&D Centre, Southampton, UK

Low Ignition Propensity (LIP) products are of interest to cigarette designers and for regulatory bodies in various parts of the world. The role of changing cigarette paper characteristics, tobacco types and cigarette circumference; on cigarette ignition propensity is therefore of interest to the tobacco industry.

During LIP testing the cigarettes in question are placed on a number of layers of a particular substrate and therefore it is the performance of the wrapped tobacco column that is of interest. Thus the tobacco types that make up the blend, the cigarette paper and the cigarette circumference can be considered as some of the broad variables in question. In order to examine the tobacco blend in greater detail a systematic statistically based mixture design was utilised involving Burley, Virginia and Oriental tobacco types. In turn the resulting blends were wrapped in a series of both banded and low permeability cigarette papers and manufactured at a given circumference. Further subsets of blends incorporating stem and a range of cigarette circumferences were also examined.

An issue that arises with any experimental design involving ignition propensity measurements is that, a part or whole, of an experimental series can be manufactured and found to give 100% LIP pass rates. Data interpretation can then, in certain instances, become more problematic. This can be potentially overcome by further testing of all the cigarettes in the series in question on a reduced number of layers of substrate; in turn this can be viewed as being somewhat wasteful and time consuming. An alternative approach was developed, in which the individual cigarettes that had been smouldered on the layers of substrate were retained, and the resulting length of non burnt tobacco column was measured and recorded as a residual length. Subsequent analyses utilising this technique gave good correlations between residual length values and LIP pass rates in the range of above 0% and below 100%. Additionally the residual length data could be used to help resolve the effect of design variable changes that consistently gave 100% pass rates.

These studies illustrated that LIP pass rates decrease with increasing levels of Burley tobacco and conversely increase with increasing levels of Oriental tobacco within a given blend. Increasing the level of stem in a blend reduced LIP pass rates. With cigarette paper, diffusion

values were critical in determining LIP pass rates, but the absolute diffusion values were found to be different between the bands of banded papers and inherently low permeability papers. With given tobacco blends and cigarette papers, cigarette circumference changes had no significant effect on LIP pass rates.

11:15 AM MONDAY

4. THREE WAVES OF TOBACCO SCIENCE: ANALYSIS, BIOMARKERS AND BEYOND - WHERE IS THE SCIENCE HEADING? Kevin REINERT, Jack Reid, and Dan Heck; Lorillard Tobacco Company, Greensboro, NC

Tobacco science has been analytically focused for many years, yielding what is found in smoke and at what levels. Of course, we have gone beyond the traditional T, N, and CO to Hoffmann analytes and further in this first wave of tobacco science. However, these efforts only clue us to the levels of constituents in smoke. Recently, we have moved beyond analytical measurements to exposure, bringing us to the second wave of tobacco science – what biomarkers are observed when laboratory surrogates (*i.e.*, in vitro) and animals (*i.e.*, in vivo) as well as humans are exposed to smoke? While these biomarkers of exposure are numerous, fairly well understood and readily measurable, we need to address the effects that these exposures create in these test systems and organisms. Current activities around understanding how these exposures manifest as effects leads us to the third wave of tobacco science – biomarkers of effects and ultimately risk. This presentation takes us through the three waves of tobacco science as it relates to our increased understanding of the risks inherent in tobacco. We also briefly discuss how these waves are expected to be integral in upcoming regulatory scenarios.

11:45 PM LUNCH

1:00 PM POSTERS

5. POST MANUFACTURE AUTOMATIC TRACK IDENTIFICATION OF MIXED POPULATIONS OF RODS FROM A DUAL TRACK MAKING MACHINE. I. TINDALL and H. Jose; Cerulean, Milton Keynes, UK

Quality assurance measurements of manufactured cigarette rods can be made by sampling from the mass flow at the exit from a making machine either by "grab sampling" or various forms of autosampling. From the analysis of defects in the rod manufacture, or through SPC trends, adjustments can be made to the making process that ensures consistency of manufacture. However in the new generation of makers, to achieve high speeds, two making tracks are combined and mix the population of rods from both tracks in the mass flow. When data is obtained from a conventional test station on these rods that indicates a defect, or requires a process change, it is not possible to pinpoint the origin of the rod and so make the correct adjustment.

A method has been devised that uses a special optical configuration and utilizes a contact image sensor (CIS) that can determine the track of origin in a twin track maker. The method is reliant on differences in contrast in a line image of the rod and utilizes a discrimination algorithm to deconvolute seam information from a surface plot of time/intensity/location.

Data is presented that shows how not only track information can be determined but also how this method can be used to determine if the rod is "front" or "back". The influence of lighting conditions and cigarette substrate colour is discussed with respect of detection efficiency. The implications for quality control in the making process and pinpointing of corrective actions at the maker level are discussed in the context of other measurement parameters such as rod density profile.

6. DETERMINATION OF PLASTICIZER PROFILE IN MONO ACETATE FILTERS USING THE MICROWAVE METHOD. James VINCENT and Ian Tindall; Cerulean, Milton Keynes, UK

Plasticizer is a vital addition to cellulose acetate tow in filter production to ensure that filters pass cleanly through the cigarette assembly process and maintain their designed filter characteristics during smoking. A microwave-based method to determine the total content of plasticizer in a filter rod has recently been announced.

This presentation will describe the further development of the technique to provide quantitative information about the distribution of plasticizer in a filter using a scanning microwave unit. This provides information about obvious process issues, such as melt hole formation and more generally about the uniformity of the product that is ultimately experienced by the consumer.

Melt holes are caused by local excesses of plasticizer that dissolve the tow. These derive most commonly from drops of plasticizer fall onto the tow from the hood of the spray unit. We show that the additional plasticizer is likely to be within the normal range of variation, so would not be detected reliably by a whole-rod measurement and describe how the microwave scan can quantify the presence of a local concentration of plasticizer and its validation using coloured drops of triacetin so that deliberately created 'process faults' could be identified and linked to the formation of melt holes.

The technique is shown to be quantitative and sufficiently sensitive to detect plasticizer concentrations well before they cause melt holes. The technique can also determine the plasticizer content of the individual tips in a filter and so identify any non-uniformity of plasticizer application even in a process that is apparently under control. The correlation between microwave measurements and GC analysis of triacetin in the corresponding tips is presented.

7. A RIGOROUS EXTRACTION METHODOLOGY FOR SNUS: APPLICATION OF ISO 10993-12 GUIDELINE TO PERMIT THOROUGH *IN VITRO* TOXICOLOGY TESTING. MARK BALLANTYNE, Mel Lloyd, and Vicky Stone; Covance Laboratories Ltd., Harrogate, North Yorkshire, UK

Smokeless tobacco products, and in particular Snus, are being increasingly considered as Potential Reduced Exposure Products, and in some quarters as smoking cessation products. Although epidemiological evidence from Sweden suggests that Snus use is substantially less hazardous than cigarette smoking, Snus is not considered as harmless.

The objective of this work described was to establish an appropriate extraction methodology for Snus such that robust safety assessment of Snus products could be conducted in the Ames, Mouse Lymphoma, *in vitro* Micronucleus and Neutral Red Uptake assays.

There is no standard method for applying Snus to *in vitro* test systems, but most workers use Snus extractions to provide treatment solutions compatible with the assay systems. We have applied certain modifications to the ISO 10993-12 guidelines (for the biological evaluation of insoluble medical devices) in order to prepare Snus extracts which allow robust safety assessment in various in vitro genotoxicity and cytotoxicity assays.

Snus samples were extracted for 24 hours at 37°C in both water and dimethyl sulphoxide (DMSO), at varying concentrations up to 500 mg (equivalent) per mL. Nicotine content was measured as 82-86% and 73% for aqueous Snus extracts at 200 and 500 mg (equivalent) per mL respectively, indicating relatively little loss in relative nicotine recovery over this concentration range. Extraction at a higher concentration was considered impractical due to excessive absorption of the extraction vehicle.

In vitro genotoxicity assays performed using Snus extracts at this highest practical concentration exceed maximum exposure concentrations required by the standard regulatory guidelines applicable for these assays. In some cases treatments resulted in biological dose limiting effects, further demonstrating the rigour of these safety assessments.

8. ANALYSIS OF PESTICIDES IN SMOKELESS TOBACCO EXTRACTS BY COMPREHENSIVE MULTI-DIMENSIONAL GAS CHROMATOGRAPHY-TIME OF FLIGHT MASS SPECTROSCOPY (GC X GC-TOFMS). Joe BINKLEY and Scott Pugh; LECO Corporation, Saint Joseph, MI

Smokeless tobacco is known to contain several thousand analytes making it an extremely difficult matrix in which to identify pesticide residues. Current methodologies incorporate the use of complex, time consuming sample cleanup techniques to eliminate much of the matrix interference prior to GC-MS analysis.

This poster will show how the increased peak capacity of comprehensive multi-dimensional gas chromatography provides the ability to effectively separate pesticides from complex sample matrix while minimizing the need for extensive sample cleanup methods. In addition, the cryo-focussing effects of thermal modulation increases detectability allowing lower levels of these pesticide residues to be determined. The use of a Time of Flight Mass Spectrometer provides the ability to acquire full mass range spectra without sacrificing sensitivity. This is beneficial for detecting not only target pesticide analytes, but also new and emerging contaminants.

The GCxGC-TOFMS data from two commercially available smokeless tobacco products will be discussed. Various QuEChERS sample preparation techniques were used for this work and will be shown in this poster.

9. DETERMINATION OF NICOTINE TRAPPING EFFICIENCY OF CAMBRIDGE FILTERS PADS USING PRODUCTS WITH DIFFERENT LEVELS OF 'TAR' SMOKED AT VARIOUS SMOKING REGIMENS. Karen B. KILBY and Tanya J. Collins; R. J. Reynolds Tobacco Company, Winstin-Salem, NC

The purpose of this study was to determine the nicotine trapping efficiency of the Cambridge filter pad (44mm) using cigarettes with different 'tar' levels smoked at various smoke regimens (35/60/2 0% vent block, 45/30/2 50% vent block, and 55/30/2 100% vent block). Eight products ('tar' levels ranging from approximately 1.0 to 39 mg/cig) and one sham were smoked for this study. Cigarettes were smoked using a Cambridge filter pad followed by two impinger traps. Five milliliters of pure isopropyl alcohol was placed in each impinger. The pad extracts and the impinger solutions for each sample were analyzed for nicotine using a GC/MS operated under selected ion monitoring conditions.

The nicotine measured on the Cambridge filter pads ranged from 0.17 to 2.65 mg/cig. The nicotine measured in the sham ports was below the detection limit. The nicotine measured in the impinger solutions was below the detection limit for all samples (<0.5 µg/cig or 0.0005 mg/cig). Thus, using the detection limit, the nicotine trapping efficiency of the Cambridge filter pad was greater than or equal to 99.7% for all products. Therefore, the Cambridge filter pad offers an effective means for quantitating nicotine in mainstream cigarette smoke for products with different levels of 'tar' smoked at various smoking regimens.

10. TSNA METHOD COMPARISON USING UHPLC-MS/MS. John A. MATHIS and Po Ying Yeung; Global Laboratory Services, Wilson, NC

Method development and optimization was performed for the quantification of tobacco specific nitrosoamines (TSNAs) in tobacco leaf. The objective was to shorten run time and compare the new method to the previous method. The evaluation of method performance indicated that the matrix effects, reproducibility, and detection limits were reduced.

In the previous method, the initial stage of the ISO/CD TS 22304 sample preparation was performed with aqueous sodium hydroxide. Using the new method, samples were prepared using a single step extraction procedure with an ammonium acetate buffer as described in earlier collaborative studies. Quantification was performed using a new ultra-high performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS) method with electrospray ionization. The UHPLC method was optimized to separate the four common TSNAs using a gradient elution profile and a reversed-phase column. Following optimization, validation experiments were performed for the updated sample preparation and instrumental analysis procedures. The results from the validation of the newly developed method indicated that along with decreased run time, the method performance improved.

The run time was reduced from 12 to 4.2 minutes. The matrix effects defined here as the degree of suppression was evaluated by comparing the response of each TSNA in the presence of tobacco matrix to the response in solution. The results illustrates that suppression was generally lower with the exception of NNN and NAT in oriental and NAB in both oriental and burley tobacco. The reproducibility of the method was reduced from up to 16% to

less than 10% RSD. The limits of detection for the two methods were significantly lower as indicated by ANOVA, P=0.003.

11. A HIGHLY SPECIFIC ANALYTICAL PROCEDURE FOR THE DETERMINATION OF NICOTINE AND COTININE IN HUMAN SERUM TO SUPPORT THE PHARMACOKINETIC EVALUATION OF NICOTINE UPTAKE FROM NON-COMBUSTIBLE TOBACCO PRODUCTS. Brian BAILEY, Jane Spink, and David Bakes; Covance Laboratories Ltd., Harrogate, North Yorkshire, UK

This poster discusses a highly specific analytical procedure for the determination of nicotine and cotinine in human serum using a combination of Oasis® weak cation exchange (WCX) solid phase extraction, hydrophilic interaction liquid chromatography (HILIC) and Atmospheric Pressure Chemical Ionisation (APCI) triple quadrupole tandem mass-spectrometric detection.

Oasis® WCX is designed to provide improved sample preparation for strong bases. The retention mechanism is mixed mode, that is, both ion-exchange and reverse phase. This is suitable for a combined extraction of nicotine and cotinine from serum as the weak cation exchange gives good retention of nicotine, a strong base, while reverse phase interactions give a second retention mechanism for improved recovery.
HILIC uses a polar stationary phase such as silica and a less polar solvent as the mobile phase, typically acetonitrile. Elution is achieved by increasing the aqueous portion of the mobile phase. HILIC gives good retention of polar bases such as nicotine and cotinine. Two modes of retention mechanism can be observed:

(i) partitioning of the analyte between a water layer absorbed to the surface of a stationary phase and the mobile phase
(ii) cation exchange of charged polar analytes with charged surface silanol groups.

APCI was used as this gives a wide dynamic range. A mass transition of 163.0 to 130.0 Da was monitored for nicotine and a mass transition of 177.1 to 80.0 Da was monitored for cotinine in positive ion mode.

A quantitative analytical procedure with sensitivity to 0.5 µg/mL using a sample volume of 50 µL and linearity to 250 ng/mL was successfully developed and validated to support the pharmacokinetic evaluation of nicotine uptake from non combustible tobacco products.

12. A COMPARISON OF RADIOACTIVE ELEMENTS IN US AND SWEDISH SMOKELESS TOBACCO PRODUCTS. Michele MOLA[1], Arif Faizi[1], Kevin McAdam[1], Brad Rodu[2], Roger Benzing[3], Gary Prior[3]; [1]British American Tobacco, Group R&D, Southampton, UK. [2]University of Louisville, James Graham Brown Cancer Centre, Louisville, KY. [3]Scientific Limited, Harwell Science and Innovation Campus, Didcot, UK

A total of 28 toxicants have been reported in smokeless tobacco products (STPs), including the radioactive elements ^{210}Po, ^{235}U and ^{238}U (1). According to the International Agency for Research on Cancer (IARC), these three elements are classified as Group 1 carcinogens (2).

In contrast to the significant body of historic information available on the levels of radioactive elements in tobacco leaf and cigarettes, there is not much available information on STPs. A limited number of studies have been carried out on STPs, and activities ranging from 6 mBq/g to 74 mBq/g for snuff and natural tobacco have been reported (3). Given the lack of comprehensive or recent data in this area, an up to date survey was conducted to reflect current STPs on the market.

70 STPs were sampled from all major manufacturers in Sweden and the US in October 2008. They consisted of 32 Swedish loose and pouched snus products and 38 US products including chewing tobacco, dry snuff, pellets, moist snuff and plug.

In order to provide a more complete picture, several commonly occurring α radioisotopes (^{232}Th, ^{230}Th, ^{228}Th, ^{234}U, and ^{226}Ra) and one β emitter (^{210}Pb) were also examined. Polonium, thorium and uranium isotopes were measured by α-spectrometry and the radium and lead isotopes were measured with a gas flow proportional counter.

The combined α activity for the measured radioisotopes ranged from <12 mBq/g to 50 mBq/g for both Swedish and US STPs, whilst the β activity of ^{210}Pb ranged from <5 mBq/g to 150 mBq/g.

(1) IARC Monographs on the Evaluation of Carcinogenic Risk to Humans, IARC Press, Lyon, France, Volume 89, 2007, 55-60
(2) www.iarc.fr
(3) Brunnemann K.D. and Hoffmann D., Chemical Composition of Smokeless Tobacco Products, Smoking and Tobacco Control Monograph No. 2, 1992, 96-108

13. COMPARATIVE QUANTIFICATION OF OXYGEN AND NITROGEN FREE RADICALS INDUCED BY CIGARETTE SMOKE IN HUMAN LUNG CELLS *IN VITRO*.
M. BENNETT; Lorillard Tobacco Co., Greensboro, NC

Oxygen and nitrogen free radical-mediated oxidative stress is implicated in several smoking associated effects such as inflammation, lipid peroxidation, protein oxidation and DNA damage and disease states such as atherosclerosis, chronic obstructive pulmonary disease, and cancer. This study sought to identify a sensitive *in vitro* model to quantify the impact of smoke fractions on endogenous oxygen and nitrogen radical load using molecular probe technology. We used the fluorescence-based molecular probes, 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate and 4-amino-5-methylamino-2',7'-difluorofluorescein diacetate to evaluate the effect of smoke on oxygen-centered and nitrogen-centered radical activity, respectively, in two human lung cell lines. The human lung adenocarcinoma epithelial cell, A549, and normal human lung immortalized cells BEAS-2B, were utilized for our comparative study. The cells were exposed to wet total particulate matter and gas/vapor phase fractions of smoke of 2R4F cigarettes. H_2O_2 and 2-(N, N-Diethylamino)-diazenolate 2-oxide salt were utilized as positive controls for oxygen and nitrogen-centered radicals, respectively. Radical activity was measured qualitatively in arbitrary relative fluorescence units with a fluorometer. Exposure to wet total particulate matter and gas vapor phase smoke fractions yielded a wide and sometimes disparate range of reactive oxygen and nitrogen species, suggesting differential susceptibility of the cells to smoke fractions. Overall, A549 cells appear more sensitive than BEAS-2B cells in this test system.

14. DETERMINATION OF WATER AND NICOTINE IN TOBACCO SMOKE BY GAS CHROMATOGRAPHY USING CAPILLARY COLUMNS INSTEAD OF PACKED COLUMNS. Jacques DUMONT; Imperial Tobacco Canada Limited, Montreal, Quebec, Canada

The standard procedure to determine water and nicotine in tobacco smoke is gas chromatography with two packed columns for the separation. The analysis time is between 4 and 6 minutes. The quality of packed columns is variable from one production to another and ghost peaks are even present on some occasions (hand-made preparation by supplier). To eliminate this problem, capillary columns were evaluated to perform routine water and nicotine determinations in tobacco smoke. Capillary columns are produced by machine and are less variable to production. The goal of this method improvement was to use the same standard concentrations, internal standards and only 1 injection for both columns by the use of an injector splitter. The procedure was optimized to reduce the analysis time. Finally, same or better method characteristics such as LOQ and separation system robustness.

Results are not statistically different with packed or capillary columns ($t\text{-}_{test}$, P_{95}) under ISO and Canadian Intense smoking regimes. The chromatogram is very simple and is acquired with the TCD for water for the first minute and then changed for the FID for the last portion of it. This approach has the advantage to reduce the disk space for data storage and to generate only 1 chromatogram which minimize the time for the data processing. The analysis time was reduced to less than 2 minutes. The Limits of Quantitation are 0.2 and 0.3 mg/cig for water and 0.05 and 0.03 mg/cig for nicotine with packed and capillary columns respectively.

15. SPECIAL FUNCTIONAL MESOPOROUS SILICA MATERIALS FOR ELIMINATING NITROSAMINES IN SMOKE. Ying WANG, Yu Zhou, Hong Ji Wang, Jing Yang, and Jian Hua Zhu; College of Chemistry and Chemical Engineering, Nanjing University, Nanjing, China

Mesoporous silica is the new candidate for reducing the health hazard of tobacco smoking due to the high surface area and uniform pore size, and their regular periodic mesoporous structure can accommodate various modifiers to form special functional materials. Two latest progresses of research are reported in this paper, one is the SBA-15 with 3D-net like morphology and another is the "in situ carbonization" of template micelles. Mesoporous silica fails to trap tobacco specific nitrosamines (TSNA) that exist in the particles suspending in smoke because the sizes of particles achieve μm, exceeding the pore of the adsorbent. So, we prepare the new mesoporous candidates with net-like morphology to filter the particles with μm size in stream, like fishing-net to hold up fish. These fibers-like materials can efficiently reduce the content of TSNA from 14% to 48% when they are added into the filter of Chinese Virginia type cigarette, and they can also trap 20-42% of the tar in smoke. In additional, these 3D net-like mesoporous silica samples kept the high activity in the adsorption of nitrosamines in solution and gas stream in laboratorial tests.

It is necessary to use template in synthesis of mesoporous silica, and these template micelles should be removed to empty the channel. We utilize these micelles as the carbon precursor to prepare the hybrid materials with a layer of carbon coated inside the channel, possessing

a much higher activity than siliceous material in the adsorption and degradation of volatile nitrosamines.

16. ROUTINE ANALYSIS OF BENZO(A)PYRENE IN CIGARETTE SMOKE BY A MODIFIED MDGC/MS SYSTEM. SHI Jiaqin, Liu Baizhan, and Xie Wenyan; Technical Center, Shanghai Tobacco Group Corp., Shanghai, China

A method for the determination of benzo(a)pyrene in cigarette smoke was developed. The particulate matter in mainstream cigarette smoke collected on Cambridge pad was extracted with cyclohexane and the extract was injected into heart-cutting multidimensional GC/MS directly, without any further clean-up step. The MDGC system was based on 6890 GC fitted with a MDGC kit MDS6890 (SGE). The 1st dimensional column was DB-5MS (30m×0.25mm id×0.50um df the 2nd dimensional column was DB-17 30m×0.25mm id×0.25um df, a deactivated silica capillary column(2m×0.53mm id) was used as the guard column for the 1st dimensional GC. The mid point restrictor (the key part of MDGC) was modified. The graphite restrictor was replaced by a 0.25 mm id fused silica capillary column, the two-hole graphite ferrule was replaced by a press-fit glass Y-splitter of zero dead volume. The stability of retention time on the 1st dimensional column was significantly improved and the accuracy of heart-cutting was ensured. The absence of graphite in the restrictor eliminated the carry-over of benzo(a)pyrene completely. Based on this system, a simple, sensitive, fast and reliable method was developed for the routine determination of smoke benzo(a)pyrene. The coefficient of variation for repeatability was 1.94%. The recovery was from 90.74% to 101.86%. The detection limit of qualitative analysis and quantitative analysis was 0.2ng/cig and 0.6ng/cig, respectively. The intra-day and inter-day repeatability of the system was satisfactory. The benzo(a)pyrene content in the 2R4F Kentucky reference cigarette smoke determined by this method was 6.60ng/cig, while it was 6.96ng/cig as reported in literature. Cigarette samples of different filters, blends and tar deliveries were determined.

17. PUFF-PROFILE MONITORING EQUIPMENT AND TEST SETTING BOTH INFLUENCE HUMAN YIELD-IN-USE MEASURES. Paul NELSON, J.A. Bodnar, M.F. Borgerding, S.A. Bowman. K.M. Harger, E.K. Round, T.J. Steichen, M.F. Stiles, and J.H. Robinson; R.J. Reynolds Tobacco Co.,Winston-Salem, NC

Previous work has shown that human yield-in-use (YIU) measurements obtained from cigarettes smoked during puff-profiling are usually greater than those obtained from cigarettes smoked outside the laboratory. The present data speak to whether these differences result from the puff-profiling hardware attached to the cigarette and/or from smoking behavior differences related to the laboratory setting. Data from two smoking behavior studies involving YIU measures demonstrate effects of both the hardware and laboratory setting.

Study 1: Thirty smokers participated in two all-day sessions smoking either 14 UB (usual brand) or 14 Test cigarettes at 30 min intervals. In a given session, 5 of the 14 cigarettes were equipped for puff-profiling measurement and the other 9 cigarettes were smoked without profiling. YIU nicotine measures were significantly higher for both UB (1.92 vs. 1.68 mg/cig) and Test (1.65 vs. 1.43) cigarettes when the puff-profiling equipment was attached.

Study 2: Forty-four smokers participated in three weekly sessions where they smoked three different cigarettes. On the day prior to their lab visit, they collected all butts from cigarettes smoked outside the lab (OL). In the lab, they smoked one cigarette attached to a puff-profiler probe (PP), waited 30 min, then smoked a second cigarette which was not profiled (UP). For each cigarette, YIU "tar" and nicotine followed the order: profiled > unprofiled > field. (Average YIU nicotine mg/cig.: 1.57 (PP), 1.44 (UP), 1.34 (OL); average YIU "tar" mg/cig.: 18.0 (PP), 16.9 (UP), 13.4 (OL))

These results indicate that human yields obtained in the laboratory overestimate typical cigarette yields to the smoker and that puff profiling also leads to an additional increase in yield estimates.

18. MAINSTREAM SMOKE CHEMICAL AND BIOLOGICAL ANALYSES FOR 3R4F KENTUCKY REFERENCE CIGARETTE. C. WILLIARD and R. Leverette; Lorillard Tobacco Co., Greensboro, NC

Most analytical and biological labs routinely analyze reference cigarettes to monitor method performance and variability. In 2007, a new reference cigarette, 3R4F, was introduced to replace the 2R4F Kentucky Reference cigarette. Currently minimal data exist for many of the analytes regarding the 3R4F reference cigarette compared to a wealth of information available for the 2R4F Reference cigarette. This poster reports 3R4F and 2R4F data for tar, nicotine, carbon monoxide, tobacco specific nitrosamines, semi-volatiles, polyaromatic amines, and benzo[a]pyrene in mainstream smoke. Biological mutagencity data obtained from Ames biological assays is also reported. Significant differences were observed between the two reference cigarettes in the TA98 Ames Assay. Differences in the chemical composition of the mainstream smoke were examined as a possible explanation for the differences observed in the TA98 Ames Assay.

19. DEVELOPMENT OF IN-VIVO MOUSE MODEL FOR SMOKING-INDUCED CARDIOVASCULAR DISEASE. M.A. EL-MAHDY, W. Johnson, C. Hemann, A. Tewari, H.M.A. Talukder, and J.L. Zweier; Center for Environmental and Smoking Induced Diseases, Davis Heart and Lung Research Institute, The Ohio State University, Columbus, OH

Animal models for cigarette smoke associated diseases are important for research studies of the mechanisms involved in disease pathogenesis. We have developed an exposure protocol that results in cardiovascular dysfunction in the exposed mice. Male C57BL/6 mice, 8-9 weeks of age, were exposed to whole body mainstream and side stream cigarette smoke generated from 3R4F reference research cigarettes that deliver 9.4mg tar and 0.726mg nicotine per cigarette under the standard Cambridge filter smoking condition. The smoking machine was programmed to puff smoke over a period of ~24 min (240 puffs), followed by a break of fresh air for ~ 20 min. The total exposure time was ~72 min per day 6 days per week for 8, 16, 32 or 48 weeks. Exposure concentrations and durations, concentrations of NO, NO_2, O_2, HCN, H_2S, NH_3, SO_2, CO, and volatile organic compounds were closely monitored to assure reproducibility of daily exposure. We observed significant changes in the arterial blood pressure and vascular endothelial function as manifestations of smoke-induced cardiovascular disease. Blood carboxyhemoglobin concentration, as an exposure marker, and CO dissociation kinetics were also measured. Since cigarette smoke contains several gases including NO, CO, and H_2S that are known signaling molecules and

can protect against or modify disease, it is important to measure their concentrations and correlate these with the induction and severity of disease.

20. MAINSTREAM TOBACCO SMOKE EXPOSURE OF APO-L-MICE. GENE EXPRESSION ANALYSES OF THE THORACIC AORTA. Brian K. NORDSKOG, G.M. Curtin, J.E. Brown, and B.R. Bombick; R.J. Reynolds, Winston-Salem, NC

The goal of this study was to identify key molecular alterations induced by mainstream smoke exposure and diet in a genetically susceptible mouse model of atherosclerosis. ApoE-deficient female mice were exposed to nose-only mainstream tobacco smoke for 18 weeks (3h/day, 5day/week) at a concentration of 0.48 mg WTPM/L +/- high fat diet. Total RNA from thoracic aortas was isolated and prepared for hybridization onto Affymetrix 430 mouse 2.0 arrays. Using a Benjamini and Hochberg False Discovery Rate for multiple testing correction ($p<0.05$), and a 2-fold threshold, ANOVA analysis identified 3124 differentially expressed genes compared to control samples. Genes having the greatest change from basal expression included: GREM1, IL1RA, TIMP1, LCN2, CLEC4D, ANXA8, RNF123, CRABP2, CXCL1 and GPR176. Ontology and pathway analyses were conducted using GeneSifter and Pathway Studio software suites. The top gene ontologies identified included immune and inflammatory processes, protein and carbohydrate binding, lysophospholipase activity and cellular locations/processes related to endocytosis, lysosomal degradation and the extracellular matrix. Compared to controls, thoracic aortas from mice consuming the high fat diet and exposed to cigarette smoke had the highest number of differentially expressed genes, whereas the chow fed mice exposed to smoke had the fewest. Based on gene expression and cluster analyses, the treatment groups clustered primarily based on diet with smoke exposure augmenting the gene expression responses. In summary, the combination of diet and smoke exposure had the biggest effect on molecular alterations in the thoracic aorta of ApoE-/- mice.

21. COMPARISON OF *IN VITRO* MICRONUCLEUS ASSAY AMONG FOUR PROCEDURES. Hiroshi FUKUDOMI[1], Maiko Ogura[2], Toshiro Fukushima[1], and Toru Tsujimoto[1]; [1]Japan Tobacco Inc., Tobacco Science Research Center, Yokohama, Japan, [2]Japan Tobacco Inc., Product Science Division, Tokyo, Japan

The *in vitro* micronucleus (MN) assay has been used as one of the tools for assessment of the genotoxic potencies of cigarettes in the tobacco industry. It is recognized that the difference in results among laboratories is larger in the MN than in the widely-used Ames assay. The variation in results is assumed to be due to several factors, mainly the diversity in procedures and/or the difference of laboratory conditions. However, there is little information regarding these aspects.

We tried comparing four procedures in terms of reproducibility and relative activity among cigarettes. Since the comparative study was conducted in the same facility with consistency of environment, the variation factors associated with the laboratory conditions were eliminated.

The reproducibility was investigated based on three replicates of the experiment using 3R4F, and the relative activity was analyzed based on two replicates of each experiment using Flue-cured, Blend and Burley cigarettes.

For the evaluation of the results, EC3SC, the effective concentration for three times the solvent control value, was used as the MN activity index.

As a result, the reproducibility in EC3SC of 3R4F with a coefficient of variation of about 20% was realized in all procedures with and without S9.

Furthermore, the order of the MN activity of the sample cigarettes was the same (Flue-cured > Blend > Burley) in all procedures with and without S9, although there were slight differences in the ratios between the MN activities of cigarettes among the four procedures with S9.

It is suggested that comparable results are obtained under these four procedures when the tests are examined in the same laboratory.

22. COMPARISON OF THE SENSITIVITY OF HUMAN BRONCHIAL EPITHELIAL CELLS TO CIGARETTE SMOKE-INDUCED INFLAMMATORY RESPONSES. Ji-Hye YOO, Han-Jae Shin, Hyung-Ok Sohn, Chul-Hoon Park, Hyeong-Seok Lee, and Chung-HO Lee; KT&G Central Research Institute, Daejeon, Korea

The aim of this study is comparing the sensitivity of both two NCI-H292 and A549 cell types to acute inflammatory responses induced by cigarette smoke. For this, we treated the cells with two kinds of smoke fractions derived from 2R4F reference cigarettes: total particulate matter (TPM) collected onto a Cambridge filter pad and gas/vapor phase (GVP) prepared by bubbling through in buffer solution. When we measured cellular cytotoxicity by neutral red uptake assay after treatment for 24 hours, TPM and GVP induced cytotoxic effect in a dose-dependent manner in the range of 10-100 ug/ml and 60-300 ug, respectively, in both cell types without any cellular difference. Additionally, when we examined acute inflammatory responses by analyzing cytokines secreted into culture media including tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8), and transforming growth factor-α (TGF-α) as well as matrix metalloproteinase-1 (MMP-1), the treatment with smoke fractions increased those marker proteins in a dose-dependent manner in NCI-H292. Meanwhile, in A549 cells only MMP-1 was observed to be increased in a dose-dependent fashion. To further investigate whether it is due to gene expressional regulation, we examined mRNA expression of those genes by RT-PCR. Collectively, our data indicate that NCI-H292 cell type is more sensitive to cigarette smoke-induced inflammatory response than A549 cells. This suggests that NCI-H292 could be useful as an in vitro evaluation tool to assess harmful effects of cigarette smoke.

23. ANALYSIS AND IMPROVEMENT OF THE SMOKE DELIVERY SYSTEM FOR AUTOMATED CIGARETTE SMOKE INHALATION EXPOSURE. Vladimir B. MIKHEEV[1], Alec K. Hitchman[1], Bruce R. Westerberg[1], and David J. Hesse[2]; [1]Battelle Toxicology Northwest, Richland, WA, [2]Battelle, Columbus, OH

Delivery of the tobacco smoke for the inhalation exposure requires simultaneous maintaining stable constituents concentration, stable critical ratios (such as CO/WTPM, CO/nicotine, etc), and stable environmental conditions (such as relative humidity and temperature), while working under wide range of smoke concentrations. CFD simulations along with smoke generation experiments were conducted in order to analyze influence of smoke

mixing-dilution-delivery system parameters on stability of chemical constituents. Particular attention was paid to the design of smoke siphoning part of the system. Earlier experiments along with CFD analysis of the original mixing-dilution-delivery system confirmed instability of aerosol mass flow with siphon flow increase caused by high turbulence and flow non-uniformity. Redesign of the mixing-dilution-delivery system followed by CFD analysis and direct smoke generation experiments allowed higher flow uniformity, and as a result better stability of smoke constituents under wide range of concentrations. Wet total particulate matter, carbon monoxide, nicotine, aldehydes (acetaldehyde, formaldehyde, acrolein, prionaldehyde, crotonaldehyde), and particle size were measured. Monitoring and recording of temperature, relative humidity, and system flow rates was provided by automated Battelle Exposure Data Acquisition and Control System.
Acknowledgement: This work was supported by Internal Battelle R&D funding

24. DETERMINATION OF AGROCHEMICAL RESIDUES IN TOBACCO COMBINING SOLID-PHASE MICROEXTRACTION AND GAS CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY. Jeong-Min LEE, Gi-Chul Jang, Jang-Mi Lee, Hyo-Keun Kim, and Keon-Joong Hwang; KT&G Central Research Institute, Daejeon, Korea

A solid-phase microextraction (SPME) method combined with gas chromatography-tandem mass spectrometry was evaluated for its effectiveness in the extraction and analysis of ten organophosphorus and pyrethroid residues in tobacco. The influence of parameters such as the type of fiber, adsorption/desorption time and the extraction temperature over the precision and accuracy of the SPME method was investigated. Among three types of fibers, polyacrylate (PA), poly-dimethylsiloxane (PDMS) and polydimethylsiloxane-divinylbenzene (PDMS-DVB), PDMS fiber was selected for the extractions of the agrochemicals, because the adsorption efficiency of the PDMS fiber seems to be more effective than other fibers. The SPME device was automated and on-line coupled to a gas chromatograph with a triple quadrupole mass spectrometer. The triple quadrupole MS-MS instrument gave a good linearity for the ten agrochemicals over the range of 0.01 μg/mL to 0.5 μg/mL. The relative standard deviations for replicates ranged from 2.7% (bromophos) to 9.1% (permethrin). The LODs of the method fully satisfied the requirements of the CORESTA GRL. The recoveries of ten selected agrochemicals obtained from the analysis of the fortified tobacco samples ranged from 80.5% to 95.1%, indicating high method accuracy. Also the reproducibility of the analytes showed a good result under 10% RSD. These results demonstrate that SPME with the mass spectrometric analytical system is a fast, simple and solvent-free method for qualifying and quantifying the selected organophosphorus and pyrethroid residues from tobacco samples.

25. UP-REGULATION OF THE 4-HYDROXYBENZOATE POLYPRENYL TRANSFERASE GENE IN CULTIVATED TOBACCO AND ITS EFFECT ON COENZYME Q_{10} LEVELS AND THE RESPONSE TO OXIDATIVE STRESS. Michael R. STIFF, David Danehower, and Arthur Weissinger; Department of Crop Science, North Carolina State University, Raleigh, NC

Coenzyme Q_{10} is an integral component of electron transport during oxidative phosphorylation within the mitochondria. In plants, CoQ_{10} is also an integral part of the 'alternative' electron transport associated with the plant response to oxidative stress. While the amount of CoQ_{10} has been increased in a model tobacco variety, to date there

have been no published reports of enhanced levels of CoQ_{10} in commercial cultivars. We transformed haploid burley and flue-cured tobaccos, TN90LC and NC55, respectively, via *Agrobacterium*-mediated transformation with the gene encoding 4-hydroxybenzoate polyprenyl diphosphate transferase from *Arabidopsis thaliana*. The haploid lines were then doubled, resulting in homozygous doubled haploid lines. We report an apparent increase in the amount of CoQ_{10} in these lines as measured by increased production of CoQ_{10} using HPLC-UV.

Lines with elevated CoQ_{10} levels will be used to test the hypothesis that increased CoQ_{10} will improve tobacco's response to oxidative stressors. These results will provide insight into the use of secondary metabolism for the improvement of crop responses to environmental oxidative stressors, an increasing concern as climates become warmer. These lines will also be included in future analyses for decreased reactive oxygen species in the cured leaf.

26. RAPID METHOD FOR THE QUANTIFICATION OF IMIDACLOPRID RESIDUES IN TOBACCO AND CIGARETTE FILLER BY HPLC. Sharad K. MEHTA, S.V. Dhalewadekar, and B.J. Rajesh; ITC R&D Centre, ITC Ltd., Bangalore, India

Imidacloprid is a newly introduced broad-spectrum insecticide.1-[(6-chloro-3-pyridyl)-methyl]-2-nitroimidazolidin, chloronicotinyl compound.It is effective for harmful & resistant pest species .

A simple HPLC method is developed for the quantitatification of Imidacloprid residues in tobacco leaves and cigarettes.The titled method using HPLC with Photo-diode Array (PDA) detector involves extraction of Imidacloprid with Dichloromethane, followed by liquid- liquid partition clean- up with 1.5% Sodium carbonate solution, concentrating the extract using rota- evaporator and diluting with Acetonitrile- Methanol mixture (1:1) and subsequent chromatographic separation on LiChrospher 100 RP- 18e,250mm x 5 micron (Merck). Gradient program is used for better resolution of Imidacloprid from tobacco impurities. Mobile phase A - {(1.5% Acetic acid solution (85%), Acetonitrile (10%) and Methanol (5%)} (pH- 3.0) and Mobile phase B- {(1.5% Acetic acid solution (20%), Acetonitrile (10%) and Methanol (70%)}. The separations are monitored by UV absorbance at 275 nm. Retention time (RT) for Imidacloprid is found to be 8.54 min at a flow rate of 1.0 ml/min. Various parameters *i.e.* mobile phase, sample cleaning techniques to remove tobacco impurities were optimized.The method has been validated by standard validation protocols i.e.Limit of detection, Limit of Quantification, Recovery, Repeatability and Reproducibility. Minimum recovery of 80% was obtained with a linear regression coefficient of 0.9998 for the range of 0.05- 10 mg/Kg Imidacloprid. Limit of detection is 0.02ppm. Titled method has advantage of easy and simple sample preparation.

27. MODIFICATION OF ALKALOID ACCUMULATION IN *NICOTINA TOBACCUM* THROUGH GENETICAL ENGINEERING. Bingwu WANG, Ray Long, Ramsey Lewis, and Rongda Qu; Department of Crop Science, North Carolina State University, Raleigh, NC

Alkaloids greatly affect the quality of commercial tobacco leaf. Four major alkaloids, nicotine, nornicotine, anabasine, and anatabine, partially share a common biosynthetic pathway in tobacco. To modify the relative content of these alkaloids in tobacco leaf, two genes: PMT (Putrescine N-methyltransferase) and QPT (Quinolinate phosphoribosyltransferase),

which are involved in the alkaloids biosynthesis pathway, are over-expressed in transgenic tobacco plants. We did not observe significant changes at total alkaloid level among the plants that over-express PMT or QPT in a replicated field test. However, two F1 hybrids from crossings between PMT and QPT over-expression lines had increased total alkaloid level in last year's field test. The experiment is being repeated this year to verify last year's results.

In addition, our preliminary results suggest that we have cloned two transcriptional factors from tobacco roots after topping. These two TFs have different expression patterns and might be involved in the regulation of nicotine biosynthesis.

28. RESEARCH ON CONTENT CHANGE OF FURFURAL AND 5-METHYL FURFURAL OF FLUE-CURED TOBACCO FROM DIFFERENT REGIONS DURING ITS PYROLYSIS AND COMBUSTION. PENG Xin-Hui, Yi Jian-Hua, Sun Zai-Jun, Pu Wen-Xuan, Dai Yuan-Gang, Wang Yao-Fu, and Peng Yu; Technology Research & Development Center, China Tobacco Hunan Industrial Corp., LTD., Changsha, Hunan, China

The ecological environment has important impact on the quality of flue-cured tobacco. In order to finding out the differences of aroma matters and its origin in the WTPM of flue-cured tobacco, furfural and 5-methyl furfural, which are selected as typical aroma matters in the WTPM of tobacco from different areas during their pyrolysis at 310°C and combustion, were studied in this paper with some statistical analysis on the relationships between them and the other chemical compositions. The results indicate that during pyrolysis the content of furfural and 5-methyl furfural are at its peak in tobacco leaves from Wannan, next is from Yuqing, the followed are from Jianghua, Sangzhi and Zimbabwe. And during combustion the content of furfural and 5-methyl furfural in WTPM are relatively higher in tobacco leaves from Zimbabwe and Wannan, the followed are from Jianghua, Yuqing and Sangzhi. It has been proved statistically that significant or even more significant differences of the content of furfural and the 5-methyl furfural of tobacco during its pyrolysis and combustion exist among different regions. The content of furfural and 5-methyl furfural of tobacco during pyrolysis are much higher than those during combustion. The contents of furfural in the WTPM of smoking during tobacco combustion are only slightly lower than that of 5-methyl furfural although that of furfural are well lower than that of 5-methyl furfural during their pyrolysis. The content of furfural and 5-methyl furfural during pyrolysis are positively relative to the content of sugar, reduced sugar and the ratio of reduced sugar to total alkaloid, but negatively relative to the content of total alkaloid and total nitrogen. And the content of furfural and 5-methyl furfural during tobacco combustion are positively relative to the content of chloride and ratio of reduced sugar to total alkaloid in tobacco leaves, but negatively relative to the content of other four components. Furfural and 5-methyl furfural in the smoking don't originate mainly from the long-playing intense pyrolysis, and they also can distinguish tobacco from different areas. Total alkaloid and chloride should be concerned properly in the course of cigarette formula, as well as characteristic high-quality tobacco production.

29. EFFECT OF CIGARETTE SMOKE EXPOSURE ON THE HUMAN METABOLOME.
J. REID; Lorillard Tobacco Co., Greensboro, NC

Metabolomics is a technique for chemically identifying and quantifying small molecule biochemical metabolite profiles, as the end product of normal metabolic pathways. Using this, we explored changes in the metabolic profiles of human alveolar epithelial carcinoma (A549) cells following separate *in vitro* exposure to mainstream whole smoke aerosol, wet total particulate matter (WTPM) and gas/vapor phase (GVP) generated from 2R4F Kentucky reference cigarettes. The A549 cells were exposed to smoke condensates at 0, 5, 25, and 50 μg/mL or directly to mainstream smoke from 2, 4, and 6 cigarettes and analyzed after 1 h and 24 h exposure times. The treated cells were analyzed for biochemical changes in approximately 600 small molecule normal metabolic products. Both WTPM and GVP exposures showed a decreased glycolysis effect, based on decreased glycolytic intermediates, with increased oxidative stress and cell damage. Variations in the Krebs and the Urea cycles were unique to WTPM exposures, while an increase in hexosamines and changes in lipid metabolism were unique to the GVP exposures. The whole smoke exposures showed dramatically altered glutathione levels, enhanced polyamine and pantothenate levels, an increase in β-oxidation of fatty acids, and an increase in phospholipid degradation, as evidenced by an increase in phosphoethanolamine. The glutathione, glutamine, and pantothenate metabolites showed the most significant changes in the A549 cells upon cigarette smoke exposure, and appeared to be dose responsive to the levels of smoke delivered. The increases and decreases in specific metabolites will be presented along with an interpretation of the associated biochemical pathways.

MONDAY AFTERNOON, SEPTEMBER 28, 2009

SESSION A ***Session Chair: Dan Heck***

2:20 PM MONDAY

30. THE INFLUENCE OF CIGARETTE BASE PAPER PHYSICAL PROPERTIES ON THEIR MEASURED CO_2 DIFFUSIVITIES AND THE RESULTANT EFFECTS ON ISO SMOKING YIELDS. Matthew HESFORD and Paul Case; British American Tobacco, Group R&D Centre, Southampton, UK

Lower or Reduced Ignition Propensity cigarettes (LIP or RIP) is a term used to describe modified cigarettes that demonstrate lowered ignition propensity under specific laboratory tests. These cigarettes must meet standard performance criteria described by the test method established by ASTM E2187-04 regulations in which lit cigarettes must self-extinguish under controlled conditions.

Studies have shown that there is a higher correlation between the ASTM performance of cigarettes and the measured diffusivity of CO_2 through the paper than there is with permeability. Therefore cigarette base-paper diffusivity is becoming increasingly important when specifying LIP paper grades.

Recently, several studies have sought to identify the base-paper parameters that influence diffusivity and the effects that the latter has on cigarette smoking yields (NFDPM, nicotine and CO). Discussed here are the results of a systematic study which was undertaken in order to investigate the above, across a range of filler and fibre levels and paper permeabilities. The cigarette paper physical characteristics (*e.g.* tensile strength, stretch, opacity and whiteness) as a function of the fibre and filler contents of the paper are also discussed. Amongst other findings, the base-paper diffusivity is shown to increase as the filler content is increased and as the fibre content is decreased. In turn, NFDPM and CO yields decrease with increasing diffusivity, as does the puff number. Tensile strengths of the papers are shown to increase as the fibre level is increased and as filler levels and paper permeabilities decrease.

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31. INFLUENCE OF POTASSIUM ON THE FORMATION OF BENZO[A]PYRENE IN TOBACCO PYROLYSIS. Yoji UWANO and Shinya Yoshida; Japan Tobacco Co., Yokohama, Japan

It is known that potassium, which is the most abundant alkali metal in tobacco leaves, affects the thermal degradation of biomass and cigarette combustion. However, there is little information on the relationship between potassium and the formation of benzo[a] pyrene (B[a]P) from tobacco. In this study, tobacco samples with various potassium contents were pyrolyzed using an infrared image furnace to evaluate their B[a]P yields, and their pyrolysis behaviors were investigated by thermogravimetric analysis (TGA). Potassium-extracted samples were prepared by washing tobacco samples with water, and potassium-added samples were prepared by adding potassium lactate, potassium carbonate (K_2CO_3) and potassium chloride (KCl) to individual tobacco samples. It was found that

the removal of potassium by water extraction increased the yield of B[a]P significantly. However, the influence of potassium addition on the yield of B[a]P was different according to the types of salts and tobacco. The addition of potassium lactate or K_2CO_3 to flue-cured tobacco decreased the yield of B[a]P, while the KCl addition increased it. The addition of potassium lactate to burley tobacco did not decrease the yield of B[a]P. As the results of the TGA, the addition of potassium lactate or K_2CO_3 to flue-cured tobacco lowered the temperature at which cell wall components such as cellulose and lignin decomposed. On the other hand, no significant change in temperature was observed in the case of the KCl addition to flue-cured tobacco and for the addition of potassium lactate to burley tobacco. These results indicate that the modification of the pyrolysis process of cell wall components by potassium influences the formation of B[a]P and the influence of potassium depends on the form of potassium that is present.

3:00 PM MONDAY

32. COMPARISON OF THE EFFECTIVNESS OF TAPE AND SIMPLE HOLDER BASED VENT BLOCKING DEVICES WHEN USED IN INTENSIVE SMOKING REGIMES. I. TINDALL, and T. Mason; Cerulean, Milton Keynes, UK, and L. Dutertre, Laboratoire National de Metrologie et d'Essais, Trappes, France

The complete blocking of ventilation features has been mandated in Health Canada standards for a number of years. In contrast some US states mandate blocking of 50% of ventilation holes during smoking and the current ISO standards require no ventilation blocking. However as smoking methods are reviewed it is becoming increasingly likely that the current ISO method will be supplemented by some form of intensive smoking regime which will include vent blocking. However some difficulties arise in the manner of vent block prescribed by the Health Canada method, namely the use of adhesive tape to block holes.

This is a time consuming activity that relies on the skill and dedication of the person taping. A conceptually simpler method, that would reduce the human element present in applying tape for vent blocking, would be to devise a holder that occludes the ventilation holes and is equivalent to the taping method.

A simple holder was devised that allowed occluding of the ventilation holes and was suitable for a range of product diameters. This holder was compared against the tape method using 15 different commercially available brands of different styles and constructions. Comparisons were made on the basis of pressure drop of taped and holder based rods and smoking yields.

Statistical equivalence was shown with a 95% confidence limit for paired t tests for pressure drop measurements and two sample t tests for smoked rods.

The R and r implications are discussed with considerations of reducing variability of intensive smoking methods explored.

3:20 PM ***Break***

3:50 PM MONDAY

33. MODULATING EFFECTS OF FE(II), FE (III) AND QUINONE/HYDROQUINONE ON TOBACCO SMOKE-MEDIATED HYDROGEN PEROXIDE FORMATION.
E.A. ROBINSON and M. Misra; Lorillard Tobacco Company, Greensboro, NC

Accumulation of iron in the lung has been demonstrated in smokers. The tar phase of tobacco smoke contains a stable radical population consisting of a mixture of quinone- (Q), semiquinone- (QH•), and hydroquinone- (QH_2) like compounds. This Q/QH•/QH_2 couple is capable of releasing iron from labile iron stores in the lung to participate in Fenton reactions that lead to the formation of H_2O_2 and HO• radical. We investigated the role of Q and QH_2 on Fe(II)- or Fe(III)-mediated H_2O_2 formation in the presence of whole mainstream cigarette smoke and particulate phase smoke. Experiments were performed based on statistical "Design Of Experiments" in order to determine the individual and combined effects of smoke sample, Fe(II), Fe(III), Q, and QH_2 on the formation of H_2O_2 as measured by the fluorogenic probe, Amplex Red. Ferritin was tested as an iron source in the presence of Q and QH_2. The data clearly showed that the amount of smoke in the sample was the dominant factor for the formation of H_2O_2. The use of ferritin as a source of iron had no effect. In the absence of Q and QH_2, Fe(III) showed positive correlation, while Fe(II) had a negative correlation with H_2O_2. In the absence of external iron, Q and QH_2 both had positive effects on H_2O_2 formation. When all four species were present, neither form of iron had a statistically significant effect on H_2O_2. Shifts in ion chromatography peaks for the iron species and ultraviolet absorbance spectra for Q and QH_2 indicate metal complexation and oxidation/ reduction in the presence of the quinone species. These results suggest an essential role of the Q/QH•/QH_2 couple in iron-mediated hydrogen peroxide formation.

4:10 PM MONDAY

34. ON-LINE DETECTION OF TOBACCO SMOKE CONSTITUENT BY SINGLE PHOTON IONISATION TIME -OF-FLIGHT MASS SPECTROMETRY FOR ON-LINE ANALYSIS OF TOBACCO SMOKE: APPLICATIONS AND DETERMINATION OF THE PHOTO IONISATION CROSS-SECTIONS OF RELEVANT COMPOUNDS.
Ralf ZIMMERMANN, Markus Eschner, Matthias Bente, Christian Deuerling, and Mohammed Saraji; Univeristy of Rostock, Germany

Recently the single photon ionisation time-of-flight mass spectrometry (SPI-TOFMS) - technology was developed and adapted for on-line monitoring of tobacco smoke constituents. One of the main advantages of the SPI-TOFMS approach is the possibility to investigate dynamic processes during the smoking process. For example, different smoking regimes were investigated (mainstream and sidestream smoke) [1], the pyrolysis behaviour of different tobacco sorts was studied [2] and the exhaled smoke/breath was monitored [3]. Together with the company Borgewaldt KC GmbH, Hamburg, Germany a prototype of a commercial on-line tobacco smoke profiler was developed (Borgwaldt Photo-TOF). This prototype is operated with an improved Electron Beam pumped rare gas Excimer VUV-Light source, EBEL. In this work some on-line cigarette smoke monitoring applications are reviewed and new results, obtained by the on-line tobacco smoke profiler prototype, are shown. Furthermore results on the determination of SPI cross sections of tobacco smoke constituent are given. These are important parameters for the quantitative analyses of the

chemical species. For the photo ionisation cross section measurements, a gas chromatograph was coupled to a SPI-TOFMS device.

[1] T.Adam, R.R.Baker and R.Zimmermann, *J. Agr. Food Chem.* 55 (2007) 2055-2061
[2] T.Adam, S.Mitschke, T.Streibel, R.R.Baker and R.Zimmermann, *Anal. Chim. Acta* 572 (2006) 219–229
[3] F.Mühlberger, T.Streibel, J.Wieser, A.Ulrich and R.Zimmermann, *Anal.Chem.* 77 (2005) 4708-4714

4:30 PM MONDAY

35. MODELING BIOFILTRATION DEODORATION OF EXHAUST AIR FROM BURLEY TOBACCO PROCESSING PLANT. SHU Ming[1], Cen Pei-Lin[2], Yang Wei-Pin[1], Feng Feng[1], Xu Wei-Min[1]; [1]China Tobacco Zhejiang Industrial Co. Hangzhou, Zhejiang Province, China, [2]Department of Chemical and Biochemical Engineering, Zhejiang Unversity, Hangzhou, Zhejiang Province, China

As environmental legislation becomes increasingly stringent, tobacco industry is making efforts to improve exhaust air treatment in China. Comparing with flue-cured tobacco processing, the exhaust gas come out of burley tobacco processing is more irritant and undesirable due to its higher ammonia and nicotine concentrations. In order to describe the basic biofiltration process of odorous pollutants in a biofilter, a kinetic model was established. This model took into account biodegradation along with mass transfer and adsorption. The model treated the damp porous medium as a two-phase system: the air phase and the biofilm/solid phase. The process was modeled based on matter balance equations. The model equations of outlet concentration and removal efficiency were put forward and verified respectively, and model parameters were estimated by partial least-square regression. The value of parameter a reflected the rate of odor removal in the biofilter. The results showed that the predicted profiles agreed well with the experimental data.

4:50 PM MONDAY

36. BIODEGRADATION OF NICOTINE IN AQUEOUS EXTRACT FROM TOBACCO BY *PSEUDOMONAS* SP. ZUTSKD. SHU Ming[1], Yang Jun[1], Zhu Chen-Jing[2], and Zhong Wei-Hong[2]; [1]China Tobacco Zhejiang Industrial Co. Hangzhou, Zhejiang Province, China, [2]College of Biological and Environmental Engineering, Zhejiang University, Hangzhou, Zhejiang Province, China

The object of this study is to evaluate the feasibility of microbial degradation of nicotine in aqueous extract of waste tobacco (AEWT) which was utilized for preparation of reconstituted tobacco. One novel nicotine-degrading strain was isolated from waste tobacco. Based on standard morphological, physiological assays and nucleotide sequence analysis of enzymatically amplified 16s ribosomal deoxyribonucleic acid, it was identified as *Pseudomonas* sp. ZUTSKD with a Genebank Database access number of EF538425. Due to the higher concentration of nicotine, NaCl, and reducing sugar in AEWT, the strain had to go through a course of adaptation. The tolerance of *Pseudomonas* sp. ZUTSKD to NaCl and nicotine was improved from 2.0 % to 3.5% and from 4.5 g/L to 5 g/L respectively. Its tolerance to glucose was up to a concentration of 40 g/L. The domesticated strain showed the nicotine degradation ability under higher NaCl and nicotine concentrations, even in the

AEWT. In addition, it was found that the addition of glucose improved the cell growth but inhibited nicotine degradation as glucose concentration increased. It could be concluded that *Pseudomonas* sp. ZUTSKD could degrade the nicotine in AEWT. However, its tolerance to reducing sugars needs to be further improved.

5:10 PM MONDAY

37. EVALUATION OF THE EFFECT OF PHYTOL ON THE FORMATION OF PAHS IN CIGARETTE SMOKE. Serban MOLDOVEANU, William Coleman III, and Niraj P. Kulshreshtha; R. J. Reynolds Tobacco Company, Winston-Salem, NC

Pyrolysis of phytol has been studied with various purposes, including the potential formation of polycyclic aromatic hydrocarbons (PAHs). When pyrolyzed in a quartz tube at 860 ± 5°C in a flow of nitrogen (30 mL/min) phytol was reported to generate benzo[a] pyrene at a level of about 3.9 mg/g from the initial material. However, in a flash pyrolysis experiment performed at 900°C for 10 s the pyrolysis products were found more similar to those of a long chain hydrocarbon that typically generate only very low levels of PAHs. Since phytol is naturally present in tobacco at levels around 100-150 μg/g dry leaf, where it is bound in the form of an ester to form chlorophyll, the compound has been considered a PAHs precursor in cigarette smoke. This study evaluated the formation of PAHs when several levels of phytol were added on 3R4F cigarettes. The resulting phytol levels were up to about 15 times higher than those expected in cigarettes. These cigarettes were smoked under two different smoking conditions, 35 mL puff of 2 s every 60 s (35/60) and intensive 60 mL puff of 2 s every 30 s (60/30). A statistical evaluation of the dependence of total PAHs and the added level of phytol showed that the hypothesis of a zero slope for the dependence line cannot be rejected (with a $P = 0.101$ for 35/60 smoking and $P = 0.626$ for 60/30 smoking). Flash pyrolysis of free phytol and of chlorophyll *a* provided results that indicated that phytol bound in chlorophyll is not likely to generate different PAHs level compared to free phytol. The study showed that phytol is not a significant contributor/precursor to the PAHs formation in cigarette smoke.

5:30 PM ADJOURN

MONDAY AFTERNOON, SEPTEMBER 28, 2009

SESSION B ***Session Chair: Jim Franke***

2:20 PM MONDAY

38. DEVELOPMENT OF TECHNICAL REGULATIONS OF TAR, NICOTINE AND CARBON MONOXIDE FOR TOBACCO PRODUCTS IN THE EUROPEAN UNION IN THE LAST DECADE TILL TODAY WITH SPECIAL FOCUS ON CIGARETTES. Jürgen HAHN; Chemisches Und Veterinaruntersuchungsamt Sigmaringen, Sigmaringen, Germany

Since 2002 the Directive 2001/37/EC of the European Parlament (Tobacco Product Regulation) regulates the maximum levels for tar, nicotine and carbon monoxide. The Directive also asks for a statement of ingredients in tobacco products and its toxicological data in burnt and unburnt form as well as for addictive effects of ingredients. The industry has to provide the required information, but presently no binding guidelines for the evaluation of toxicological data for tobacco products exist.

The presentation will give some information about the rules and the function of organisations like national standardisation institutions, International Organisation for Standardisation (ISO), World Health Organisation (WHO) and some regulative committees like the Regulative Committee for Tobacco and Tobacco Product of the European Commission and the European Network of Government Laboratories for Tobacco and Tobacco Products. The relation between the different organisations will be discussed. Furthermore I will give an overview of the sampling and testing procedures used in the European Union.

2:40 PM MONDAY

39. THE SUBMISSION OF AVAILABLE TOXICOLOGICAL INFORMATION ON THE INGREDIENTS IN TOBACCO PRODUCTS IN THE EUROPEAN UNION. Anja THIELEN[1] , Gerhard Scherer[2], Wolf-Dieter Heller[1]; [1]Deutscher Zigarettenverband, Berlin, Germany, [2]Analytical-Biologisches Forschungslabor GmbH, Munchen, Germany

Article 6 of the European Union Tobacco Products Directive 2001/37/EC requires that manufacturers and importers of tobacco products submit a list of all ingredients used in the manufacturing of their tobacco products itemized by type and brand name. It calls for the list to be supplemented with the toxicological data available to the manufacturer and importer. For this purpose, the Commission developed drafts of two harmonized ingredient reporting forms based on a common EU format and improved definitions. Consequently, there are at the moment two tables for ingredients. Table 1 is supposed to provide a list of all ingredients used in a given product with their exact quantities. Table 2 is to be used for the submission of available toxicological information on, and the addictive properties of, the ingredients in burnt and unburned form. This table may include a broad range of different methods, such as in-vitro toxicity tests and inhalation studies, but also cardiovascular toxicity or addictive properties.

The fact that the toxicological evaluation takes into account both the combustion products and the potential addictive effects of ingredients is a new development. The presentation provides an overview on Table 2 and the problems or complications that may arise.

3:00 PM MONDAY

40. ESTABLISHMENT OF FUNCTIONAL RELATIONSHIPS FOR PREDICTING MAINSTREAM SMOKE CONSTITUENT YIELDS FOR CONVENTIONAL CIGARETTES FROM JAPANESE MARKET. Masahiro FUJIWARA, Takatsugu Hyodo, and Kazue Minagawa; Japan Tobacco Inc., Yokohama, Japan

Some papers about functional relationships for predicting mainstream smoke constituent yields using tar or other parameters as independent variables have been previously published. Since these functional relationships can predict the mainstream smoke constituent yields in cigarettes with reasonable range, these are useful methods for monitoring the mainstream smoke constituent yields. For the long-term use of the prediction formulas, stability should be checked because they are generated by a data set obtained in a specific time range. The objective of this study was to check the stability of the prediction formulas and to compare predicted values and measured values, using the data obtained at different times. In this study, data obtained in 2002, 2005 and recent data from Japanese market were used. Their mainstream smoke constituent yields were all analyzed in the same laboratory under ISO smoking conditions. The stability of the prediction formulas was examined by comparing slopes and Y-intercepts in simple linear regression models between the formulas derived from the data of 2002 and the ones derived from the data of 2005. The measured values for the brands obtained recently were compared with the predicted values derived from the prediction formulas using the data of 2005. Slopes and Y- intercepts of the prediction formulas derived from the data of 2002 and the ones from 2005, show no significant differences for most of the smoke constituents. Comparison of predicted values by the prediction formulas derived from the data of 2005, and measured values obtained from recent products, shows that most of the smoke constituent yields could be predicted within reasonable range.

3:20 PM ***Break***

3:50 PM MONDAY

41. COMPARATIVE ANALYSIS OF GENE EXPRESSION PROFILE OF TOBACCO TRICHOME IN DIFFERENT ECOLOGICAL REGIONS. CUI Hong and Ji Hao; National Tobacco Cultivate Physiology and Chemistry Research Center, He Nan Agricultural University, He Nan, Zhengzhou, China

To identify the difference of gene expression of tobacco trichomes in different ecological regions, microarray analysis were performed between trichomes collected from tobacco leaves of different ecological region. The same tobacco variety (*Nicotiana tabacum* L, k326) was choosed to plant in Henan and Yunnan in China respectively. The entire set of 2831 trichome cDNAs were amplified and spotted in high density on glass microscope slides. Each glass slide held three copies of the entire array. To ensure the reliability of the results, two microarray slides (six replicates) were used for each experiment. Two independent

RNA preparations were made for each analysis, and labeling of the cDNA (Cy3 versus Cy5) was reversed on the second slide. The result showed that 445 genes were differentially expressed (with ratio values ≥2 or ≤0.5) in total, in which 239 were up-regulated in Hennan and other 206 were up-regulated in Yunnan. Though founction analysis, we found that carbolic metabolism related genes seemed highly expressed in Yunnan , while genes related to chloroplast development , pigment metabolism and tepenoid metabolism pathway were highly expressed in Henan. Besides ,we also found that there were a great deal of resistance and defense genes in trichomes that probably respected to their specific circumstance. In addition, 31 novel genes were also detected in the experiment. The functions of these novel genes and their importance in tobacco trichome development and substance metabolism needs to be studied in the future.

4:10 PM MONDAY

42. CLONING AND EXPRESSION OF CALCIUM-DEPENDENT PROTEIN KINASE GENE FAMILY FROM *NICOTIANA TABACUM*. Guanshan LIU[1,2], Yuhe Sun[2], Shuaishuai Tai[2], Weifeng Wang[2], and Jia Chen[3]; [1]Key Laboratory for Tobacco Quality Control, Ministry of Agriculture, China, [2]Tobacco Research Institute of CAAS, Quingdao, Chandong, China, [3]State Key Laboratory of Plant Physiology and Biochemistry, Beijing, China

To further study the function of calcium-dependent protein kinase (CDPK) gene family in tobacco (*Nicotiana tabacum*), we initiated a project to isolate CDPKs from tobacco (*Nicotiana tabacum*), describing the sequence characteristics, evolutionary relationship and gene expression. RT-PCR, RACE and bioinformatic methods were used to isolate CDPKs from tobacco; a phylogenetic tree was created using the MEGA4.0 program; the expression patterns of three full-length CDPK genes were studied by RT-PCR. After all aforementioned efforts, we were able to obtain 8 additional tobacco CDPK genes, of which 3 possessed complete ORFs; phylogenetic analysis divides the CDPK gene family into four subfamilies, and two putative tobacco and Arabidopsis orthologous CDPK genes might correspond to well-conserved functions; three full-length tobacco CDPK genes were detected in all tobacco organs tested, but the expression patterns of them were significantly different. Eight non-redundant tobacco CDPK genes were isolated in this study, along with the previously characterized CDPK genes, revealed that at least 15 members of the CDPK family are existing in tobacco. This work provides a foundation for a genome-wide study of this important gene family in tobacco.

4:30 PM MONDAY

43. CLONING, STRUCTURAL FEATURES, AND EXPRESSION ANALYSIS OF RESISTANCE GENE ANALOGS IN TOBACCO. GAO Yu-Long, Xu Zhao-Li, Jiao Fang-Chan, Yu Hai-Qin, Xiao Bing-Guang, Li Yong-Ping, Lu Xiu-Ping; Yunan Institute of Tobacco Science, Yuxi, China

Using degenerate primers based on the conserved NBS domain and PK region, we isolated 100 RGAs from tobacco variety Nicotiana repanda. BLASTx search against the GenBank database revealed that 27 belong to the NBS class and 73 belong to the PK class. Cluster analysis and multiple sequence alignment of the deduced RGAs protein sequences indicate that the cloned tobacco RGAs of the NBS class can be divided into two types: TIR and non-

TIR types. Both types possess 6 conserved motifs (P-loop, RNBS-A, Kinase-2, RNBS-B, RNBS-C, GLPL).Based on their sequence similarity, the tobacco RGAs of the PK class were assigned to 8 subclasses, with the kinase region containing 10 conserved motifs. We examined their expression after infection with either TMV or the tobacco black shank pathogen (*Phytophthora parasitica* var. *nicotianae*). The expression levels of 4 RGAs of the PK class were found to be significantly elevated by TMV and of 1 RGA of the PK class and 3 RGAs of the NBS class were up-regulated by *P. parasitica* var. *nicotianae*. The expression of two RGAs of the PK class was induced by *P. parasitica* var. *nicotianae*. Infection of tobacco by either TMV or *P. parasitica* var. *nicotianae* enhanced the expression of *NtRGA2*, a RGA of the PK class. The present study shows that RGAs are abundant in the tobacco genome and the identification of tobacco RGAs induced by pathogens should provide valuable information for cloning related resistance genes in tobacco.

4:50 PM MONDAY

44. GENETIC ANALYSIS AND MOLECULAR MARKING FOR RESISTANCE OF TOBACCO CUCUMBER *MOSIAC VIRUS*. Yuanying WANG, Jingyuan Fan, Caihong Jiang, Wansheng Chen, Min Ren, and Haizhou Hu; Tobacco Research Institute of Chinese Academy of Agricultural Sciences, Qingdao, China

Cucumber mosaic virus (CMV) in tobacco cause significant losses in China. The resistant varieties to CMV is considered to be the most economical and effective methods to control the disease. The objective of this study is to explore the genetic mechanism and molecular marks for this resistance so that new approach can be identified for tobacco virus resistance breeding. The study was carried out using F_1, F_2, BC_1 populations from a cross between CMV resistant varieties and CMV susceptible varieties. They were identified by artificial inoculation on seedlings in greenhouse. Results showed that the resistant character of both resistant parents is controlled by single recessive gene. DNAs of F_2 population were extracted to develop into a resistant pool and a sensitive pool based on the BSA (bulk segregant analysis). 135 pairs SSR primers were used for molecular marker analysis. The results showed that the genetic distance between $SM1_{350}$ and resistant genes from "tiebazi" is 8.64 cM, and the genetic distance between $SM2_{270}$ and resistant genes from "taiyan-8" is 3.92 cM. The Specific fragment's Sequences that were Amplified with SM1 and SM2 were composed of 197 bp and 203 bp respectively. The molecular marks for this resistance has been used in tobacco breeding.

5:10 PM MONDAY

45. IDENTIFICATION AND SSR MARKING OF RESISTANCE GENE TO TOBACCO CUCUMBER *MOSAIC VIRUS*. Jingyuan FAN, Yuanying Wang, Caihong Jiang, Wansheng Chen, Min Ren, and Haizhou Hu; Key Laboratory for Tobacco Quality Control, Minisrty of Agriculture, China, Tobacco Research Institute of China Academy of Agricutural Sciences, Qingdao, Shandong, China

The study was carried out using F_1, F_2, BC_1 populations from a cross between CMV resistant varieties tiebazi/taiyan-8 and CMV susceptible varieties NC82/zhongyan15. The disease resistance was evaluated by artificial inoculation on seedlings in greenhouse and the inheritance of resistance were analyzed . Results demonstrated that the resistant character

of both resistant parents is controlled by single recessive gene. DNAs of F_2 population were extracted to develop into a resistant pool and a sensitive pool based on the BSA (bulk segregant analysis). 135 of pairs SSR primers were used for molecular marking analysis. The results showed that the genetic distance between $SM1_{350}$ and resistant genes from "tiebazi" is 8.64 cM, and the genetic distance between $SM2_{270}$ and resistant genes from "taiyan-8" is 3.92 cM.

5:30 PM ADJOURN

TUESDAY MORNING, SEPTEMBER 29, 2009

SESSION A ***Session Chair: Ray Robertson***

8:30 AM TUESDAY

46. FORMATION OF RADICALS FROM CIGARETTES UNDER DIFFERENT SMOKING CONDITIONS. E.A. ROBINSON and A.J. Dyakonov; Lorillard Tobacco Company, Greensboro, NC

It is well known that changes in the smoking regimen used to generate cigarette smoke result in changes in analyte delivery which are sometimes quite large. However, the effect of these changes in smoking regimen on free radicals in the smoke is largely unknown. To study this effect, we used the spin-trap BMPO, 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline-N-oxide, which is selective for oxygen-centered free radicals, to trap vapor phase free radicals from cigarette smoke using a standard 35 ml puff and a series of different puff frequencies (1 s, 10 s, 60 s, and 120 s). We found that the relative amounts of two radical species trapped by BMPO and detected by electron paramagnetic resonance spectroscopy changed dramatically depending on the puff frequency used. We also examined the free radicals produced in individual puffs by tandem mass spectrometry of the BMPO-trapped species and found that there was a shift to lower molecular weight free radical species between the second and ninth puffs. These changes could result from the differences in combustion of the original tobacco within the first puffs and combustion of the tobacco with tar-deposited in later puffs.

8:50 AM TUESDAY

47. IDENTIFICATION OF GAS-PHASE FREE RADICALS BY TANDEM MASS SPECTROMETRY (MSMS). E.A. ROBINSON and J.D. Johnson; Lorillard Tobacco Company, Greensboro, NC

Free radicals are typically studied by electron paramagnetic resonance (EPR) spectroscopy, often using spin traps to stabilize the radicals long enough to allow their analysis. A serious limitation associated with the use of EPR to analyze complex mixtures, such as cigarette smoke, is its inability to provide detailed structural information outside the radical center. In order to address this limitation, we have completed a series of tandem mass spectrometry experiments in which gas-phase radicals generated in cigarettes smoked under intense and Cambridge Filter pad conditions were trapped and subsequently identified. Seven spin traps (3AP, BMPO, DMPO, DEPMPO, DIPPMPO, POBN, and PBN) were assessed for their ability to trap primary radicals in both polar and non-polar solvents as well as for their ability to undergo efficient volatilization and ionization for MS analysis. Rapid identification of adducted radicals was achieved through the use of less common precursor ion scans (MSMS). Basic knowledge of the likely product masses coupled with the tunable selectivity of various tandem mass spectrometric methods affords a simple and selective way to study gas phase radicals.

9:10 AM TUESDAY

48. DETECTION OF REACTIVE OXYGEN SPECIES BY DMPO SPIN-TRAPPING METHOD IN AQUEOUS EXTRACT OF CIGARETTE SMOKE AND COMPARISON WITH MODEL REACTION SYSTEMS. Yuichiro TAKANAMI; Japan Tobacco Inc., Yokohama, Kanagawa, Japan

It is well known that an aqueous extract of cigarette smoke generates reactive oxygen species. The methods for detection of these species, to study their generation, are critical. We have already reported a method for analysis of hydrogen peroxide at TSRC2008. This time we tried to detect superoxide anion radicals and hydroxyl radicals by electron spin resonance (ESR), using a DMPO spin-trapping technique. The particulate phase of cigarette mainstream smoke was extracted with phosphate buffer and the extract was analyzed with ESR. The model reaction system for generating superoxide anion radicals used in this study was a hypoxantine-xantine oxidase system and for hydroxyl radicals a hydrogen peroxide-ferrous ion system. We added several radical scavengers to the extracts and the model systems, and monitored the respective ESR signals. We detected DMPO-OH signals from the extract; however, the signals were eliminated by adding superoxide dismutase and not by adding catalase. The modification of the signals was similar to the model system for superoxide anion radicals and not for hydroxyl radicals. It is suggested that the signals of DMPO-OH are derived from degradation of DMPO-OOH, which is a reaction product of DMPO with superoxide anion radicals. This supports the idea that the extract of smoke generates superoxide anion radicals and not directly hydroxyl radicals.

9:30 AM TUESDAY

49. ANALYSIS OF HYDROGEN SULFIDE, CARBONYL SULFIDE, METHANETHIOL, CARBON DISULFIDE, METHYL THIOCYANATE AND METHYL DISULFIDE IN MAINSTREAM VAPOR PHASE CIGARETTE SMOKE. Ji-Zhou DONG and S. M. DeBusk; R. J. Reynolds Tobacco, Winston-Salem, NC

A method is described for the simultaneous analysis of hydrogen sulfide, carbonyl sulfide, methanethiol, carbon disulfide, methyl thiocyanate and methyl disulfide in mainstream vapor phase (MVP) cigarette smoke using a gas chromatography/mass spectrometry (GC/MS) technique. The fresh MVP smoke was collected in a gas bag, followed by injection of a 50 μL gas sample into the GC inlet via an automatic six-port valve. The separation was done on a CP-PoraPLOT Q column and the MS was operated in SIM mode. It was found that while carbonyl sulfide and carbon disulfide are very stable in the gas bag, hydrogen sulfide, methanethiol, methyl disulfide and methyl thiocyanate are extremely reactive and their levels increase or decrease dramatically with the storage time in the gas bag. These results suggest that there is an absolute need to analyze the smoke sample as quickly as possible. A precise time after the smoke collection is a key factor in order to obtain a reproducible result. In this study, all the samples are injected within 2 minutes after MVP smoke was collected in the bag. Under smoke conditions of 60 mL puff of 2 second duration for every 30 seconds, 12 brands of commercial cigarettes and Kentucky Reference 2R4F cigarette were analyzed. Average values of three replicates of the 2R4F cigarettes were 31.6 μg/cigt hydrogen sulfide, 40.7 μg/cigt carbonyl sulfide, 25.6 μg/cigt methanethiol, 2.2 μg/cigt carbon disulfide, 23.7

μg/cigt methyl thiocyanate and 17.6 μg/cigt methyl disulfide. All other types of analyzed cigarettes show a similar quantitative distribution for these analytes.

9:50 AM ***Break***

10:20 AM TUESDAY

50. A NEW HIGH PERFORMANCE LIQUID CHROMATOGRAPHY - FLUORESCENCE DETECTION METHOD FOR THE DETERMINATION OF PHENOLIC COMPOUNDS IN CIGARETTE SMOKE AND SMOKELESS TOBACCO PRODUCTS. Jingcun WU and Bill Rickert; Labstat International ULC, Kitchener, Ontario, Canada

A widely used method for the analysis of phenolic compounds in cigarette smoke is HPLC with fluorescence detection using acetonitrile as the mobile phase. The current global shortage of this compound prompted a search for a substitute to reduce cost and potentially improve the method. In this study, a simple, fast and sensitive HPLC method was developed using methanol instead of acetonitrile. A short (150mm, 3μm particle size) column with a pentafluorophenyl propyl (PFP) ligand in the stationary phase was chosen in order to increase separation efficiency and reduce solvent consumption. The limit of detection (LOD) and limit of quantification for the method are at the ng/mL level for most of the phenols with good linearity ($R^2 \geq 0.999$) and precision (RSD<10%). Mainstream yields of phenolics from 3R4F are comparable to those obtained by Health Canada method with the advantage that o, m, and p-cresols can be determined and reported separately. The results for o, m, and p-cresols under ISO conditions are 2.05 ± 0.28, 1.76 ± 0.21, and 4.23 ± 0.30 μg/cig respectively and 35.5 ± 4.72, 25.6 ± 0.87, and 51.6 ± 1.90 μg/cig in sidestream smoke. The results for phenol, o, m, and p-cresols in 2S3 smokeless reference product are 4.73 ± 0.22, 1.25 ± 0.08, 3.18 ± 0.10, and 1.68 ± 0.13 μg/g respectively.

10:40 AM TUESDAY

51. NEW METHODOLOGIES FOR QUALITATIVE AND SEMI-QUANTITATIVE DETERMINATION OF CARBON-CENTERED FREE RADICALS IN CIGARETTE SMOKE USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY AND GAS CHROMATOGRAPHY-MASS SELECTIVE DETECTION. Anthony GERARDI and William Coleman, III; R. J. Reynolds Tobacco, Winston-Salem, NC

Several approaches were explored to develop a high throughput procedure for relative determination of 14 different carbon-centered free radicals, both acyl and alkylaminocarbonyl type, in cigarette smoke. Two trapping procedures using cyanoproxyl radical (3-CNP) were designed for this study: a) trapping in solution and b) trapping on a solid support which was a Cambridge filter pad. Fresh whole smoke and gas phase smoke from mainstream cigarette smoke from Kentucky Reference Cigarettes 2R4F, as partitioned via an unadulterated Cambridge filter pad, were insulted into each trapping system in separate experiments. The 3-CNP coated Cambridge filter pad approach was shown to be superior to the impinger procedure as described in this study. Gas chromatography coupled with mass selective detection (GC-MS) was employed for the first time as an alternate means of detecting several relatively highly concentrated radical adducts. Liquid chromatography tandem mass spectrometry (LC-MS/MS) with either precursor ion monitoring (PIM),

selected reaction monitoring (SRM) or selected ion monitoring (SIM) was used for detecting the large array of radicals, including several not previously reported: formyl, crotonyl, acrolein, aminocarbonyl, and anilinocarbonyl radicals. Relative quantitation was achieved using external calibration standards of 4-[1-pyrrolindino]benzaldehyde and nicotine. It was determined that the yield of carbon-centered free radicals for reference cigarette 2R4F was approximately 265 nmoles/cigarette at 35 cc puff/60 sec interval/2 sec duration smoking conditions.

11:00 AM TUESDAY

52. QUALITATIVE AND RELATIVE QUANTITATIVE DETERMINATION OF CARBON-CENTERED FREE RADICALS IN WHOLE SMOKE FROM VARIOUS CIGARETTE TYPES. Anthony GERARDI and William Coleman, III; R. J. Reynolds Tobacco, Winston-Salem, NC

Free radicals in cigarette smoke may contribute to harm associated with cigarette smoking, more specifically, contribute to oxidative stress and subsequent biological activity. Recently, a high throughput procedure for relative determination of 14 different carbon-centered free radicals, both acyl and alkylaminocarbonyl type, was developed. This procedure used the radical scavenger 3-cyanoproxyl radical (3-CNP), which was diluted in acetone and spiked onto a 44 mm fiberglass Cambridge filter pad, which had the acetone subsequently evaporated. Fresh whole smoke from various cigarettes was insulted onto the 3-CNP coated Cambridge filter pads. Cigarettes evaluated afforded a representative range of standardized 'tar' yields (14, 10, and 5 mg/cigarette, respectively by the Cambridge filter method), and also included a carbon filtered prototype, as well as Kentucky reference cigarettes 2R4F, 3R4F, and 1R5F. Cigarettes were exposed to coated filter pads using 2 different smoking regimes: 35 cc puff/ 60 sec interval/ 2 sec puff duration / 0% vent block (35/60/2/0) and 55 cc puff/ 30 sec interval/ 2 sec puff duration / 100% vent block (55/30/2/100). Liquid chromatography tandem mass spectrometry (LC-MS/MS) with precursor ion monitoring was used for detecting the large array of radicals. High resolution mass spectrometry was used to confirm several previously proposed 3-CNP:radical (3-CNP-R) adducts, the formyl and ethyl radical adducts. The range of carbon-centered free radical concentration was related to 'tar' delivery and was found to be 41-348 nmoles/cigarette with the 35/60/2/0 regime, and 349-647 nmoles/cigarette with the 55/30/2/100 regime.

11:20 AM TUESDAY

53. DEVELOPMENT OF AN ANALYTICAL METHOD FOR THE SIMULTANEOUS DETERMINATION OF TRACE METALS AND MERCURY IN MAINSTREAM CIGARETTE SMOKE BY ICP-MS. Yutaka KUROKI, Shinya Yokoyama, Hisayuki Takahashi, and Masahiro Fujiwara; Japan Tobacco Inc., Yokohama, Kanagawa, Japan

Usually trace metals and mercury in mainstream smoke are analyzed quantitatively by applying AAS or ICP-AES after trapping of compounds from smoke in each smoking machine and after sample preparation using wet digestion (Health Canada Method). Because of this very time and cost consuming sample preparation, our study was focused on the development of an analytical method for the simultaneously determination of each compound with only less sample preparation. After trapping the trace metals from

mainstream smoke in an electrostatic precipitation (EP) tube using an electrostatic collector, the compounds were extracted with methanol. The methanolic extract was acidified with nitric acid and directly applied to ICP-MS, without further sample preparation. Mercury was trapped from smoke using an impinger containing an oxidizing agent (potassium permanganate) and addition of a reducing agent. Trapping of trace metals and mercury was carried out simultaneously. All compounds were determined using ICP-MS. Validation experiments (Linearity, spike recovery tests, reproducibility, etc.) showed that this method was appropriate for the determination of trace metals and mercury in mainstream cigarette smoke. Data obtained by analyzing 3R4F cigarettes are in good agreement with previous analyzed values.

11:40 AM TUESDAY

54. REDUCING THE NITROSAMINES LEVEL OF SMOKE BY ZEOLITES. Jian Hua ZHU, Ying Wang, Ling Gao, Yang Xu, and YI Cao; College of Chemistry and Chemical Engineering, Nanjing University, Nanjing, China

Zeolite is a group of porous aluminosilicates with regular nano-sized pores and it has the ability of selective adsorption. Recently, we found strong interactions between some zeolites and nitrosamines present in cigarette smoke. The objective of this study was to compare the efficiency of different zeolites in adsorbing nitrosamines under both laboratory and cigarette smoking conditions. For zeolites to be used in cigarette filters, instantaneous adsorption of volatile nitrosamines in gas stream was studied by varying temperature, contact time, pore structure, surface acidity-basicity of zeolite and the constitution of the adsorbates. NaY possessed the highest capacity among the commercial zeolites in laboratorial test, and it trapped 80% of nitrosamines in the mainstream smoke. Contrarily, zeolite NaA has a small capacity owing to its pore size of 0.4 nm but its adsorption strength was very high due to the plenty of cations that provide the strong electrostatic interaction for nitrosamines. We sprayed the zeolite onto a Chinese Virginia cut tobacco at 3 wt% and made filter-tipped cigarettes. The smoke results showed that TSNA in the sidestream and mainstream smoke were reduced by 25-32% and 35-60%, respectively. These reductions were achieved without significantly altering the tar and nicotine levels of the cigarettes. Besides, various zeolites with different pore structure and morphology were put into the filter trip to examine their performances in reducing the nitrosamines level of mainstream smoke, exploring the property-function relation. In conclusion, it is feasible to use zeolites in cigarettes to reduce the nitrosamine level of cigarette smoke.

12:00 PM LUNCH

TUESDAY MORNING, SEPTEMBER 29, 2009

SESSION B ***Session Chair: Brian Nordskog***

8:30 AM TUESDAY

55. COMPARISON OF THE MUTAGENICITY OF DIFFERENT SMOKE PREPARATIONS AND WHOLE SMOKE EXPOSURES IN THE AMES ASSAY. R. LEVERETTE; Lorillard Tobacco Co., Greensboro, NC

The Ames Assay is frequently used to determine the mutagenicity of cigarette smoke particulates. Assessing only the particulate fraction of smoke ignores any contribution of gas vapor phase (GVP) to the overall mutagenicity of whole smoke (WS). Collecting smoke fractions through the use of an impinger containing ice-cold phosphate buffered saline (PBS) allows the mutagenicity assessment of any captured hydrophilic compounds. The preferred method of exposure would be a direct exposure to whole cigarette smoke, without any intermediate smoke preparation that may alter the smoke's composition. Systems that allow this type of direct smoke exposure are available for the Ames and other in vitro cell-based assays. A comparison between exposures of PBS bubbled with whole smoke (WS-PBS) and gas vapor phase (GVP-PBS) with whole smoke exposures was conducted. Pre-incubation exposure conditions (S9+/S9-) for WS-PBS and GVP-PBS were optimized for plated cell density (5 X 10^{10} cells / mL), exposure time (120 minutes) and number of cigarettes smoked through a set volume of PBS (4 cigarettes through 20 mL). Whole smoke exposures were performed using the VITROCELL® VC10 smoking robot, dilution system and the Ames exposure chambers (35mm plates). Results show significant differences in response depending on smoke preparation and method of exposure. TA98 (S9+) WS-PBS gave a 2-fold higher response (18,700 revertants per cigarette) when compared to the measured whole smoke response (9,800 revertants per cigarette). This appears to be the result of a longer and more direct exposure of the WS-PBS versus the whole smoke. Correcting for the particulate deposition onto the Ames plate surface in the whole smoke exposure system results in a revertants per cigarette response comparable to those seen in the WS-PBS and GVP-PBS pre-incubation exposures.

8:50 AM TUESDAY

56. ***IN VITRO*** **EFFECTS OF MENTHOL ON CYTOCHROME P450 ENZYMES, CYTOTOXICITY AND GENOTOXICITY ENDPOINTS.** R. LEVERETTE; Lorillard Tobacco Co., Greensboro, NC

Menthol is used extensively in the tobacco, food, and drug industries. Menthol has been shown to be negative in a battery of genetic toxicology methods (Ames, SCE, micronucleus, Comet) and is generally regarded as safe (GRAS). However, menthol has been shown to have inhibitory effects on CYP450 enzymes important in the metabolism of cigarette smoke constituents. Nicotine is metabolized by CYP2A6, an enzyme that has been shown to be inhibited by menthol, but the effect menthol at levels received by a smoker remains unclear. *In vitro* enzyme inhibition assays using recombinant human CYP enzymes known to metabolize cigarette smoke constituents (2A6 & 2A13, nicotine; 2E1, nitrosamines; 1A2, arylamines) demonstrated enzyme inhibition (IC50) at menthol levels greater than what

would be expected physiologically in a smoker. IC50 (μM) values for 2A6 were 63.81 ± 1.03 and 153.5 ± 1.03 for (+) and (-) menthol isomers, respectively. These experimental IC50 values appear to be in excess of an estimated level (7 – 15μM) of menthol a smoker could receive upon smoking one typical mentholated cigarette, suggesting minor effects, if any, on the metabolism of nicotine *in vivo*. In addition to enzyme inhibition studies, the effects menthol may have in a series of cytotoxicity (membrane permeability, mitochondrial trans-membrane potential, and nuclear size) and genotoxicity (phospho-H2AX) endpoints were also examined *in vitro*. A549 (human lung carcinoma) cells were exposed with smoke preparations spiked with increasing levels of menthol (0 - 1000μM). Endpoints were measured using a Cellomics ArrayScan High Content Imaging system (Thermo Scientific). Results indicate menthol had no apparent effect on the cytotoxicity endpoints and there appeared to be a slight protective effect in the phospho-H2AX genotoxicity endpoint.

9:10 AM TUESDAY

57. PROTEOMIC ANALYSIS OF CIGARETTE SMOKE-EXPOSED RAT LUNG TISSUES IN A SHORT-TERM STUDY. C.A. CARTER and M. Misra; Lorillard Tobacco Company, Greensboro, NC

A short-term 5 day nose-only smoke exposure study was conducted in Fischer 344 rats to identify smoke-induced lung protein changes. Groups of 10 male and female 5 wk old rats were assigned to one of four exposure groups. Animals received filtered air (Air Control) or 75, 200 or 400 total particulate matter (TPM) mg/m^3 of diluted 2R4F cigarette smoke. Exposures were conducted for 3 hrs/day, for 5 consecutive days. Half of the harvested lung tissue was processed for pathology and half for proteomics. Mean body weights of male rats exposed to 400 mg/m^3 TPM cigarette smoke were significantly decreased compared to controls. Body weight gains were significantly reduced in males at the 200 and 400 TPM mg/m^3 doses compared to controls, while body weight gains were significantly reduced in females at all smoke concentrations. Pathology showed that inflammation was minimal and did not vary significantly between groups. Lung tissue lysates from control vs. treated animals were screened for 650 proteins using antibody-based microarray technology. Subsequently, the 18 top proteins were evaluated by Western blot in lung lysates. Altered proteins play various critical physiological roles in cell function. Major changes depended on dose and gender and included the following proteins: Jun N-terminus protein-serine kinase (stress-activated protein kinase) (JNK), heat shock protein 70, glycogen synthase-serine kinase 3, focal adhesion proteins Rac 1 and vinculin, TGF-beta-activated protein-serine kinase 1, protein-serine phosphatases, protein kinase C isoforms α, δ, ζ, and proteins related to apoptosis and cell cycle. Protein arrays are powerful in elucidating pathways involved in short-term smoke exposures. Changes in identified proteins may induce lung functional changes and serve as an early indicator of lung damage.

9:30 AM TUESDAY

58. HIGH CONTENT SCREENING ANALYSIS OF SMOKE TOXICITY IN HUMAN LUNG A 549 CELLS REVEALS BIOMARKERS OF OXIDATIVE STRESS AND DAMAGE. M. MISRA, C.A. Carter, and R.D. Leverette; Lorillard Tobacco Company, Greensboro, NC

Tobacco smoke-induced oxidative stress is associated with atherosclerosis, COPD, inflammation, and cancer. We hypothesized that smoke exposure would induce oxidative

stress and damage in various cellular compartments that can be visualized and quantitated by High Content Screening for use in *in vitro* toxicology assays. The dose-dependent effect of wet total particulate matter (WTPM) and gas-vapor phase (GVP) smoke fractions of 2R4F cigarettes on membrane permeability, 8-iso-prostoglandin F2α (8-iso-PGF2α), mitochondrial membrane potential (ΔΨm), caspase-3, nuclear size, cell number, H2AX-phosphorylation, interleukin-8 (IL-8), and heme oxygenase-1 (HO-1) were evaluated in human lung A549 cells. WTPM treatment, but not GVP, induced a dose-dependent release of 8-iso-PGF2α into cellular medium indicating lipid peroxidation and damage that paralleled an increase in cell permeability. WTPM and GVP exposures yielded a dose-dependent increase in H2AX-phosphorylation, a DNA double-strand break marker, which correlated with cell death as indicated by the lower cell count. Oxidative stress is an inducer of apoptosis that involves changes in mitochondrial mass and ΔΨm. WTPM and GVP induced apoptosis evidenced by a dose-dependent decrease in ΔΨm and nuclear size with concomitant induction in caspase-3 and reduction in cell number. IL-8, an inflammatory cytokine and mediator, is responsive to oxidative. Both WTPM and GVP fractions increased IL-8 release into cellular media. HO-1 exerts a cytoprotective defense mechanism against oxidative insults. Both WTPM and GVP fractions induced dose-dependent increases in HO-1 expression. The use of multiparameter High Content Screening technology proved to be a useful tool in evaluation and development of smoke-induced oxidative stress related biomarkers for *in vitro* assays.

9:50 AM ***Break***

10:20 AM **TUESDAY**

59. CIGARETTE SMOKE EXPOSURE RESULTS IN HYPERTENSION, LEUKOCYTE-SPECIFIC REACTIVE OXYGEN SPECIES (ROS) GENERATION, ENDOTHELIAL DYSFUNCTION, AND CARDIAC HYPERTROPHY IN MICE. J.L. ZWEIER, M.A. Hassan Talukder, W. Johnson, S. Varadharaj, J. Lian, P. Kearns, L. Druhan, X. Liu, and M.A. El-Mahdy; Center for Environmental and Smoking Induced Diseases, Davis Heart and Lung Research Institute, The Ohio State University, Columbus, OH

Background: Cigarette smoking is a major risk factor for atherosclerotic cardiovascular diseases. While the association between smoking and atherosclerosis is well-established, the underlying mechanisms are incompletely understood. We report an in vivo mouse model of smoking-induced cardiovascular pathology. Methods: Male C57BL/6 mice (8-weeks) were exposed to whole body mainstream smoke (MS) by SCIREQ "InExpose" smoking system (60 min/day, 5 days/week) for 8, 16, or 32 weeks. Mice were sacrificed 24-hour after the last MS exposure. Age-matched, air exposed mice served as controls. Blood pressure was measured and cardiac MRI was performed. In vitro vascular ring experiments were performed for vascular reactivity. Blood from control and smoke exposed mice were investigated for nitric oxide (NO) decay rate and ROS generation. Results: Smoke exposed mice had significant weight loss and developed hypertension after 16-week MS exposure. At 32-week of MS exposure (n = 4-8): acetylcholine-induced vasorelaxation was impaired; left ventricular (LV) mass was significantly larger ($P<0.01$ vs. control mice) with increased heart to body-weight ratio while LV end diastolic volume was decreased. NO decay rate in the whole blood was increased; and white blood cells demonstrated higher ROS generation. Conclusions: Thus, MS exposure induced leukocyte activation, endothelial dysfunction,

hypertension, and mild cardiac hypertrophy. This MS exposure mouse model should enable further determination of molecular and cellular mechanisms of smoking-induced cardiovascular diseases.

10:40 AM TUESDAY

60. CIGARETTE SMOKE EXPOSURE DOSE-DEPENDENTLY ALTERS THE ACTIVITY, COUPLING, PHOSPHORYLATION, AND EXPRESSION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE IN ENDOTHELIAL CELLS. J.L. ZWEIER, Tse-Yao Wang, Lawrence J. Druhan, and Chun-An Chen; Center for Environmental and Smoking Induced Diseases, Davis Heart and Lung Research Institute, The Ohio State University, Columbus, OH

Inhibition of endothelial function is a critical mechanism of cigarette smoke (CS) induced cardiovascular disease. We observe that CS extract (CSE) alters endothelial function by an endothelial nitric oxide synthase (eNOS) dependent mechanism. We show that NO generation from endothelial cells, measured by electron paramagnetic resonance spin-trapping, is decreased by 44%, 58%, or 85% with 10%, 20%, or 50% CSE treatment, respectively. Addition of the eNOS cofactor tetrahydrobiopterin, BH4, (100 uM), which is required for eNOS coupling and NO generation, to low level (< 20%) CSE treated cells largely restored eNOS activity and there was no change in eNOS protein levels. However, with CSE concentrations ≥50% there was a large (>75%) decrease in eNOS protein following treatment. Additionally, threonine 495, phosphorylation which negatively regulates eNOS, was time dependently dephosphorylated by treatment with CSE. Thus, low level CSE treatment leads to reversible inhibition of eNOS via oxidation of BH4, and exacerbates the potential eNOS-dependent oxidative stress via dephosphorylation of threonine 495. Conversely, at high CSE concentrations eNOS is irreversibly inhibited via a reduction in total eNOS protein. Thus, CSE exposure results in dose-dependent alterations in eNOS activity, coupling, phosphorylation and expression with progression from reversible to irreversible loss of endothelial function. These results suggest that treatment with BH_4 or other antioxidants may be able to reverse smoking induced endothelial dysfunction.

11:00 AM TUESDAY

61. PROTEOMIC ANALYSIS OF BIOMARKERS FOR SMOKING INDUCED DISEASES IN A MOUSE MODEL. Arun K. TEWARI, Mohamed A. El-Mahdy, and Jay L. Zweier; Center for Environmental and Smoking Induced Diseases, Davis Heart and Lung Research Institute, The Ohio State University, Columbus, OH

Cigarette smoke (CS) exposure is considered a major risk factor for cardiovascular and pulmonary diseases. CS exposure leads to an increase in oxidative stress and inflammatory injury. In the current study, we have performed measurements and proteomic analysis of 4-hydroxy-2-nonenal (HNE) modified plasma proteins in CS exposed mice. Male C57BL/6 mice were exposed to whole body mainstream and side stream CS generated from 3R4F reference research cigarettes that deliver 9.4mg tar & 0.726mg nicotine per cigarette under the standard Cambridge filter smoking condition. Mice were exposed to CS for 8, 16 and 32 weeks and plasma samples were collected for analysis. Gel electrophoresis, western blotting and mass spectrometry were used as proteomic tools to analyze the plasma samples. Our data indicate that there is a marked increase in HNE modified proteins in CS exposed mice

compared to non-exposed (control) mice that progresses with exposure paralleling the incidence and progression of cardiovascular disease. Ceruloplasmin and inter-alpha-trypsin were identified as HNE modified proteins. Enhanced accumulation of HNE modified proteins with CS exposure serves as a marker of oxidative stress and related cardiovascular injury. These oxidative protein modifications appear to be promising biomarkers predictive of cardiovascular disease.

11:20 AM TUESDAY

62. EFFECT OF TEMPERATURE AND ATMOSPHERE ON THE MUTAGENICITY AND CYTOTOXICITY OF SMOKE CONDENSATE IN TOBACCO PYROLYSIS. Yasunari OTSU, Yoshimi Nishio, and Shinya Yoshida; Japan Tobacco Inc., Research Center, Yokohama, Kanagawa, Japan

Total particulate matter (TPM) generated by the combustion of tobacco leaves shows *in vitro* biological activities. Tobacco leaves are subjected to a wide range of temperatures and oxygen concentrations during cigarette smoking. It is known that the mutagenicity of the TPM varies according to the pyrolysis temperature, but the influence of the pyrolysis condition on the cytotoxicity is less known. To develop an understanding of the mutagenicity and cytotoxicity of the TPM, we investigated the relationship between the activities and the pyrolysis conditions.

Two types of tobacco leaf (Burley and Flue-cured) were individually pyrolyzed using an infrared image furnace under the following conditions: pyrolysis temperatures (350-800°C) in 100% N_2 and pyrolysis atmospheres (0-20% of O_2 in N_2) at 800°C. The pyrolyzed products were assayed by *Salmonella* mutagenicity testing using TA98 with metabolic activation and neutral red uptake cytotoxicity testing.

The mutagenicity and the cytotoxicity which were calculated on a TPM basis increased with increasing temperature and reached a plateau around 500°C. The variability of the mutagenicity is more extreme than that of the cytotoxicity. When calculated on a nicotine-free dry particulate matter (NFDPM) basis, the variability of the mutagenicity shown on a TPM basis was still observed. In contrast, the cytotoxicity did not have much variability. Moreover, the pyrolyzed products showed decreases in both activities on a TPM and NFDPM basis with increasing oxygen concentration in the atmosphere.

The results revealed that not only the mutagenicity but also the cytotoxicity were affected by the temperature and atmosphere in tobacco pyrolysis. Further investigation including chemical analysis may provide the information to understand the relationship between the biological activity and the constituents in the TPM.

11:40 AM LUNCH

TUESDAY AFTERNOON, SEPTEMBER 29, 2009

SESSION A ***Session Chair: Ed Robinson***

1:30 PM TUESDAY

63. AN IMPROVED METHOD FOR VAPOR PHASE ANALYSIS USING ATD GC/MS. Jeremy K. STEACH; Eastman Chemical Co., Kingsport, TN

The determination of vapor phase components in mainstream cigarette smoke has been analyzed by several analytical methods. These methods have utilized impingers, direct sampling, gas bags, and adsorbent traps to capture vapor phase components prior to analysis with various chromatographic instruments. The current method uses carbon-packed adsorbent traps for collection of the vapor phase components directly from the mainstream cigarette smoke. These traps are analyzed by using automated thermal desorption (ATD) coupled to a GC/MS.

To improve the current ATD GC/MS method, all puffs were collected onto the adsorbent traps for analysis, while the previous method only collected puffs 3-8. The breakthrough and carryover of the adsorbent traps and cold trap were used to determine the amount of vapor phase that could be collected. The improved method was validated using 2R4F Kentucky reference cigarettes and cellulose acetate filters containing activated carbon. The carbon filter rods were made with a constant level of carbon and with both dalmatian and cavity constructions. The removal of several vapor phase components was monitored over a 24 week period. Results for the improved ATD GC/MS method and the effect of carbon filter aging will be discussed.

1:50 PM TUESDAY

64. ACCURACY OF TAR YIELD DETERMINATION AND INTENSE SMOKING REGIMES. France COTE and Jules Verreault; Imperial Tobacco Canada Limited, Montreal, Canada and F. Kelley St. Charles, St. Charles Consultancy, Winston-Salem, NC

Total Particulate Matter (TPM) is determined by weight difference of the Cambridge Filter Pad (CFP) holder before and after smoking. The holder is opened, wiped and the CFP transferred into a flask for extraction. We have suspected that water could be lost between the after smoking weighing and CFP extraction. This could result in an overestimation of tar yields, especially at the more intense smoking regimes which generate higher water yields. A modification to the weighing step has been performed to reduce the delay between weighing and extraction. CFP were weighed directly in the extraction flask. The effect of the modified weighing step on smoke yield determination was established for ISO, Canadian intense and Massachusetts smoking regimes. Yields of TPM, tar, nicotine, CO and water were determined for three Canadian cigarettes (ISO tar: 4 to 14 mg; tip ventilation: 10 to 61%; blend nicotine: 2.2 to 2.5%) using the new and standard weighing steps. At intense smoking regimes, tar yields obtained using the standard weighing step were significantly higher by 14 to 26% when compared to the modified method. For the ISO regime, a less significant effect was observed. Nicotine yields were not affected. The results suggest that the standard method does not take into account the potential water loss between the pad

weighing and its extraction. Modification of the weighing step can significantly improve the accuracy of tar yield determination at high smoking regimes.

2:10 PM TUESDAY

65. THE PERFORMANCE OF SUPERSLIMS CARBON FILTERS AT DIFFERENT SMOKING REGIMES. Tony MCCORMACK and Mike Taylor; Filtrona Technology Centre, Jarrow, Tyne & Wear UK

A series of papers have been presented at previous conferences examining the characteristics of filter cigarettes containing activated carbons. All these studies related to standard circumference cigarettes, but comparatively little is known about the performance of superslim carbon filters. Superslim products are one of the most rapidly growing segments of the cigarette market and so the purpose of this work was to explore the performance of carbon filters when used in superslim products. Their lower circumference means that the smoke velocities passing through the filter are approximately double those of standard products, resulting in shorter contact times. Smoke velocity is an important parameter governing the performance of filters in terms of both particulate retention and vapour adsorption. This paper examines the extent to which the efficiency of carbon is affected when used in superslim filters.

Experimental findings are presented of the relative retentions by non-ventilated filters containing coconut-based carbons of twelve vapour phase compounds – notably carbonyls and hydrocarbons – under both ISO and Canadian Intense smoking regimes. Filters containing carbons of two different activities were tested over a range of carbon weights using a methodology similar to that described at previous TSRC conferences. Conclusions will be drawn concerning the interaction between these various factors and comparisons will also be made with the results from standard circumference products.

2:30 PM TUESDAY

66. SIMULTANEOUS ANALYSIS OF TWENTY UNDERIVATIZED FREE AMINO ACIDS IN TOBACCO BY LIQUID CHROMATOGRAPHY / ELECTROSPRAY IONIZATION ION TRAP TANDEM MASS SPECTROMETRY. Yi-Fei HUANG, Feng Li, and Jing Hu; China Tobacco Guangdong Industrial Co. Ltd., Guangzhou, China

Rapid and accurate analysis of amino acids is of great significance to the blend formulation, processing and quality control of tobacco products. 20 underivatized free amino acids in tobacco are simultaneously analyzed by liquid chromatography-electrospray ionization ion trap tandem mass spectrometry at cation mode, using a reversed-phase HyPURITY C_{18} column (200 mm × 2.1 mm, 5 μm) , and 99% H_2O-1% acetonitrile-0.1% nonafluoropentanoic acid (NFPA) and 10% H2O-90% acetonitrile-0.1% NFPA for gradient elution. Tobacco samples are injected directly after extracted with 0.03 M HCl and filtered. No other pretreatments are needed. The limits of detection of 20 amino acids are 0.01 μM ~ 0.05 μM (S/N=3:1), the correlation coefficients are above 0.9977 in all cases, the relative standard deviations of extracted ion chromatographic areas are 0.78% ~ 4.93%. The developed method is efficient, sensitive and selective and has been successfully used in determining amino acids in various tobacco samples. Pipecolic acid was analyzed for the first

time along with other common amino acids in tobacco. The quantitative results of amino acids were applied in the evaluation of quality consistence of the cigarettes manufactured by different branch factories. Moreover, it is found that the relation between the contents of asparagine and proline can be used to estimate whether the tobacco analyzed is burley.

2:50 PM ***Break***

3:20 PM **TUESDAY**

67. A NOVEL METHOD FOR ANALYZING SOLANESYL ESTERS IN TOBACCO LEAVES USING ATMOSPHERIC PRESSURE CHEMICAL IONIZATION / MASS SPECTROMETRY DETECTOR (APC/MSD). Naoyuki ISHIDA and Michinori Yokoi; Japan Tobacco, Inc., Yokohama, Kanagawa, Japan

Solanesol found in tobacco leaves is one of the trisesquiterpenols that includes a long terpene skeleton and one hydroxyl group, which is likely to be modified chemically. Accordingly, the hydroxyl group gives numerous derivatives such as solanesyl esters to the solanesol. However, the detailed composition of the solanesyl esters has not yet been clarified, owing to the lack of a feasible method for the analysis. In order to resolve this problem, non-aqueous reversed phase chromatography (NARP) and atmospheric pressure chemical ionization / mass spectrometry (APCI/MS) were applied as the analytical method. NARP is known as a technique for separating non-volatile compounds with low polarity, and APCI/MS enables low polarity compounds to be ionized forcibly and induced into a mass analyzer. Consequently, several kinds of solanesyl esters, which include higher fatty acids, were identified. Quantification was performed using acetone and acetonitrile as the mobile phase with a linear gradient and applying the following conditions to the MS: capillary volatage 4000 V, corona current 10 uA, drying gas flow 5 ml/min, fragmentor voltage 200 V, nebulizer pressure 60 psi, and vaporizer temperature 500°C. Additionally, mass spectrometry data was acquired by selected ion monitoring at 613.5 m/z to represent the specific m/z found in solanesyl esters. Calibration was carried out in the range of 0.1-15 ug/ml with regression coefficients of over 0.995 on almost all solanesyl esters. The result of the analysis showed the different amounts and compositions of solanesyl esters among tobacco leaves.

3:40 PM **TUESDAY**

68. Estimation of Gas-Particle Partitioning of Menthol in Mainstream Cigarette Smoke Under Several Different Smoking Conditions. John LAUTERBACH; Lauterbach & Associates, LLC, Macon, GA

Both gas-particle partitioning of semivolatile compounds in mainstream cigarette smoke (MSS) and the common semivolatile cigarette flavor, menthol, continue to be topics in the current literature. Those who have done detailed analyses on the MSS gas-vapor phase (GVP) know that menthol will go through the Cambridge filter pad (CFP). However, current methods for smoke menthol continue to assume that menthol loss through the CFP is negligible. Moreover, Kalaitzoglou and Samara [Beitr. Tabakforsch. Int. 21 (2005) 331–344] found that 10% to 90+% of MSS naphthalene is in the GVP depending on filter ventilation levels under ISO smoking. Since the reported vapor pressure of menthol is about

ten times that of naphthalene at ambient temperature, one would expect a similar situation for menthol. However, we know that does not occur and that percent of menthol transferred into MSS as measured by amount trapped on the CFP depends is inversely proportional to both the concentration of menthol on the tobacco and the concentration of glycerol on the tobacco (Cook et al., 53 TSRC #90). Consequently, estimates of gas-particle partitioning of menthol were made based on recent studies on the gas-particle partitioning of nicotine using Health Canada Intensive, Massachusetts DPH, and ISO smoking conditions. These estimates show that the amount of menthol in the MSS GVP under intensive smoking conditions should be very low.

4:00 PM TUESDAY

69. STUDY ON CHARACTERIZATION OF CUT TOBACCO PARTICLE SIZE DISTRIBUTION. SHEN Xiao-Feng[1,2], Du Jin-Song[2], Li Yue-Feng[3], Li Hua-Jie[3], Li Shan-Lian[2], and Luo Deng-Shan[2]; [1]Hongyunhonghe Tobacco Group Co. Ltd. Kunming, China, [2]Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou, China, [3]Technology Center of China Tobacco Fujian Industrial Corporation, Fujian, China

Cut tobacco particle size distribution (PSD) is a key factor influencing cigarette quality. In a sieve analysis, PSD is represented by the ratios of the cumulative mass above each or a definite screen mesh to the total mass. PSD is indicated in a discrete way rather than a distributive profile. Moreover, the method fails to describe the uniformity of the PSD, which is also an important factor influencing cigarette quality. In this work, six cut tobacco samples (cut tobacco after drying, cut stem, DIET, and cut tobacco after mixing, after air-conveying, after cigarette making) were sieved by a Retsch AS400 shaker. The characteristic equation of cut tobacco PSD was set up via statistic method and the eigen values were acquired. Furthermore, the values obtained by the sieve test and actual size measurements were compared. Finally the characterization of the PSD was set up. The results showed that: 1) the PSD of cut tobacco could be expressed by the characteristic equation $F = \exp(-Bx^c)$ 2) The $x_{0.50}$ value was the characteristic size; a lager $x_{0.50}$ and C value implied a longer average size and a narrower range of the size distribution, thus a better uniformity.

4:20 PM TUESDAY

70. CHARACTERIZATION OF A NOVEL NICOTINE-DEGRADING *BACTERIUM RHODOCOCCUS SP. Y22* AND ITS METABOLIC PATHWAY. DUAN Yanqing[1], Zeng Xiaoying[1], Zhe Wei[1], Gong Xiaowei[2], Yang Jinkui[2], and Li Qinghua[1]; [1]Technology Center, Hongyun Honghe Tobacco Co., LTD., Kunming, China, [2]Laboratory for Conservation and Utilization of Bio-Resources, Yunnan University, China

A novel nicotine-degrading bacterium, strain Y22, was isolated from tobacco plantation soil and identified as *Rhodococcus* sp. based on 16S rDNA sequence and on morphological and biochemical features. The isolate could utilize nicotine as sole source of carbon, nitrogen and energy, and produce blue pigment during nicotine degradation. The optimal culture condition of strain *Rhodococcus* sp. Y22 for nicotine degradation is 28°C, pH 4-7 and 1.5 g/L nicotine concentration. The resting cells induced by nicotine showed higher activity for decomposing of pure nicotine than that without induced by nicotine, and characterizations of their nicotine degradation were different, so their nicotine catabolic pathways may be

different. Furthermore, it could degrade the nicotine completely in tobacco leaves after treated for 9 h, which indicates the isolate might apply to tobacco industry for decrease nicotine in tobacco.

4:40 PM ADJOURN

TUESDAY AFTERNOON, SEPTEMBER 29, 2009

SESSION B Session Chair: Buddy Brown

1:30 PM TUESDAY

71. QUANTIFICATION OF NITROGEN CONTENT OF TOBACCO BY COMBUSTION (DUMAS) AS AN IMPROVEMENT OVER THE DIGESTION (KJELDAHL) METHODOLOGY. N.J. GALE; Group R&D, British American Tobacco, Southampton, UK

The concentrations of proteins and other nitrogen containing compounds in tobacco are of particular interest as these compounds can lead to the formation of toxicants such as hydrogen cyanide and nitrous oxides in mainstream cigarette smoke. Traditional analytical techniques for the quantification of total nitrogen and of protein nitrogen employ a Kjeldahl sample digestion process and subsequent Continuous Flow Analysis (CFA). The digestion process is time-consuming and can yield variable results, particularly for the low levels of total nitrogen and protein nitrogen normally found in tobacco.

The Dumas method involves complete oxidative combustion of samples at 850°C, catalytic reduction of the nitrogen oxides and subsequent detection of the total nitrogen thus formed by a pre-calibrated thermal conductivity cell. A Nitrogen/Protein Determinator (LECO Instruments UK Ltd) was used to automate this process, reducing the time taken for the single analysis of a weighed sample to just three minutes. It was found that the method also lends itself to the determination of total nitrogen in liquids.

The total nitrogen content of tobacco from Ky2R4F Reference Cigarettes measured using the automated Dumas method was found to be approximately 0.5% higher than that obtained using Kjeldahl/CFA. This is probably due to two main factors. First, unlike Kjeldahl digestion, the Dumas result includes nitrogen bound in nitrates, and second there is an intrinsic possibility of incomplete digestion of the tobacco using the Kjeldahl/CFA method. The automated Dumas technique achieved superior analytical precision with a coefficient of variation of 0.5% compared to 8% by Kjeldahl.

1:50 PM TUESDAY

72. ENANTIOMER COMPOSITION OF NORNICOTINE DETERMINED BY NICOTINE DEMETHYLATION. Bin CAI, F. Fannin, A. Jack and L.P. Bush; Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY

Nornicotine, one of four main alkaloids in tobacco, is the major precursor of N'-nitrosonornicotine (NNN). Nornicotine is present in both *R*- and *S*- enantiomer in tobacco and each forms the corresponding NNN enantiomer. *CYP82E4 (E4)* and *CYP82E5 (E5)* are the two main genes responsible for nicotine demethylation and nornicotine formation in *N. tabacum*. Their roles in the enantiomer formation of nornicotine are not known. There are several literature sources that report inconsistent results of the relative amounts of *R*- and *S*- nornicotine enantiomers present in tobacco. The objective of this study was to investigate the nornicotine composition changes associated with nicotine demethylation. In conventionally derived tobacco plants that had less the 3% demethylation, the *R/S* ratio of nornicotine was usually greater than one. The *R/S* ratio decreased as demethylation

increased. This difference in the *R/S* ratio was expressed in lamina, midrib, stem and roots. RNAi nicotine demethylation silenced plants had less demethylation than conventionally derived low converter plants, however the *R/S* nornicotine ratio decreased. RNAi silenced converter plants had a greater amount of R-nicotine than the converter control but a lesser amount of *R*-nornicotine than converter control plant. *E4* is the major gene for demethylation with *E5* having a lesser impact. Our results infer that either *E4* and/or *E5* activity for enantiomer selectivity is altered by RNAi silencing or there are related genes of the *CYP82E* family which may be responsible for *R*-nornicotine formation.

2:10 PM TUESDAY

73. THE EFFECT OF A GREEN BURLEY GENOTYPE ON TSNA ACCUMULATION. Anne JACK[1], Ramsey Lewis[2], Carol Wilkinson[3], Richard Hensley[4], Xiaolong Li[1], Neil Fannin[1] and Lowell Bush[1]; [1]University of Kentucky, Lexington, KY, [2]North Carolina State University, Raleigh, NC, [3]Virgina Polytechnic Institute, Blackstone, VA, [4]University of Tennessee, Greeneville, TN

Green tobacco types, such as flue-cured and Maryland, have lower TSNAs than burley. This could be because of the lower nicotine conversion in the green types, the lower nitrogen rates used on green tobaccos, or factors inherent in the burley genotype. The objective of this study was to test TSNA accumulation and suitability for burley usage of green and burley types under burley and flue-cured nitrogen rates. The study was grown and air-cured at four sites, in a split plot design with nitrogen as main plots and varieties as subplots. Nitrogen rates were those recommended at each site; 220-250 lbs N/acre for burley and 60-72 lbs N/acre for flue-cured. Varieties included a burley (TN 90LC), a burley high converter check (TN 90H), and three green types: a flue-cured (K 326), a green x yellow F_1 (TN 90 x MD 609) and a green burley segregant (GB3) which had a burley phenotype, except for color. Within a given nitrogen rate, GB3 did not have significantly lower TSNAs than TN 90LC. However, with a more realistic comparison, TN 90LC at the high nitrogen rate vs. GB3 at the low rate, NNN and total TSNAs were lower for GB3. Total alkaloids, reducing sugars, nitrate and nitrite were all similar, but total nitrogen was lower for GB3. Yield was similar, but grade index was much lower for GB3 at all sites. However, GB3 was a very dark green plant, and cured green. There could be a place for a green burley with a lower nitrogen requirement and a potential for reduced TSNAs, but a suitable variety would have to be developed.

2:30 PM TUESDAY

74. AFLP ANALYSIS OF GENETIC DIVERSITY OF TOBACCO GERMPLASMS. DU Chuanyin, Han Zhizhong, Yang Xuliang, Wang Xigong, Zhou Jian; Shandong Weifang Tobacco Corporation, Weifang Shandong, China; Tian Jichun, State Key Laboratory of Crop Biology, Shan Dong Agricultural University, Tai'an Shandong, China; Liu Hongxiang, Qingzhou Tobacco Research Institute of China National Tobacco Corporation, Qingdao, Shandong, China

In order to probe the phylogenetic relationship of different tobacco types, the genetic diversity of 90 cultivars included in 7 tobacco types were analyzed with AFLP fluorescence technique. The results were as the follows: the genetic diversity index of the wild tobacco

was the highest while the cigar tobacco type the lowest in the 7 types. The genetic diversity index among different types was 0.0801 while the genetic diversity index within each type was 0.1233.The genetic differentiation coefficient (Gst) was 0.3921, the value of gene flow (Nm) was 0.7753. Cluster analysis based on the genetic identity by unweighted pair-group method using arithmetic average (UPGMA) clearly separated the 7 tobacco types into three groups, namely the flue-cured tobacco, the sun/air-cured tobacco and the oriental tobacco were assembled into one group, the burley tobacco and the cigar tobacco into one group, the wild as well as the rustica tobacco fell into an unattached group. The genetic diversity level and the genetic base of the 90 tobacco cultivars were relatively low.

2:50 PM ***Break***

3:20 PM TUESDAY

75. SEQUENCE CHARACTERIZATION AND BASIC EXPRESSION ANALYSIS OF GRAS GENE FAMILY IN TOBACCO USING NTLS GENE. Shuaishuai TAI, Yuhe Sun, Guanshan Liu, Weifeng Wang, and Daping Gong; Key Laboratory for Tobacco Quality Control Ministry of Agriculture, China, Tobacco Research Institute, Chinese Academy of Agricultural Sciences, Qingdao, Shandong, China

Members of the GRAS gene family encode transcriptional regulators that have diverse functions in plant growth and development such as gibberellin signal transduction, root radial patterning, axillary meristem formation, phytochrome A signal transduction, gametogenesis and plant response to biotic and abiotic stress. In the current investigation, we isolated Nicotiana tabacum Lateral Suppressor (NtLS) gene which is responsible for development of lateral shoot in tobacco. A full-length cDNA of NtLS gene was cloned by performing Rapid Amplification of cDNA Ends (RACE) PCR. Bioinformatic analysis identified 21 putative full-length genes of GRAS gene family. The expression pattern of the NtLS gene and the other GRAS genes was studied by qRT-PCR and RT-PCR, respectively. Here, we provide a fairly complete overview of this gene family, describing the gene structure, gene expression, protein motif organization, and phylogenetic analysis. Phylogenetic analysis divides the GRAS gene family into eight subfamilies, which have distinct conserved domains and functions.

3:40 PM TUESDAY

76. CLUSTER ANALYSIS OF FLUE-CURED TOBACCO LEAVES FROM DIFFERENT PRODUCTION REGIONS ACCORDING TO THE CHEMICAL COMPONENTS CORRELATING WITH AROMA TYPES. CHANG Aixia[1], Zhang Jianping[2], Du Yongmei[1], Wang Shusheng[1], Jia Xinghua[1], Fu Qiujuan[1], Zhang Jun[2], Liu Hongxian[1]; [1]Tobacco Research Institute of CAAS, Qingdao, China, [2]Shanghai Tobacco Corporation, Shanghai, China

To ensure the optimal use of tobacco leaves derived from, different production regions, several chemical components including metal ions, organic acids, polyphenols and neutral aroma constituents of Flue-cured tobacco leaves grown abroad and 15 provinces of China were analyzed according to aroma types and by simple correlation. The results showed that there were 22 kinds of chemical indices such as total alkaloid, total chlorine, the ratio of reducing sugar to nicotine, the ratio of total nitrogen to nicotine, lactic acid, oxalic acid,

methyl palmitate, methyl oleate and linoleate, geranyl acetone, megastigmatrienone-1, megastigmatrienone-2, megastigmatrienone-4 were highly significant or significant. Cluster analysis of the 22 chemical indices and tobacco leaves of different origin showed that when T=12.4, tobacco leaves from 18 regions were divided into two groups. One group representing leaves from abroad and Shanxi Ankang of China and the second group representing leaves from domestic regions, except Ankang. When T=5.6, tobacco leaves from 18 regions were divided into seven groups: Group 1, Zimbabwe and Canada; group 2, Brazil and Shanxi Ankang; group 3, Fujian, Hubei Enshi, Chongqing Qianjiang and Jiangxi Gannan; group 4, Liaoning, Sichuan and Yunnan; group 5, Guangxi , Hunan and Guizhou; group 6, Henan and Shandong; group 7, Heilongjiang Mudanjiang and Jilin.

4:00 PM TUESDAY

77. RESPONSES OF ANTIOXIDATION ENZYMES TO CHILLING STRESS IN TOBACCO SEEDLINGS. Yong-Ping LI, Yun-Ye Zheng, and Wen-Guang Ma; Yunnan Tobacco Research Institute, Yunnan, Yuxi, China

Chilling is one of the major abiotic stresses limiting yield and quality of many important crops. For better understanding of chilling stress responses in tobacco (Nicotiana tabacum), growth rate and antioxidant enzymes of two tobacco seedlings, cv. MSk326 (chilling sensitive variety) and Honghuadajinyuan (HHDJY, chilling tolerant variety), treated with chilling temperature (5 ^{o}C) were studied. The results showed that the growth rate of shoot was higher than that of root, suggesting that root growth was more easily affected by chilling stress. Chilling stress increased peroxidase (POD) activity and reduced superoxide dismutase (SOD) activity in shoot of HHDJY, and catalase (CAT) activity was little affected. In root of HHDJY, chilling stress increased SOD and CAT activities, and had little effect on POD activity. Chilling treatment increased SOD activity in shoot and CAT activity in root of MSk326. The results also showed that tobacco seedlings might have the capacity of recovering from chilling injury for a short term. The relationship between the growth rate and antioxidant enzyme activity was analyzed using stepwise regression and it was indicated that regression equations containing CAT could be used in predicting seedling growth rate of tobacco under chilling condition.

4:20 PM TUESDAY

78. STUDY OF INFLUENCING FACTORS ON HEAVY METALS IN TOBACCO PLANTING SOIL AND FLUE-CURED TOBACCO. YANG Jie[1], Chen Jiang-Hua[2], and Li Jin-Ping[3], Zhang Yan-Ling[1], Zhang Shi-Xiang[1]; [1]Zhengzhou Tobacco Research Institute of CNTC, Henan, China, [2]China National Leaf Tobacco Corporation, [3]Hubei Tobacco Science Institute

Taking Shennongjia area as an example, 9 heavy metals (Cr, Ni, Cu, Zn, As, Se, Cd, Pb, Hg) in tobacco planting soils and flue-cured tobacco leaves and the relationship with soil properties were investigated. The results showed that Cr, Ni, Cu, As, Cd and Hg levels in soils around Shennongjia area were significantly correlated with soil organic mater, while Pb levels were mainly influenced by soil pH; The availability of Ni, Zn, Cd and Pb decreased with increase of soil pH, and the availability of As, Cr and Se increased with the content of organic mater; The Ni, Cu, Cd and Pb contents in tobacco leaves showed differently

correlated with their contents in soils or soil properties. The regression analysis illustrated that the contents of Ni, Cu, Pb in tobacco leaves were determined by their availability. Cd content in tobacco leaves was influenced by both its availability and organic matter in soils with Zn by its total level in soils and soil pH, and soil organic matter maybe the only factor determine its level in tobacco.

4:40 PM TUESDAY

79. GENETIC DISSECTION OF IMPORTANT TRAITS IN BURLEY TOBACCO. Changchun CAI, Liguang Chai, Yi Wang, Fangsen Xu, and Guoping Lin; Burley Tobacco Experimental Station of CNTC, Hubei Tobacco Research Institute, Wuhan, Hubei Province, China

In order to dissect the genetic control of important traits of burley tobacco, a first genetic linkage map of burley tobacco in China based on molecular markers including amplified fragment length polymorphism (AFLP) and sequence-related amplified polymorphism (SRAP) was built using one DH (doubled haploid) population derived from a cross between two high-quality burley tobacco cultivars Burley37 and LABurley21 respectively with high and low nicotine content in this study. QTL analysis of important traits including chemical components of central leaf after air-cured involving nicotine content, total nitrogen content, total sugar content, total potassium content and agronomic traits involving plant height, stalk circumference, distance of knot, number of total leaf , length of central leaf and width of central leaf was performed. The results showed that the genetic linkage map was constructed by 112 AFLP loci and six SRAP loci which were assembled into 22 linkage groups (A1 – A22) with a total genetic distance of 1953.6 cM and an average distance of 20.5 cM between two adjacent loci. There were 25 distortion-segregation loci (17%) mainly clustered in A1, A11 and A14. QTL analysis indicated that 14 main QTLs were detected. Out of them, 2, 2, 3 and 3 QTLs for nicotine content, total nitrogen content, total sugar content and number of total leaf respectively were detected. The number of QTLs for plant height, stalk circumference, distance of knot and length of central leaf was equally one. But no QTL was detected for width of central leaf and total potassium content. Importantly, BtNic1 and BtTn1 respectively controlling nicotine content and total nitrogen content showed a good co-segregation. The 14 main QTLs accounted for 10.7% to 26.4% of phenotypic variation of traits. In the end, application of this study in tobacco breeding and its next work were discussed.

5:00 PM ADJOURN

WEDNESDAY MORNING, SEPTEMBER 30, 2009

COMBINED SESSION ***Session Chair: Balazs Siminszky***

8:30 AM WEDNESDAY

80. DEVELOPMENT OF A METHOD FOR THE MASS SPECTROMETRIC ANALYSIS OF 4-HYDROXY-1-(3-PYRIDYL)-1-BUTANONE (HPB) IN VARIOUS CELL LINES. J.E. TARRANT, D. Skinner, and A. Flores; Lorillard Tobacco Co., Greensboro, NC

The study of 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB) releasing adducts can provide additional insight into better understanding any potential toxicological effects of tobacco. HPB has generally been used to monitor the exposure of the tobacco specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). In the past, the analytical determination of HPB has centered on blood samples or large tissue samples collected during autopsy or surgery. Typically, the hemoglobin or DNA was isolated and any adducts formed from the diazohydroxide metabolites of NNK were converted to HPB via acid hydrolysis. This presentation describes a method for quantitatively measuring HPB in cells used for *in vitro* exposure. Two cell lines A-549 (human alveolar basal epithelial carcinoma cells) and BEAS-2B (immortalized human bronchial epithelial cells) were used to establish the method capabilities. Each whole cell population was lysed and hydrolyzed. The hydrolysate was partitioned into methylene chloride and derivitized with pentafluorobenzoyl chloride to be analyzed by GC/MS-NCI. The presented method possesses a sensitivity of 60 nM or 10 ng/ml (equivalent to 60 fmols or 10 pg per injection) of HPB. This new approach can monitor the intermediate metabolites and the formation of HPB releasing adducts from *in vitro* exposure of NNK. The method does not require the isolation of genomic DNA or analysis of costly and potentially unavailable tissue samples.

8:50 AM WEDNESDAY

81. CHARACTERIZATION OF REFERENCE AND COMMERCIAL MOIST SNUFF SAMPLES BY USE OF TWO GC-MS SCAN TECHNIQUES. John H. LAUTERBACH; Lauterbach & Associates, LLC, Macon, GA and Deborah A. Grimm; Coordinated Instrumentation Facility, Tulane University, New Orleans, LA

There have been several reports over the years of toxicological activity in extracts of commercial moist snuff samples as determined with several in vitro assays. However, the activity reported has been weak when compared with results obtained on cigarette smoke condensate using the same assays. Moreover, DMSO extracts of ostensibly similar moist snuff products have given dissimilar results in such assays. Furthermore, it was unlikely that the toxicological results were related to levels of the GothiaTek® analytes. Consequently. A new approach was taken. Commercial samples of several flavored and so-called straight moist snuff products were obtained at retail. They were tray-dried in a low-humidity environment at ambient temperatures to remove much of the moisture and volatile flavors. We then characterized all nine commercial products and the 2S3 reference moist snuff with two GC-MS scan techniques: 1) the Direct Silylation Scan which is known to provide identifications and semi-quantitative data, on acids, humectants, sugars, and certain other compounds (Moldoveanu *et al.*, 46th TCRC, Paper #28); and 2) the HFP (hexafluoroisopropanol) Scan, which allows the analysis of the more volatile compounds,

starting with low molecular weight ketones and extending up to neophytadiene and some sterols (Dong *et al.*, 47th TCRC, Paper #16). Both GC-MS techniques were performed on an Agilent 6890 GC coupled with an Agilent 5972 MS with a DB-5 MS column (25 m X 0.25 µm film thickness and 0.25 mm ID). Differences in the composition of the less volatile compounds in the products will be discussed with respect to the the type of flavors used.

9:10 AM WEDNESDAY

82. INFLUENCE OF PUFF VOLUME ON ADSORPTION EFFICIENCY OF ACTIVATED CARBON FOR VOLATILE ORGANIC COMPOUNDS IN CIGARETTE SMOKE. Noritoshi FUJITA and Ken-Ichi Itabashi; Japan Tobacco Co., Yokohama, Kanagawa, Japan

The adsorption efficiency (E) of activated carbon (AC) for volatile organic compounds (VOCs) in cigarette smoke decreases with increasing puff volume. Although it is known that the amount of VOCs and the velocity through the AC increase at the same time, the main factor for the decrease of E has not been elucidated. Therefore in this study, the influence of the puff volume on E was evaluated by using the puff-by-puff method to understand the adsorption behavior in detail.Smoking tests were carried out with various puff volumes. The relative concentration of each VOC was analyzed with and without the AC by gas chromatograph. E and the adsorbed amount of VOC (W_{ad}) were calculated from those concentrations.

It was found that the E of VOCs with high vapor pressure decreased with increasing puff numbers, and also decreased with increasing puff volume. Whereas, the E of VOCs with low vapor pressure hardly changed with increasing puff numbers and puff volume. The adsorption capacity of the AC is lost gradually due to the adsorbed VOCs with increasing puff numbers. Therefore these adsorption behaviors were considered in terms of W_{ad}. Regardless of the puff volume, a larger filter inlet amount of VOC (W_{in}) gave a larger W_{ad}. But the tendency was different for each VOC, which was attributed to the difference of the physicochemical interaction between the VOCs and the AC. Consequently, it was confirmed that the main factor in the decrease of the adsorption efficiency by increasing puff volume was not the velocity through the AC but the filter inlet amount of VOC, although the relation between W_{in} and W_{ad} varies for each VOC.

9:30 AM WEDNESDAY

83. DETERMINATION OF PRESSURE DROP RESPONSE FROM TRIACETIN PLASTICIZER APPLICATION ON CELLULOSE ACETATE FILTERS. Kevin NORFLEET; Celanese Acetate LLC, Narrows, VA

The triacetin plasticizer commonly applied to most commercial cigarette filters has a well known effect on rod firmness levels. However, little focus has been directed to the impact of plasticizer on rod encapsulated pressure drop. Work at Celanese has found the effect to be statistically significant and quantified the impact of plasticizer content on the final filter rod pressure drop. Additionally, a theoretical explanation for the phenomenon has been developed. By holding cellulose acetate tow delivery constant and varying only the level of plasticizer application, this work effectively measures the singular effect of plasticizer on rod encapsulated pressure drop across a variety of circumferences, filament

sizes, deniers and weights. Increased plasticizer leads to a slight increase in pressure drop at standard circumferences. As circumference decreases the effect becomes more substantial. A predictive equation has been developed that will help more accurately predict the yield capability of acetate tow on a rodmaker.

9:50 AM ***Break***

10:20 AM WEDNESDAY

84. SWITCHING FROM USUAL BRAND CIGARETTES TO A TOBACCO HEATING CIGARETTE OR SNUS - A MULTI-CENTER EVALUATION OF BIOMARKERS OF EXPOSURE AND HARM. M.W. OGDEN, M.F. Stiles, B.A. Jones, T.J. Steichen, and W.T. Morgan; R.J. Reynolds, Winston-Salem, NC

A randomized, multi-center, 3-group study was conducted in subjects who smoke (n=131) and were switched to a Tobacco Heating (TH) cigarette, Snus (S) or a Tobacco Burning (TB) cigarette (5 mg 'tar' by the Cambridge filter method), with a non-treatment group of never-smokers (NS) (n=32). Clinical confinement, with biomarker evaluation, was conducted in smokers at baseline, 12 and 24 weeks and in NS at baseline only. Samples for 24-h urine and blood were collected and analyzed for tobacco-related biomarkers. Urinary biomarkers included those for total nicotine equivalents (NicEq-T), NNK, benzene, acrolein, crotonaldehyde, 1,3-butadiene, acrylamide, polycyclic aromatic hydrocarbons (PAH), aromatic amines (AA), and urine mutagenicity (UM), among others. Blood biomarkers included carboxyhemoglobin (COHb), 4-aminobiphenyl-Hb adducts (4-ABP-Hb), C-reactive protein (hsCRP), sICAM-1, fibrinogen, homocysteine, platelets, sister chromatid exchange (SCE) in peripheral lymphocytes and circulating endothelial precursor (CEP) cells, among others. Smokers on usual brand (UB) at baseline constituted the intent to treat (ITT) sample. Usage of study product and other tobacco/nicotine forms was tracked daily via telephone diary and compliance was computed. Mean compliance >50% in week 24 defined the per protocol (PP) sample (n=88; with dual use noted particularly in the Snus group). For all urinary biomarkers listed, mean values (mass/24-h) in ITT smokers exceeded ($p<0.05$) those in NS. For listed blood biomarkers, mean values in ITT smokers exceeded ($p<0.05$) those in NS except for hsCRP, fibrinogen, platelets and CEP cells (these are not discussed further). Among matched PP subjects at week 24 (vs. UB baseline), the following significant differences were noted: In urine, TH<UB for NicEq-T, NNK, AA, PAH (4 of 6), acrylamide, butadiene, crotonaldehyde, benzene, UM; and, in blood, for 4-ABP-Hb and sICAM-1. TH>UB only for acrolein in urine. In urine, Snus<UB for NNK, AA, PAH (4 of 6), acrylamide, butadiene, crotonaldehyde, acrolein, benzene, UM and, in blood, for COHb and sICAM-1. Switching from UB cigarettes to TH cigarettes or Snus (even non-exclusively) significantly reduced exposure to several important tobacco toxicants.

10:40 AM WEDNESDAY

85. STUDIES ON POST-SYNTHESIZED AMINE-FUNCTIONALIZED MATERIAL FOR REDUCING VOLATILE CARBONYL COMPOUNDS IN CIGARETTE SMOKE. Cong NIE[1], Le Zhao[1], Bin Peng[1], Xuehui Sun[1], Huimin Liu[1], and Xuewu Yan[2]; [1]Zhengzhou Tobacco Research Institute of China National Tobacco Corporation, Zhengzhou, China, [2]Nanjing University of Science and Technology, Nanjing, China

A novel amine-functionalized material for the reduction of volatile carbonyl compounds in cigarette smoke has been prepared by post –synthesis method. Different carriers, amine-functionalized reagents and their appropriate volumes were investigated. The amine-functionalized material prepared using Al_2O_3 as a carrier, 3-aminopropyltrimethoxysilane (APTES) as amine-functionalized reagent at a rate of 1g/5mmol was found to be most effective for reducing volatile carbonyl compounds.

The effects on decreasing volatile carbonyl compounds in cigarette smoke were investigated using a cigarette attached with a dual-filter composed of the material. Compared with the control cigarette, the formaldehyde, aldehyde, propylaldehyde and crotonaldehyde deliveries of the experimental sample were reduced by about 55.2%, 25.0%, 20.7% and 17.8%, respectively, while the nicotine and tar deliveries were almost the same. It indicated that the novel amine-functionalized material had good selective filtration behavior for decreasing volatile carbonyl compounds in cigarette smoke.Moreover, the smoking quality of the experimental cigarette was similar to the original one.

11:00 AM WEDNESDAY

86. RAPID RESOLUTION LIQUID CHROMATOGRAPHY AS SECOND DIMENSION IN A COMPREHENSIVE TWO-DIMENSIONAL SYSTEM FOR ANALYZING TOBACCO EXTRACTS. Li DING[1], Fuwei Xie[1], Degke Hou[1,2], Huimin Liu[1], Shusheng Zhang[2]; [1]Zhengzhou Tobacco Research Institute of China National Tobacco Corporation, Zhengzhou, China, [2]Chemistry Department of Zhengzhou University, Zhengzhou, China

In order to achieve rapid separation in the second dimension, a comprehensive liquid chromatography system was set up, wherein an Agilent 1200 high performance liquid chromatography served as the first dimension and an Agilent 1200 rapid resolution liquid chromatography served as the second dimension, and they were connected to a 10-port-2-position valve via two storage loops. For tobacco extracts by methanol and water, different columns, mobile phases and switching cycles were compared for better resolution. A comprehensive liquid chromatography-diode array detector analytical system, coupling a phenyl column and a C18 column, with a 1.5-minute switching cycle and two 50-μL storage loops, was chosen. The results shown that the tobacco extracts which could not be separated perfectly by conventional one-dimensional liquid chromatography were well separated by this system.

11:20 AM WEDNESDAY

87. COMPLETE NUCLEOTIDE SEQUENCE AND GENOME ORGANIZATION OF TOBACCO VEIN DISTORTING VIRUS. MO Xiaohan[1] and Chen Jianping[2]; [1]Yunnan Tobacco Science Institute, Yuxi, Yunnan, China, [2]Institute of Virology and Biotechnology, Zhejiang Academy of Agricultural Sciences, Hangzhou, China

Tobacco bushy top disease is caused by *Tobacco bushy top virus* and *Tobacco vein distorting virus* (TVDV). In this study, the viral complex of tobacco bushy top disease was purified and back-inoculated to healthy tobacco successfully. The complete nucleotide sequence and genome organization of TVDV, one of the causal agents, were determined. The results indicate that TVDV genome is 5920 nts long and encodes 6 open reading frames (ORF). TVDV ORF0 codes for a protein of 28.1 kDa which is the putative gene silencing suppressor. TVDV ORF1 codes for a protein of 72.3 kDa. TVDV ORF2 codes for a protein of 66.8kDa which is the putative RNA dependent RNA polymerase (RdRp). TVDV ORF3 codes for the coat protein (CP) with a molecular mass of 22.4 kDa. TVDV ORF4 codes for the putative movement protein with a molecular mass of 17.4 kDa. TVDV ORF5 codes for the readthrough domain of the CP. An intergenic non-coding region of 201 bases locates between ORF2 and ORF3. The genome organization of TVDV is typical of the members of the genus *Polerovirus* (The family *Luteoviridae*). Phylogenetic trees constructed according to the amino acid sequences of RdRp and CP confirmed that TVDV was a new member of the genus *Polerovirus*. This is the first report of the full-length genome sequence of TVDV.

11:40 AM ADJOURN