

# CORESTA CONGRESS 2018



## ABSTRACTS

*Presenter's name is underlined when the main author (listed first) is not presenting the paper*

# CORESTA CONGRESS 2018

## PLENARY SESSION

### INVITED SPEAKER

#### **From genome to variety: explorations and practices of CNTC**

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Owing to rapid developments in genome sequencing technologies and other omics research tools, crop improvement by whole genome analysis has become routine. However, due to the polyploidy and complexity of its genome and limited number of markers available, molecular breeding of tobacco still lags behind other crops. In this presentation, I will share our experiences on improvement of tobacco varieties conducted by the China tobacco industry. In 2010, the China National Tobacco Corp. (CNTC) initiated the “Tobacco Genome Project” with the aim to promote the green development of China’s tobacco agriculture by developing tobacco varieties with higher quality and less harmful constituents.

In the past few years, we sequenced the genome of *Nicotiana tabacum* (cultivar Honghua Dajinyuan) using a strategy combining BAC-to-BAC and whole genome shotgun with around 763-fold coverage. Combining optical maps and a genetic map consisting of a set of 3,360 SNP markers, around 66% of the assembled scaffolds were anchored to pseudochromosomes. Then genomes of more than 250 tobacco varieties and landraces were resequenced and a haplotype map of genomic variations was constructed. A comprehensive tobacco gene expression atlas covering more than 100 tissues/organs under different developmental stages was presented by a custom designed gene expression array. We built a series of MS-based, multi-platform metabolomics methods, which can qualify and quantify over 500 metabolites in tobacco. A new tobacco mutant population induced by ethyl methane sulfonate mutagenesis was constructed for functional genomics applications. The genome editing system was also developed in tobacco and can be used to study gene functions on a genome scale.

The large scale genetic resources mentioned above combined with genome analyses allowed us to identify a number of genes involved in black shank and virus diseases resistance, as well as biosynthesis of flavonoid, carotenoid, precursors of NNN and phenol, etc. Understanding the functions of these genes and the performances when assembling them will be useful to conduct molecular breeding of new tobacco varieties.

# **CORESTA CONGRESS 2018**

## **PLENARY SESSION**

### **PRIZE**

#### **Technical upgrading and reconstruction of China's tobacco industry**

YUAN Xingsi

*Former Vice-President of China National Tobacco Corporation, former President of the Zhengzhou Tobacco Research Institute of CNTC, and Chief Editor of 'Acta Tabacaria Sinica' (retired)*

In the early 1980s, the newly founded China National Tobacco Corporation (CNTC) had an ambitious plan to modernize its factories. CNTC provided advanced cigarette making and packing machines to its cigarette factories. The upgrading of cigarette manufacturing was succeeded by factory reconstruction with the aim of synchronizing primary processing with advanced cigarette manufacturing techniques, promoting production automation and logistics automation. As a result, modernized and state-of-the-art cigarette factories were set up one after another.

In the tobacco production sector, the tobacco processing capacity was badly inadequate. To build new processing plants, CNTC had to make a choice between two technologies, bundle leaf drying or threshing green leaf then drying strips and stems separately. Green leaf threshing was new and there was concern regarding its adaptability to the conditions in China at that time. In the meantime, a feasibility study was undertaken. The study was carried out on an industrial scale prototype line designed especially for that purpose. The study placed emphasis on investigating the influence of the use of strips on the cigarette factory.

The study report released by Zhengzhou Tobacco Research Institute (ZTRI) concluded that green leaf threshing benefited both the tobacco processing plant and the cigarette factory in general. However, during the transition period the cigarette factory had to handle both bundle leaves and strips.

CNTC approved the report, and new processing plants were then set up at a fast pace, and covered all major tobacco growing areas by the end of last century.

Nowadays, the modernization of China's tobacco industry is no longer an ideal. It has become a reality.

# **CORESTA CONGRESS 2018**

## **INTERGROUP PAPERS**

### **IG 01**

#### **Production of very low nicotine Burley tobacco: short term feasibility from an agronomy viewpoint**

JACK A.M.; FISHER C.R.

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The U.S. Food and Drug Administration (FDA) has issued an Advance Notice of Proposed Rulemaking (ANPRM) to obtain information for developing a product standard that would lower nicotine to non-addictive levels. They have requested comments on a maximum limit of around 0.3-0.5 mg/g. We do not believe that this limit is technically feasible on a nationwide scale in the short term with the varieties currently available. Nicotine levels are extremely variable between crops. We examined data collected over decades in the Minimum Standards Program, focusing on the two checks. Nicotine levels were mostly 35-55 mg/g, but they were as high as 72 mg/g, and as low as 18 mg/g. These data are weighted means for the whole plant; the leaf grade, which comprises most of the weight, is at least 12-15 % higher. LA (low alkaloid) mutants reduce nicotine to about 10 % of the wild type: 3.5-5.5 mg/g in the average crop, which is still tenfold higher than the limit in question. Agronomic practices can reduce nicotine further, but results are notoriously variable. Data we have collected over the years show: a) not topping reduced nicotine to 55-60 % of topped tobacco, b) close spacing reduced it to 65-95 % of standard spaced tobacco, c) drastically reducing nitrogen fertilizer reduced it to 40-94 % of standard fertilized tobacco, d) early harvesting reduced it to 64-78 % of standard harvested tobacco. We know that irrigation reduces nicotine, but we do not have any data. We are currently testing the combined effect of all these factors. We do not yet have any data, but it is unlikely that it will reduce nicotine to below 10 % of standard-grown tobacco, and even more unlikely that it will do so consistently. Several groups are working on molecular nicotine reduction. It is possible that they will achieve the 0.3-0.5 mg/g nicotine levels, but it could be years before such varieties are available to growers on a commercial scale, if the industry maintains its current testing procedures.

## IG 02

### **Cigarette Variability Task Force study designs, statistical considerations, initial observations, and limitations**

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*[Presented as a representative of the CVAR TF]*

The objective of the Cigarette Variability Task Force (CVAR TF) is to examine commercial cigarette variability through testing of select cigarette smoke and tobacco filler constituents plus select physical characteristics. To meet this objective, the CVAR TF developed studies in three phases: Phase 1 short-term - three separate samples of each product on different production days within one week; Phase 2 medium-term - quarterly samples of each product within one year; and Phase 3 long-term - annual samples of each product across three years. Commercial cigarette products from international markets were chosen to represent a range of cigarette designs (e.g. blend type and tar level) and were distributed for analytical testing for select constituents (analytes) recommended or required by organizations such as the World Health Organization (WHO), the U.S. Food and Drug Administration (FDA), Health Canada, and STMA in China. The products selected are considered to be high production brands for the region in which they are sold. Product collections were from typical production runs with no additional controls imposed for purposes of the study. As the intent of the study is to examine commercial cigarette variability, several factors were incorporated in the study design to minimize the influence of testing (analytical) variability on the interpretation of results. Study design, statistical considerations, initial observations and limitations will be discussed.

# **CORESTA CONGRESS 2018**

## **WORKSHOPS**

### **PRESENTATIONS**

#### **WORKSHOP SCIENCE & INNOVATION**

##### **APSTW 01**

###### **The modern tobacco agronomist – utilizing tradition and change to sustain an industry**

VANN M.C.

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The role of the tobacco agronomist has changed remarkably over the last half-century. No longer are agronomists simply called upon to conduct applied research or to provide expertise and training in applied agriculture, but rather all of these things and much more. The responsibilities of the tobacco agronomist have transitioned to those which require in-depth knowledge of the global tobacco industry, the complexities of governmental oversight and regulation, new and emerging technologies that are useful to the industry as a whole, the intricacies of agricultural ecosystems and sustainability, and the various socio-political issues that are unique to a diversity of leaf origins. This presentation will focus on specific transitions in duties and initiatives that serve to sustain the global tobacco industry, and will provide insight as to where and how agronomists will navigate the next half-century.

##### **APSTW 02**

###### **New trends in tobacco crop protection: a Zimbabwean case study**

DIMBI S.; MAVUKA R.

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For decades, synthetic agrochemicals were the major components in tobacco crop protection. Most of these products had the advantage of efficacy, a knock-down effect and a broad spectrum of activity. Thus, for example, methyl bromide, used as a soil fumigant, could take care of all soil insect pests, nematode pests and soil pathogens at the seedling production stage. Equally other fumigants such as Ethylene dibromide (EDB),

1,3-Dichloropropane (1,3D) were relied upon heavily to protect transplants after which the equally effective organophosphate and carbamate group of pesticides could be used to protect the crop till harvest. However, due to issues of increased regulatory demands, environmental and worker protection concerns and requirements for sustainability in tobacco production, most of these chemical groups are no longer acceptable for use. Focus has been shifted to greener crop protection agents and other innovative methods that reduce levels of residues in the final leaf product and protect the environment and workers. In this paper we show the trends in tobacco crop protection in Zimbabwe by highlighting the changes in pesticide classes used and the integration of other pest control strategies over the past two decades.

## **APSTW 03**

### **Scientific research and product standards: building trust in the e-vapour category**

THOMPSON J.M.

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Electronic vapour products (EVPs) have the potential to substantially reduce the harm caused by smoking at both the individual and population level. Despite this, the number of smokers that have tried and switched to EVPs remains low. This is driven in part by the frequent misreporting of scientific studies in the media, concerns over the lack of data evaluating long-term risks, and the ability of products to satisfy smokers.

As manufacturers' innovations address the latter to encourage more smokers to move to EVPs and improve the user experience, such as through nicotine salts and flavourings, these raise broader concerns amongst regulators including youth smoking and abuse liability. To that end, two things become critical: (1) the scientific evidence-base substantiating EVPs as reduced harm relative to smoking, and (2) development of robust product quality, manufacturing and safety standards that ensure product quality, efficacy and safety (both relative to smoking and in absolute terms).

Both of these are underpinned by robust validated scientific methods. Advances in 21<sup>st</sup> century toxicology tools, combined with clinical studies, behavioural studies, and population modelling offer an opportunity for a weight-of-evidence approach to assess the harm reduction potential of EVPs. The public health potential of these products will only be fully realised if scientists (both industry and academic), regulators, and the public health community work cooperatively to develop robust scientific methodology for harm reduction, address data gaps, and develop high product quality standards that minimize any chemical, mechanical, thermal or electrical risks.

As science inspires and validates product innovations, it will increase trust in the category amongst consumers and enable regulators to meet public health goals in reducing the harms of smoking.

## **APSTW 04**

### **Assessing the impact of tobacco harm reduction**

ERKKILA B.E.

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The last decade has seen exponential growth in the number of products on the market as consumers around the globe transition away from traditional cigarettes. However, instead of sitting back and waiting for the public health gains to roll in, it is necessary that we predict, and hopefully shape, what the landscape may look like in the future. In the absence of the quantity and quality of long-term epidemiological data that exists for cigarettes, we must drive the innovation of validated signals of harm from emerging products and apply established risk assessment techniques to their evaluation. The fitness of existing biomarkers and a process for the efficient development and qualification of novel biomarkers should involve coordination between industry, academia, and regulators, and draw from best practices in the pharmaceutical arena. The practice of human health risk assessment is a quantitative method to predict the potential harm from an agent, it is often used by regulatory agencies to set standards for consumer products as well as environmental and occupational exposures. The nascent field of tobacco product risk assessment has evolved over recent years, but we are far from consensus on the appropriate methods, inputs, assumptions, and interpretation of the relative and absolute risks. Continuing dialogue amongst parties with relevant experience, including regulators, academics, industry scientists, and the greater public health community will inform standardization of methods as well as identify and resolve existing data gaps. In addition, robust study into best practices for communicating relative and absolute risk to the consumer are paramount to the continued uptake of emerging products. We cannot assume that innovation for innovation's sake will ultimately lead to significant public health gains, there must be continued vigilance and dialogue to ensure that harm reduction products deliver on their promise.

## **WORKSHOP CROP PROTECTION**

### **APW 01**

#### **CPAs, tobacco production, integrity and compliance**

PRAT M.

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Crop Protection Agents (CPAs) have played an important role in increasing crop yield and quality across agriculture, including tobacco production. However, the segment of crop production has experienced significant changes over the years, with regards to the types of CPAs used, the associated residues, and the analysis of CPA residues. This presentation will outline these changes in marketed leaf tobacco and offer a view on the future of CPAs in tobacco production.



## **APW 02**

### **Pesticide fate in soil and plants**

GANNON T.W.

*North Carolina State University, Raleigh, NC, U.S.A.*

Understanding pesticide fate and behavior is imperative to optimize efficacy, reduce residue levels and ensure environmental and human health are not adversely affected. Pesticide fate depends on numerous factors including physical and chemical properties of the pesticide as well as characteristics of the system including, but not limited to, application timing, crop stage, environmental conditions and edaphic attributes. Pesticide absorption by shoots and roots is influenced by pesticide physicochemical properties, notably molecular size, composition, and lipophilicity but also varies significantly with other factors including adjuvant inclusion, formulation, plant species and environmental conditions. Lipophilic pesticides are readily absorbed into cuticular waxes but limited movement occurs beyond this region; however, hydrophilic pesticides are slow to be absorbed into cuticular waxes but increase as they approach pectin and cell wall layers. Once absorbed, pesticide redistribution in plants is governed by a number of factors. Systemic activity is often used to describe longer distance redistribution involving xylem and phloem transport compared to contact pesticides which are not transported significant distances from the site of absorption. Environmental conditions most favorable for plant growth generally result in maximum absorption and translocation; however, foliar absorption and translocation may be impacted more with hydrophilic pesticides compared to lipophilic pesticides which may impact efficacy and pesticide residues. Once a pesticide reaches the soil surface, it is subject to absorption by roots, adsorption by soil colloids or organic matter and other processes. The aforementioned pesticide fate topics and processes will be discussed as they relate to optimizing efficacy, reducing pesticide inputs and residues in tobacco production systems while ensuring environmental and human health are not adversely affected.

## **APW 03**

### **Maximizing the spray droplet to effectively deliver pesticides**

LEGLEITER T.

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Numerous factors influence the efficacy of a pesticide on the intended target pest, including the delivery method of the pesticide to the target pest. Pesticides in the agronomic realm are most often delivered using spray droplets produced by a broadcast nozzle. The spray droplet as a delivery method for pesticides can result in either an effective or ineffective deliver depending on a number of biological, physical, and environmental factors. Droplets as a delivery vehicle can be an inefficient process with 50 to 75 % of the released droplets not achieving deposition onto the intended target in many cases. Factors that influence the ultimate fate of a droplet include the number of

droplets released or spray volume, variability and range of droplet sizes, distance between the droplet release and target, and the environment in which the droplet is released. Effectively delivering the droplet to the target is only a small step in achieving efficacious control of the target pest as the quantity and quality of deposits as well as the biological features of the target and environment all influence the ultimate effectiveness of the pesticide. Each of the factors that influence the spray droplet from its creation from the spray sheet at the nozzle orifice to its final destination will be discussed for various pesticide, pest, and environmental scenarios.

## **APW 04**

### **Biocontrol: what place in crop protection?**

KANE K.; MAUMENE C.

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Since the early 2000s, the number of registered active substances to control pests has been constantly decreasing due to societal and regulatory pressure. The number of new active ingredients currently being developed worldwide is also decreasing. Meanwhile the emergence and development of new resistances amplify the reduction of solutions that can be used by farmers. The need for innovation is therefore crucial for the whole of agriculture, particularly in the area of biocontrol, which is considered to be one of the possible responses to changes in expected crop protection practices. Based on French regulation, products and agents of biocontrol include macro-organisms, micro-organisms, chemical mediators such as pheromones and kairomones and natural substances extracted from plants, animals or minerals. The French authorities have the will to encourage biocontrol thanks to incentive measures, in particular those whose toxicological and eco-toxicological profile is most favourable. They publish a list ("List of biocontrol products" [Memo 2018-205 on 03-15-2018]) of products, updated every two months, which contains 73 active substances and 416 commercial products. Only six usages are covered for the tobacco crop.

The International Biocontrol Manufacturers' Association (IBMA) considers that 5 % of the crop protection agents' market is taken up by biocontrol products and the ambition is to reach a two-digit growth in the coming years. If societal expectation and, even more, the regulatory pressure on conventional pesticides, represents a real engine for the development of biocontrol, three main obstacles are regularly identified: (i) efficacy, (ii) simplicity of implementation, (iii) and price. Experience shows that these hurdles can be overcome and lead to the development of solutions on several tens, or even hundreds of thousands, of hectares. There are three examples: anti-slug based on ferric phosphate, trichogrammes on corn, nonanoic acid on vine, potato and tobacco.

# WORKSHOP

## TOBACCO BIOTECHNOLOGY – FROM GENOME TO VARIETIES

### APW 05

#### Tobacco genomic resource

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*Nicotiana tabacum*, as a model plant system, has played important roles in the development of molecular plant biology. Recently, because of its active metabolites, it has attracted more attention in biochemistry and biotechnology. However, the tobacco genome is large and challenging to sequence because it is an allotetraploid, formed by hybridization of diploid parents *N. sylvestris* and *N. tomentosiformis*. Although several groups have already released their draft genomes, production of a genome sequence with sufficient quality is still difficult and challenging.

Here we report on a tobacco genome sequence of the HongDa cultivar, which has been produced by the combination of BAC-to-BAC and whole genome shotgun technologies. The final assembly achieved an N50 size of 1.61 Mb and enabled the anchoring of 70 % of the genome to the chromosomes, which is a big improvement compared with most of the current tobacco genomes. Meanwhile, 71,456 gene modes were annotated, which is also more comprehensive than other versions.

In order to support usage of tobacco genomic resources, relative data and a number of tools have been made available by the China Tobacco Genome Database developed by our group. These include, but are not restricted to, genome browser, transcriptome expression level, metabolism and many useful analysis tools (BLAST, WEGO, Primer3 etc.).

### APW 06

#### Modification of tobacco chemical constituents by molecular breeding

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Tobacco plant chemistry affects consumer perception of derived products and also the potential for health risks associated with their use. Some cured leaf chemical components contribute positively to taste and aroma, while others have negative impacts and may also be designated as 'harmful or potentially harmful' tobacco product constituents. Variability in some measured chemical traits can be partially attributed to genetic variation. Naturally existing and *de novo* genetic variation created via mutagenesis,

genetic engineering, or gene editing can be used in the development of new tobacco cultivars with modified chemical profiles. A range of molecular breeding methodologies has been used to modify alkaloid profiles, and to reduce levels of tobacco specific nitrosamines (TSNAs) and several other harmful constituents in cured leaf. Increasing amounts of genomic DNA sequence information for tobacco and other plant species enhances potential for identification of additional genes affecting cured leaf chemistry. However, complexities associated with outcomes of genetic engineering or so-called 'new breeding methodologies' can complicate wide scale industry adoption of potentially useful new cultivars.

## **APW 07**

### **Contribution of genomic research to develop disease resistance in tobacco**

JULIO E.

*Imperial Tobacco Limited, Leaf Research, La Tour, 24100 Bergerac, France*

Diseases still represent a major concern for tobacco growers all over the world, causing millions of dollars of losses each year. The most economically important are fungus like blue mold, soil borne bacteria like bacterial wilt, or aphid-transmitted viruses like potato virus Y (PVY).

Besides the economic losses related to the attack of different pathogens on tobacco, the impact of the use of crop protection agents (CPAs) is an important issue. A careful use of CPAs is essential to ensure compliance with guidance levels set by industry (CORESTA Guide No. 1). To limit the use of CPAs, it is essential to develop resistant cultivars, and to multiply the sources of resistance in a moving environment where pathogens are able to resist CPAs or bypass old resistance genes.

In recent years, there has been an acceleration in the discovery of resistance genes, thanks to the significant progress of -omics and next generation sequencing methodologies. In this presentation, different kinds of strategies developed to identify new genes or markers in tobacco will be presented, and an example of this acceleration will be given through the story of resistance to PVY, a virus against which breeders can now fight with the strong and multiple tools available.

## **APW 08**

### **Recent developments and practical applications of new breeding technologies**

STRICKLAND J.A.

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The global tobacco industry has enjoyed a relatively stable leaf supply over decades due, in part, to incremental germplasm improvements through traditional breeding, common agronomic practices and harmonized regulatory frameworks. However, this stability is

becoming increasingly fragile as governments continue to expand their authority over tobacco products sold within their borders. Regulatory standards can, in some instances, be addressed simply through product design changes. However, FDA's recently proposed regulations for smokable and smokeless products sold in the United States are so stringent that genetic modifications may be the only way to achieve such drastic limits. Given that the industry continues to oppose GMO utilization, New Breeding Technologies (NBTs) not involving DNA integration may offer the best solution to create tobaccos that could be used to create products meeting regulatory requirements within time limitations initially suggested by the FDA.

NBTs have improved in efficacy and precision over the past decade. For example, EMS created mutant lines (ZYVERT® Technology) have shown NNN reductions of up to ~75 % in leaves and moist smokeless tobacco products. ZYVERT® Technology required ~10 years and 8 generations for stable line creation. In contrast, CRISPR editing of select nicotine biosynthetic genes has taken ~3 years and 3 generations for stable line creation.

NBT effectiveness in developing commercial germplasm is enhanced by advancements in genome sequencing, analyses of transcriptomes, metabolomes and proteomic datasets of multiple tissues collected at various time intervals. Whole genome Axiome® SNPchip characterization also facilitates rapid trait introgression. However, regulatory harmonization in regards to the non-GMO status of NBTs is needed to avoid costly segregation practices and global trade disruptions.

## **WORKSHOP RISK ASSESSMENT**

### **STW 01**

#### **Quantitative risk assessment to compare health risks of chemicals in consumer products**

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Risk assessment is a tool for evaluating public-health concerns, informing regulatory decisions, and developing approaches for cost-benefit analyses. Governmental agencies throughout the world routinely employ risk assessments to predict morbidity or mortality risk in individuals exposed to chemicals or micro-organisms in the environment, or to consumer products (e.g. food additives, pesticides, tobacco). With quantitative risk assessment (QRA), risk characterization brings together the assessments of hazard, dose response, and exposure to provide health risk estimates for the exposure scenarios of interest. Traditional health-related QRA approaches typically begin by screening available data in a deterministic QRA intended to be protective of human health. This approach uses relatively simple mathematical models to produce point estimates of risk (e.g. average or reasonable worst-case) from which risk estimates may then be compared for individual chemicals, chemical mixtures, consumer product ingredients, or

remediation approaches. Probabilistic risk assessment (PRA) uses more sophisticated mathematical modeling approaches that rely on distributions of data as inputs, resulting in a calculated probability distribution of the relationship between exposure and risk. Because risk assessment approaches may vary from organization to organization, the methods used to create the final risk characterization must be transparent, clear, reasonable, and consistent. This presentation will discuss the current state of the science pertaining to QRA and how it can be used to characterize exposures and estimate and/or compare human health risks associated with different types of consumer products, including tobacco products. It will include a description of the data and methods available for conducting each step of the QRA process and the potential uncertainties/challenges with each step.

## STW 02

### **Quantitative risk assessment (QRA)-based prioritization of mainstream cigarette smoke toxicants**

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In 2008, seven representative toxicants in mainstream cigarette smoke were screened and validated by China Tobacco to indicate the harmfulness of mainstream cigarette smoke, which included carbon monoxide (CO), hydrogen cyanide (HCN), 4-(N-methyl-nitrosamine)-1-(3-pyridyl)-1-butanone (NNK), ammonia (NH<sub>3</sub>), benzo[a]pyrene (B[a]P), phenol, and crotonaldehyde. A hazard index based on these seven representative toxicants was then used to regulate the cigarette products of CNTC. In order to more accurately evaluate the harmfulness of cigarettes smoke, a quantitative risk assessment (QRA)-based method of harmful toxicants in cigarette smoke was developed, which consisted of hazard identification, dose-response evaluation, exposure evaluation and risk characterization. Based on the toxicological data in the database of the International Agency for Research on Cancer (IARC), the California Environmental Protection Agency (Cal/EPA), and the U.S. Environmental Protection Agency (US EPA), the toxic potencies of the seven representative toxicants were identified. Exposure evaluation of the seven representative toxicants was performed based on the delivery of mainstream cigarette smoke and parameters of smoking behavior of the Chinese populations (cigarettes smoked per day, exposure frequency, duration of exposure). The quantitative risk of the seven representative toxicants in mainstream cigarette smoke was evaluated by three methods, including incremental lifetime cancer risk (ILCR), hazard quotient (HQ), and margin of exposure (MOE), and a weight-based hazard index of the seven representative harmful toxicants in mainstream cigarette was proposed.

## **STW 03**

### **Purpose-driven risk assessment of tobacco products**

ERKKILA B.E.

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Quantitative risk assessment (QRA) of human health is used to inform decision makers. Generally, the purpose of a QRA is to provide actionable information to researchers, regulators and the public health community. However, the ultimate decision maker for traditional and reduced risk tobacco products is the user, and QRA should integrate proper risk communication from the problem formulation stage.

The preponderance of evidence, including our recent State of Smoking poll ([https://amadashboards.com/kp/eu\\_smokefree/](https://amadashboards.com/kp/eu_smokefree/)), indicates that misperception of risk is common across the globe. This is true for both understanding the absolute risks of tobacco products as well as appreciating the relative risks across product categories. Academics and the public health community at large are often themselves guilty, claiming that certain products are “95 %-safer” without the support of rigorous QRA.

As our goal is to improve public health, purpose-driven risk assessment is user-focused and relies on product characterization and use patterns representative of the population in question. These assumptions should be country, region, and sub-population specific, and must be based on recent analytical, survey, and topography data. In addition, the estimates of absolute and relative risk should not exist solely to inform regulators but should be the basis for the development of risk communication strategies. If smokers were armed with sufficient information about risks they would be in a position to make decisions which could greatly improve their health.

## **STW 04**

### **A framework for toxicological risk assessment of combustible tobacco products in the substantial equivalence pathway**

EHMAN K.

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For the U.S. Food and Drug Administration (FDA) to determine that a tobacco product is substantially equivalent, a New Product must have either the same characteristics as a Predicate Product, or if it has different characteristics, it must be demonstrated that the New Product does not raise different questions of public health. This presentation provides a toxicological risk assessment approach for combustible tobacco products that can be applied in the substantial equivalence pathway to demonstrate that differences between a New Product and Predicate Product do not raise different questions of public health. More specifically, the presentation focuses on the applicability of health-based occupational exposure limits (OELs) and a threshold of toxicological concern (TTC)

approach to evaluate added or increased ingredients as well as their potential pyrolysis products. Occupational exposure levels that are based on health effects are derived through well-established risk assessment practices that rely on integrated analysis of critical health effects, dose-response relationships, and extrapolation methods. The resulting OEL is an inhalation-specific level that an adult can experience without adverse health effects over a working lifetime (i.e. 8 hours of continuous exposure per day, 5 days a week, 40 years). Thresholds of toxicological concern (TTC) have been extensively applied in various industries, including food and beverage, cosmetics, personal and household products, pharmaceutical impurities and medical devices. A TTC of 1.5 µg/person/day is used across regulated industries as an acceptable level for lifetime exposure (i.e. 70 years) to chemicals, including mutagenic compounds, and is applicable to all routes of exposure, including inhalation. In the absence of tobacco-specific guidance documents on toxicological risk assessment, two scientifically-valid approaches to evaluating ingredients for potential risk are presented while taking into consideration the inherent toxicity of tobacco products.

## **WORKSHOP BIOMARKERS**

### **STW 05**

#### **The role of biomarkers in assessing individual health risks of tobacco products**

SARKAR M.

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Assessment of the individual health risk of a modified risk tobacco product (MRTP) is an important component of the regulatory decision-making process for granting authorization of such products. Cigarette smoke contains a mixture of thousands of chemicals, many of which harm the body causing a broad range of diseases such as lung cancer, emphysema and heart disease. The scientific evidence indisputably establishes that smoking cessation leads to significant reduction in these disease risks. Studies have consistently shown that an exposure-response relationship exists between cigarette consumption and disease risk. Thus, reducing exposure to the chemicals from cigarette smoke by switching to a MRTP should reduce the risks of diseases in adult smokers unable or unwilling to quit smoking. Biomarkers can serve the purpose of establishing reduction in health risks from switching to MRTPs. Biomarkers of exposure to specific chemicals from cigarette smoke can unequivocally demonstrate reduction in exposure. The current portfolio of biomarkers of chronic inflammation and oxidative stress provide further insights into the likelihood of reduction in disease risks since these two mechanisms are a common thread across many of the smoking related diseases. The Center for Drug Evaluation and Research (CDER) has developed a “Biomarker Qualification Program” to qualify biomarkers that have “the potential to advance public health by encouraging efficiencies and innovation in drug development.” This presentation will provide an



overview of various biomarkers that can be useful in the regulatory decision process. A synopsis of the CDER Biomarker Qualification Program and potential application of a similar concept for biomarkers available to assess the health risks of tobacco products will also be presented. The role of CORESTA in advancing this proposal to the Center for Tobacco Products will be discussed.

## STW 06

### **Biomarkers of tobacco smoke exposure in the U.S. population: result and resources from the National Health and Nutrition Examination Survey (NHANES)**

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Tobacco smoke exposure is the primary cause of disease and premature death in the U.S. population and a major contributor to negative health impact globally. Tobacco smoke harms the health of active users and non-users with indirect exposure. Biomarkers of exposure to tobacco smoke are crucial for characterizing exposure and evaluating the health impact of exposure, especially as the tobacco product marketplace changes and non-combustible tobacco products are used more widely. The objective of this presentation is to (1) underscore the importance of analytical methods in ensuring the long-term accuracy and precision of reported biomarker data, (2) summarize tobacco-related biomarker data available as part of the NHANES, and (3) highlight the usefulness of combinations of tobacco-related biomarkers for characterizing exposures. The presentation will include examples of the U.S. Centers for Disease Control and Prevention (CDC) quality assurance/quality control (QA/QC) efforts to ensure long-term stability of analytical methods so that exposure trends across decades can be reliably evaluated. Additionally, the availability of tobacco exposure biomarkers in NHANES will be discussed. The presentation will primarily focus on selective smoke exposure biomarkers, including 1-hydroxypyrene, thiocyanate, acrylonitrile metabolite N-acetyl-S-(2-cyanoethyl)-L-cysteine (CYMA), and 2-Amino-9H-pyrido[2,3-b]indole (AaC). These tobacco smoke exposure biomarkers will be compared with cotinine for distinguishing among different types of tobacco product use. For example, sample-weighted median urinary CYMA levels were much higher among exclusive smokers 147 [25th, 75th percentile: 69.6, 245] compared with non-users (1.41 [0.93, 2.20] µg/g creatinine). Urinary CYMA is an excellent biomarker of exclusive smoking vs. non-users, as indicated by its sample-weighted area under the ROC curve (AUC; 0.958 [95 percentile: 0.947, 0.969]), and which was comparable to serum cotinine's (0.970 [0.960, 0.980]). In conclusion, urinary CYMA is an excellent biomarker of smoke exposure, especially in situations where smoke exposure is of primary interest and is to be assessed independently from nicotine exposure.

## STW 07

### Identifying suitable biomarkers of biological effects (BOBEs) for evaluating reduced risk products

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Switching from conventional cigarettes (CCs) to reduced risk products (RRPs), such as e-cigarettes, heat-not-burn and oral tobacco products, could be an effective approach for tobacco harm reduction (THR), provided that the use of RRPs effectively entails a substantial risk reduction. While the application of biomarkers of exposure (BOEs) in human studies is a well-accepted approach to prove reduced exposure to toxicants when using RRPs compared to CCs, the application of biomarkers of biological effects (BOBEs) is less well established. The utilization of suitable BOBEs could significantly improve the RRP evaluation process and place regulators' decisions on a firmer foundation.

In this presentation, five criteria for rating the suitability of BOBEs are described, including (1) association with disease, (2) extent and consistency of differences between smokers and non-smokers, (3) dose-response relationship, (4) reversibility and (5) kinetics after smoking cessation. Furthermore, the effect size, which is an important determinant of the sample size required for clinical studies is calculated for a variety of BOBEs indicative for different biological and clinical endpoints.

It is concluded that the presented rating process is a useful tool for selecting suitable BOBEs to be applied in clinical studies for the evaluation of RRPs.

## STW 08

### Biomarker qualification using adverse outcome pathways (AOPs)

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Smoking is a leading cause of numerous human disorders including pulmonary disease, cardiovascular disease and cancer. Disease development is primarily caused by years of exposure to cigarette smoke constituents, many of which are known toxicants. Switching smokers to modified risk tobacco products (MRTPs) has been suggested as a potential means to reduce the risks of tobacco use, by reducing such exposure.

Although several clinical studies have shown significant reductions in toxicant exposure in smokers who switch to e-cigarettes or tobacco heating products (THPs) further work is required to demonstrate whether this leads to a reduction in the risk of developing smoking related diseases.

Demonstrating product-related reduced risk in the short to medium term is a challenge, biomarkers of potential harm (BoPH) have been suggested as a potential way of

demonstrating risk reduction in a shorter timeframe. However, as there are few, if any biomarkers qualified for the context of use of “smoking harm reduction” further work is required to identify the biomarkers as well as qualify them as ‘fit for purpose’.

A novel approach is to utilise adverse outcome pathways (AOPs) for the development of smoking related diseases. AOPs are a cause and effect paradigm, which characterise important steps in the biology of disease development. AOPs begin with a Molecular Initiating Event (MIE), which is the first action of a stressor in the biological process and subsequent Key Events (KEs), which lie downstream of the MIE, and are linked to an Adverse Outcome (disease). As each KE requires a robust measurement method, this process integrates chemical, *in vitro*, *in vivo*, and clinical data to facilitate weight-of-evidence based risk assessment.

The aim of this presentation is to briefly describe the process required for AOP development, and with examples, show how a more simplistic approach to biomarker qualification could be achieved by tethering biomarker “context of use” to AOP KEs. This approach would better define how BoPHs could be used, and subsequently, focus the qualification process for their specific use in weight-of-evidence based risk assessment.

**CORESTA CONGRESS 2018**  
**AGRONOMY & LEAF INTEGRITY and**  
**PHYTOPATHOLOGY & GENETICS**

**ORAL PRESENTATIONS**

**AP 01**

**FDA advance notice of proposed rulemaking for nicotine level of combusted cigarettes – feasibility for compliance via tobacco plant genetics?**

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On March 16, 2018, the U.S. Food and Drug Administration (FDA) published an advance notice of proposed rulemaking (ANPRM) entitled “Tobacco Product Standard for Nicotine Level of Combusted Cigarettes.” The publication of an ANPRM solicits information and public comments from stakeholders – industry, public health advocates, etc. – regarding a particular issue. After all of the requested information has been submitted, the FDA must consider the information in fashioning a proposed rule, or it may decline to issue a proposed rule. This presentation centers on one aspect of the referenced ANPRM – establishing a nicotine level standard in combustible cigarettes. Specific potential nicotine levels mentioned in the ANPRM are: 0.3, 0.4, and 0.5 mg nicotine/g of tobacco filler. Can levels this low be achieved in the tobacco plant and maintain tobacco leaf yield and quality? Low nicotine tobacco lines containing the *nic1/nic2* loci have been available for several years, but agronomic data from large field trials is scarce and *nic1/nic2* will not achieve the lowest levels mentioned in the ANPRM. Molecular biology techniques have been employed to elucidate information for many of the major nicotine biosynthesis pathway genes as well as transporters, transcription factors and non-coding RNAs. By providing an overview of these various genetic aspects and their feasibility, insight into some of the challenges facing the tobacco industry will be presented. Challenges are to not only comply with the potential nicotine level standard, but maintain both flavor characteristics acceptable to adult tobacco consumers as well as crop yields and tobacco leaf quality tobacco producers require.

## AP 02

### **A newly characterized regulatory mechanism modulates the *NIC2* locus that controls nicotine biosynthesis**

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The AP2/ERF family transcription factors (TFs) have emerged as key regulators of specialized metabolite biosynthesis, including nicotine in tobacco. Several group IX AP2/ERFs form physically linked gene clusters, likely originated from gene duplication events. It is unclear whether the duplicated TF genes are functionally redundant and co-regulated by the same transcriptional circuit, or if they have evolved through gene divergence to possess unique functions, e.g. regulation of one another. The tobacco *NIC2* locus comprises at least ten AP2/ERFs that are homologous to the *ORCA* cluster that regulates vinca alkaloid biosynthesis in medicinal plant *Catharanthus roseus*. Overexpression of the *NIC2* ERFs, e.g. *ERF189* and *ERF221*, induce expression of nicotine pathway genes in tobacco. The *ORCA* and *NIC2* locus ERFs commonly respond to the phytohormone, jasmonic acid (JA). Question thus arose as to whether AP2/ERFs from different clusters are functionally equivalent and interchangeable. We discovered that the individual *NIC2* or *ORCA* ERFs are capable of activating other ERFs within the cluster. The intra-cluster regulation of ERFs implies that the individual components within a cluster are not simply redundant duplication of one another. The positive amplification loops help plants to make sufficient precursors required for the spatial-temporal biosynthesis of alkaloids. We also found that *NIC2* ERFs can activate the *C. roseus ORCA4* promoter and *ORCA5* can regulate *NIC2* ERFs. Furthermore, *ORCA3* and *ORCA5* can up-regulate the nicotine biosynthetic pathway, significantly activating the *PMT* and *QPT* promoters. Similarly, the tobacco *NIC2* ERFs can activate the *C. roseus STR* promoter. Moreover, *ORCA5* overexpression in tobacco induced expression of *PMT* and *QPT*, resulting in increased nicotine accumulation, whereas overexpression of a *NIC2* ERF in *Catharanthus* activated *STR* expression. The mutual activations of two distinct metabolic pathways by the *ORCA* and *NIC2* clusters support our hypothesis that the AP2/ERFs are functionally equivalent and interchangeable. Knowledge gained from this study advances our ability to regulate nicotine biosynthesis.

## AP 03

### Transcriptomic and metabolomic analysis of very low nicotine tobacco lines

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Nicotine is the most abundant alkaloid in cultivated tobacco (*Nicotiana tabacum*), typically constituting more than 90 % of total alkaloids. Recently, the U.S. Food and Drug Administration issued an advance notice of proposed rulemaking to obtain information for consideration in developing a tobacco product standard to set the maximum nicotine level in cigarette filler. Cigarettes that can meet a very low nicotine filler level regulation are dependent upon technical achievability. Historically, *nic1nic2* mutant lines (approximately 95 % nicotine reduction) have been the unique resource for developing low alkaloid traits. However, these mutants produce plants having very poor leaf quality making them commercially unfavourable. In comparison, our data showed nicotine levels in Putrescine N-methyltransferase (PMT) RNAi experimental lines were reduced more than 95 % and had a better grade index after curing compared to *nic1/nic2* mutant controls. To understand grade index/leaf quality and its correlation with nicotine levels, transcriptomics and metabolomics studies were performed using seven Burley and four flue-cured lines at different plant growth time intervals. We will discuss our findings on differential gene expression of these 11 lines and changes in key metabolite markers.

## AP 04

### Regulation network of nicotine biosynthesis in tobacco: the non-coding players

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Several key gene encoding enzymes of nicotine biosynthesis and catabolism pathways have been characterized in tobacco. Non-coding RNAs (ncRNAs) are transcripts with no or low protein coding potential. ncRNAs include microRNAs (miRNAs) with a size of 20-24 nucleotides (nt), long non-coding RNAs (lncRNAs) with a size longer than 200 nt, and circular RNAs (circRNAs) that are covalently closed RNA molecules. The past few years have witnessed an exciting increase in the richness and complexity of ncRNA-mediated regulatory circuitries. However, there is still little knowledge about the role of ncRNAs in nicotine metabolism. The objective of this study was to identify tobacco-specific ncRNAs (miRNAs, lncRNAs, circRNAs), and build the regulatory network between ncRNAs and key

genes of the nicotine metabolism pathways. The genome-wide identification of ncRNAs was performed with RNA sequencing data that was obtained from the root samples of control and topping-treated tobacco (*Nicotiana tabacum*). The co-expression analysis was verified by quantitative Real Time PCR. Nicotine content was measured according to the standard continuous flow protocol (YC/T160-2002) described by the State Tobacco Monopoly Administration of China. 837 miRNAs, 7423 lncRNAs and 5622 circRNAs were identified from the tobacco genome. Five tobacco-specific ncRNA-coding RNA interactions were predicted for nicotine regulation. For example, *nta-eTMX27* (lncRNA) targeted to *nta-miRX27* (miRNA), which down-regulated the activity of quinolinate phosphoribosyl transferase 2 leading to the decrease of nicotine content in the fresh leaves. *nta-miR37* (miRNA) targeted to *nta-ciR1* (circRNA) which was generated from aspartate oxidase 2 (AO2). This interaction up-regulated the expression of AO2 to increase the nicotine level. This is the first report about the regulation of nicotine biosynthesis by ncRNA in tobacco. The understanding of the regulatory module of ncRNAs could be helpful for intensive genetic improvement aimed at modifying the nicotine content.

## AP 06

### Effects on the enzyme activity and microbial community of soil under different fertilizations

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Different fertilizations have significant different effects on soil enzyme activity and micro-organisms. In this paper, high-throughput sequencing was used to analyze the tobacco planting soil with long-term different fertilizations in Guizhou Province. The results showed that the pH value of organic fertilization was much higher than that of inorganic fertilization. The pH value of organic fertilization increased by 6.89 % more than that of the inorganic fertilization. Organic fertilizer significantly increased soil organic matter content and urease enzyme activity. However, different fertilization had no effect on total soil nitrogen, total phosphorus, total potassium and available nitrogen. The operational taxonomic units (OTUs) were significantly diverse in different fertilizations. The OTU of organic fertilization was significantly higher than that of inorganic fertilization, with the rate increasing up to 43.55 %. The cluster analysis of soil micro-organisms at the genus level showed that the organic fertilization and organic + inorganic fertilization were more closely related, while organic fertilization was at a distance from the other fertilizations. The relative abundance of *Nitrospirae* in inorganic fertilization was significantly higher than that of the other two fertilizations, while the relative abundance of *Actinobacteria* and *Chloroflexi* in organic fertilization was significantly higher than that of other fertilizations. In conclusion, long-term fertilization with organic fertilizer alleviated soil acidification, increased organic matter content and improved microbial structure balance and diversity, thus enabling sustainable soil development in the future.

## AP 07

### Float water alkalinity adjustment in organic seedling production

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Float water bicarbonate ( $\text{HCO}_3^-$ ) concentration in excess of 2.0 meq/L (100 ppm) can result in stunted, unusable seedlings unless neutralized. In conventional greenhouse systems, sulfuric acid is used for this purpose; however, it is not currently approved for use in organic production. Research was conducted to evaluate the following organic acidifying compounds: 30 % acetic acid, 50 % liquid citric acid, 45 %  $\beta$ -Hydroxytricarballic acid, and 99.5 % granular citric acid. One additional treatment included continuous float water aeration, which has been documented as a long-term option for bicarbonate reduction. A non-treated control (no acidification) was included as a negative control treatment. Sulfuric acid (35 %) was included as a conventional grower standard. Float water samples were collected at five-day intervals after seeding and titrated to quantify bicarbonate concentration. Following titration, acidifying materials were added to each bed at rates determined by titration results. Twenty-four hours after acidification, float water samples were again collected to measure the effects of each material. Results indicate that acetic,  $\beta$ -Hydroxytricarballic, and sulfuric acid can rapidly neutralize bicarbonates to an acceptable concentration and that re-application may be required as soon as five days later for neutralization. Alternatively, the effects of both citric acid sources are not sufficient for suitable bicarbonate adjustments, thus indicating that application rates should likely exceed those evaluated in this study. Lastly, results from aerated treatments indicate that oxygen supplementation could produce a long-term reduction in bicarbonate concentration that is also complemented by an increase in nitrate-nitrogen concentration and seedling vigor.

## AP 08

### Growth and nutrient uptake of Burley tobacco fertilized with alternative sources of potassium

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Burley tobacco has a relatively high requirement for potassium (K) with a typical crop removing over 250 kg  $\text{K}_2\text{O}/\text{ha}$ . High levels of chloride (Cl) in the cured leaf can have a negative impact on cured leaf quality, limiting the amount of muriate of potash (MOP) that can be applied to tobacco fields. Tobacco growers in the United States must rely on more expensive sources of K such as sulfate of potash (SOP) or sulfate of potash magnesia (SOPM). Poly 4 is an evaporite mineral currently being evaluated for its potential as a



fertilizer source of K (14 % K<sub>2</sub>O), sulfur (19 % S), calcium (12.1 % Ca) and magnesium (3.6 % Mg). With a relatively low Cl content Poly-4 may be a suitable source of K for Burley tobacco crops. A field study was established in 2017 near Lexington, Kentucky, on a McAfee silt loam soil (fine, mixed, active, mesic Mollic Hapludalfs). Initial soil testing at the site indicated a low level of plant available K and resulted in a recommendation of 336 kg K<sub>2</sub>O /ha for Burley tobacco production. Burley tobacco was grown with different sources (SOP, MOP, SOPM, and Poly-4) of K fertilizer applied at the recommended rate. Cured leaf yields and leaf K levels were both increased by all fertilizer sources relative to the unfertilized check. Fertilization with MOP as the sole source of K resulted in leaf chloride levels that exceeded 1 %. Fertilization with Poly-4 resulted in slightly elevated Cl levels compared to SOP only, but chloride levels remained below 1 % in the leaf at harvest. Poly-4 appeared to be agronomically suitable for Burley tobacco production.

## AP 09

### **Chloride application: effects to nutrient assimilation, agronomic performance, and cured leaf chemistry of flue-cured tobacco**

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Chloride (Cl<sup>-</sup>) application in excess of 34 kg ha<sup>-1</sup> has generally been discouraged in the production of U.S. flue-cured tobacco, due to the negative effects the nutrient can have on cured leaf yield, quality, and smoke sensory. However, fertilizer blending errors or misapplication sometimes result in the exposure of flue-cured tobacco to sources of potassium that contain a high Cl<sup>-</sup> concentration, resulting in the possibility of excess Cl<sup>-</sup> absorption. Research was conducted in 2016 and 2017 to quantify the effects of Cl<sup>-</sup> application to nutrient assimilation, cured leaf yield, quality, value, and chemistry. Chloride application rates ranged from 0 to 112 kg ha<sup>-1</sup>, increasing in intervals of 11 kg ha<sup>-1</sup>, which were sidedress applied 10 days after transplanting. Potassium chloride (0-0-60-47 % Cl<sup>-</sup>) was the source of Cl<sup>-</sup> and was blended with potassium sulfate (0-0-50) and calcium sulfate (18 % SO<sub>4</sub><sup>2-</sup>) to ensure that K<sub>2</sub>O and SO<sub>4</sub><sup>2-</sup> application rates were consistent among treatments (168 and 60 kg ha<sup>-1</sup>, respectively). Liquid urea-ammonium-nitrate (28 % N) was utilized as the source of N fertilizer and was split-applied 10 days after transplanting and at layby. Cured leaf Cl<sup>-</sup> concentration was in excess of one percent as application rates increased from 34 to 112 kg Cl<sup>-</sup> ha<sup>-1</sup>, thus demonstrating strong concern with applications beyond the recommended maximum. Despite relatively high Cl<sup>-</sup> concentration, traditional toxicity symptoms and stunting were not observed nor were cured leaf yield and value negatively affected. Cured leaf quality was reduced as application rate increased beyond 67 kg Cl<sup>-</sup> ha<sup>-1</sup>. In addition, reducing sugar concentration increased with Cl<sup>-</sup> application, although total alkaloid concentration was not affected. Flue-cured tobacco producers should apply no more than 22 to 34 kg Cl<sup>-</sup> ha to ensure acceptable leaf quality and usability by leaf dealers and manufacturers, as smoke quality issues are likely to result from excess leaf Cl<sup>-</sup>.

## AP 10

### Testing performance of tobacco varieties in water stress conditions

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Increasing climate instability could lead to local agricultural constraints, presenting a risk for volumes and quality of tobacco supply. Selecting and breeding tobacco varieties for the ability to perform in unfavourable water conditions becomes a pressing need. Today, in addition to the compilation of crop performance over multiple years in relation to climatic data, there is a need for tools to select the best drought-accommodating germplasm in a consistent, reliable manner. We aimed at identifying and validating a tool for reliable tobacco performance testing under defined water stress conditions, and we scored a panel of eight commercial Virginia varieties for drought tolerance. Three different approaches were tested: 1) tailored classical greenhouse conditions, 2) PhenoFab digital phenotyping (Keygene, Wageningen, NL), 3) Rainout system (LandLab, Quinto Vicentino, IT). The water regime was set to mimic drought episodes that can occur in specific areas. Depending on the system, different crop parameters were monitored, such as growth dynamics, final yield, and quality. The metrics and the absolute differences vary from one approach to the other. For instance, the PVH2254 variety displayed the highest Water Use Efficiency index in digital phenotyping, and the PVH2299 variety produced 11 % more cured biomass compared to the K326 variety in Rainout experiments. Interestingly, the ranking for drought tolerance in the different varieties within the different experiments is consistent. For instance, K346 was identified as the most sensitive to water stress conditions, and some tested hybrids showed higher drought tolerance than K326. As result of the study, we show that it is possible to obtain promising performance trends in the different selected test systems. Moving forward, these tools can provide information allowing a reasoned management of the environmental factors.

## AP 11

### Breeding of an extremely low nicotine Burley tobacco line and its characteristics

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Worldwide, regulation on tobacco and cigarettes is increasing more and more. In addition, the U.S. Food and Drug Administration (FDA) has recently announced a plan for regulation of nicotine content in tobacco. In this situation, not only flue-cured varieties, the major raw material, but also Burley varieties, the supplementary raw material, have been required to lower nicotine content.

Effective methods for reducing nicotine in the cigarette manufacturing process have been studied. However, the nicotine content in tobacco is affected by genetics, environmental conditions and cultural practices. Cured leaves with less than 0.5 % nicotine content have a poor smoking taste. But, due to the recent tightening of nicotine regulation, it is necessary to breed a new variety with less unfavourable substances. The objective of this study was to breed a variety of Burley tobacco which had low nicotine content, was resistant to potato virus Y (PVY) and black shank. The new Burley tobacco line, BLN-5, was developed by crossing a low alkaloid line and a KT&G breeding line KB110 with PVY resistance. The KRATF's drop test method for rapid estimation of alkaloid content and isatin coloration method for identifying low nicotine converters of tobacco were used in the selection procedures, respectively. The agronomic traits of BLN-5 line were very similar to those of KB108 as standard cultivar. It showed vigorous growth in the field and was almost the same as KB108 in days to flowering. The yield of cured leaf was approximately 1-2 % less than that of KB108 but its other agronomic characteristics were very similar to those of KB108. BLN-5 line was also resistant to PVY and black shank. In the performance test, the nicotine content of BLN-5 was approximately 86 % lower than that of KB108. Also, it had wider leaves than KB108 and it was almost the same as KB108 in days to flowering.

## AP 12

### Identification of tobacco ANGUSTIFOLIA (AN) gene promoter activity

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Narrower and thicker upper leaves of tobacco (*Nicotiana tabacum*) are the primary cause of its lower usability in southwest China. The ANGUSTIFOLIA (AN) gene controls the polar elongation of leaf cells in leaf width direction and the trichome-branching pattern in the plant. The tobacco K326 AN gene (*NtAN*) originated from diploid species *N. tomentosiformis* (*NtAN-T*) cloned in our previous study. The promoter activity of the *NtAN-T* gene was studied in this report. Firstly, the possible transcriptional regulation sites of the *NtAN-T* gene promoter sequence, which we had cloned 2779bp in our previous experiment, were analysed by bioinformatics analysis. Many light responsive regulatory sites (such as AE-box, ATCT-motif, Box 4, Box I, G-Box, GAG-motif, Gap-box, I-box and TCCC-motif), hormonal response regulatory site (such as TGA-element) and environmental stress reactions sites (such as TCA-element, ABRE, TC-rich repeats and CGTCA-motif) were found in this promoter sequence. Secondly, the full-length sequence of the *NtAN-T* gene promoter was cloned into the plant expression vector pCAMBIA 1301, the recombinant expression vector was then transferred into tobacco K326 sterile seedling leaf, and positive transgenic plantlets were detected; twelve positive transgenic plantlets were confirmed. These study results will provide the experimental basis for further study of the function of this promoter and for the control of *NtAN-T* gene expression.

## AP 14

### **RNA-Seq analysis of Orobanche resistance in tobacco: development of molecular markers for breeding recessive resistance from Wika tobacco variety**

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*Orobanche* spp. (broomrape) is an obligate root parasite that can attack a wide spectrum of plants, including tobacco. It is responsible for economic losses in Europe since 2002 and its incidence in many tobacco growing countries is increasing. Preventive and curative methods exist, including the use of agrochemicals, however dissemination is important due to the high multiplication rate of the parasite and very small seeds.

The tobacco variety Wika shows lower or later germination of broomrape seeds. This seems to be conditioned by a single recessive gene. Artificial testing in Petri dishes was developed to evaluate the ability of tobacco plantlets to stimulate seed germination. Different lines derived from Wika, with susceptible control lines, were tested and studied by RNA-Seq. Candidate markers including SNPs or genes differentially expressed between susceptible and resistant lines were identified. A F2 population segregating for Wika recessive tolerance was then used for validation and mapping. All the candidates mapped on chromosome 14 of the tobacco genetic map. The *Nicotiana* collection of varieties from Imperial Tobacco was also tested with these markers, highlighting or confirming other potential donors.

KASP™ genotyping or markers for conventional gel electrophoresis are now available to pilot the transfer of Wika recessive tolerance into elite lines.

RNA-Seq technology combined with good experimental testing has proven again its high efficiency to identify useful markers for tobacco breeding.

## AP 15

### **Chemical changes in ultra-low nicotine tobacco produced by grafting with *Solanum melongena* L.**

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Ultra-low nicotine tobacco production has been given broad attention due to the possible regulation of nicotine content in cigarette tobacco. A pot experiment was carried out in Xiangcheng County, Henan Province, in 2017, to study the feasibility of producing very low nicotine tobacco leaves and to investigate the changes in chemical composition of

low nicotine tobacco obtained by grafting with eggplant (eggplant as rootstock, flue-cured variety Yunyan 87 as scion). Three treatments were set up as follows: (1) both scion and rootstock with flue-cured tobacco as control, represented by “tobacco/tobacco”; (2) tobacco as scion, eggplant as rootstock without soil covering the bottom of the scion, represented by “tobacco/eggplant without soil covering”; (3) tobacco as scion, eggplant as rootstock with soil covering the bottom of scion to stimulate root production, represented by “tobacco/eggplant with soil covering”. The results showed that the tobacco-eggplant grafting did not cause significant morphological, botanical and agronomic changes to the tobacco plant and tobacco leaf. Nicotine level in grafted tobacco leaves decreased dramatically with average nicotine content in fresh tobacco leaves being lowered to 0.06 %, and in flue-cured leaves lowered to 0.8 %, which represented a 94 % and 95 % decrease compared with tobacco/tobacco control. The contents of nornicotine and anabasine were also decreased, although the percentage decrease was less for anabasine, indicating that the anabasine may have a certain degree of biosynthesis capacity in leaves in addition to roots. Anatabine contents were not detectable in both fresh leaves and cured leaves of tobacco/eggplant without soil covering. Covering soil at the bottom of scion stimulated adventitious root emergence and growth which was able to undergo alkaloid biosynthesis, resulting in a significant increase of nicotine content in the grafted tobacco leaves compared with no soil covering grafted tobacco. The content of chlorophyll in tobacco/eggplant tobacco leaves was significantly higher and matured slower than the control plants. The contents of amino acids, the precursors of alkaloids, increased significantly in grafted tobacco, and higher levels of protein, starch, total nitrogen and lower levels of sugar contents were also observed. This study provided technical support for selectively and dramatically reducing and regulating nicotine content in cured tobacco through agricultural approaches.

## **AP 16**

### **Flue-cured tobacco tip leaf yield, quality, value, and color distribution as influenced by cultivar and harvest schedule**

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Changes in consumer preference and export strategy strongly influence the buying practices of tobacco leaf dealers and cigarette manufacturers. In current times, a dark-colored (orange to red) style of flue-cured tobacco is preferred over a brighter style (lemon-yellow). Research was conducted in four environments from 2009 to 2013 to determine the effect of flue-cured tobacco cultivar and tip leaf harvesting schedule to leaf yield, quality, value, and color distribution as designated by USDA grading standards. Two cultivars were evaluated within each environment, K326 and NC196, with tip leaf harvest schedule as follows: 7 days under-ripe (-7), 3 days over-ripe (+3), 13 days over-ripe (+13), 23 days over-ripe (+23), and 33 days over-ripe (+33). Tobacco cultivar did not affect the measured parameters, thus indicating that K326 and NC196 are likely to have similar maturity and ripening patterns when produced under similar growing conditions.

The effect of tip leaf harvest schedule was significant. Cured leaf yield was greatest between -7 and +13 day treatments and declined as harvest was delayed to +23 and +33 days over-ripe due to advanced senescence. Harvesting +3 to +23 days over-ripe reduced the percentage of leaf graded as under-ripe (G, V, KL, KV, and KM) and increased the percentage of ripe to over-ripe grades (KF, F, and K), thus improving cured leaf quality and value. Cured leaf quality and value were reduced in the +33 day treatment due to excess leaf deterioration which sometimes resulted in non-descript (N) grades that have low usability. Results indicate that producers may find it beneficial to delay final harvest by as long as two to three weeks in order to produce the style of over-ripe, full-flavored leaf that is desired by purchasing entities while avoiding the significant yield and value losses that are often associated with over-ripe leaf characteristics.

## **AP 18**

### **A non-destructive rapid method for blend grade verification using visible-near infrared hyperspectral imaging, advanced data processing and classification algorithms**

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The main objective of this study was to investigate the potential of hyperspectral imaging as a non-destructive, rapid, quality control method for grading cured tobacco bales. Cultivated tobacco plants were harvested and cured. Cured tobacco bales were brought to the stemmery and mixed into blend grades. Blend grades were then graded traditionally based on visual, physical and sensory characteristics. Hyperspectral images of cured tobacco bales were acquired using a visible near-infrared (VNIR) hyperspectral pushbroom imaging system (400-1000 nm). Multivariate calibration models were built using end-member extraction and linear discriminant analysis (LDA). The LDA model using Mahalanobis distance metric showed clear discrimination between the different tobacco grades. The relative classification accuracy of this method for flue-cured and Burley tobacco grades was 93 % versus the traditional grading method. This study demonstrates that hyperspectral imaging can be used as a reliable, rapid, non-destructive quality control method for grading cured tobacco bales.

## AP 19

### **A review of the innovation based on Proficiency Tests conducted by the CORESTA Agrochemical Analysis Sub-Group and its consequences for daily result interpretation**

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The objectives of the CORESTA Agrochemical Analysis Sub-Group are to perform regular proficiency testing of multi-residue methods for the analysis of Crop Protection Agents (CPA's) in tobacco, to undertake joint experiments to resolve identified issues, and to work on Technical Guidelines and Technical Notes for method development and improvement. Annual studies include more than 20 participations from tobacco industry and independent commercial laboratories, authorities and universities. Information about these studies and the consequences deserve to get a wider recognition by the industry. Results from Proficiency Tests (PTs) conducted since 2005 using the FAPAS testing scheme will be reviewed and then assessed.

Calculating the weighted average of squared z-scores for all studies over time will be used to assess long term laboratory performance (in general and per lab). Additionally, statistical evaluation of variability of analytical results will demonstrate the consequences of lab performance when comparing results to CORESTA's Guidance Residue Levels (GRLs).

The results indicate that continuous ring trials improve the quality of participating laboratories significantly over time. Big differences in improvement were found between regular and occasional participants. However, when calculating the weighted average of squared z-scores for all studies, a significant decrease is observed over years indicating overall significant progress in quality.

The best performing laboratories show average weighted squared Z-score above 2, where other laboratories can have scores above 5. The consequences of these values for the interpretation of results will be demonstrated. The variability of the sampling will be compared with the laboratory variability.

The joint experiments, scientific dialogue and exchange of available knowledge result in continuous innovation and more reliable analyses of CPAs. The quality of the laboratory in the PTs has an important impact on the evaluation of the samples in view of CORESTA GRLs.

## AP 20

### Impact of reduced drift spray nozzles on herbicide deposition and efficacy

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The use of the growth regulator herbicide dicamba has significantly increased over the past two growing seasons with the introduction of dicamba-resistant soybean. The use of dicamba post-emergence in soybean has shown to be vital in controlling resistant broadleaves such as Palmer amaranth (*Amaranthus Palmeri*) and waterhemp (*Amaranthus rudis*). In conjunction with the increased use of dicamba for post-emergence applications there have been increased cases of off-site movement of dicamba onto non-target high input and sensitive crops such as soybean, tobacco, and grapes. In an effort to reduce off-site movement of dicamba, the use of pre-orifice and air-induction spray nozzles that produce extremely coarse to ultra-coarse droplets are required with the newly released dicamba formulations. The increase in droplet size in herbicide applications can potentially lead to decreased deposition and decreased herbicide efficacy. Research was conducted at Purdue University and the University of Kentucky to evaluate the deposition of dicamba herbicides onto target weeds using pre-orifice and air induction nozzles. A fluorescent tracer dye was used to track deposition onto target plants in conjunction with spray cards to determine droplet deposition density and volume. In all research droplet deposition density was reduced with the pre-orifice and air induction nozzles on the spray cards as compared to a single stage or non-air-induction nozzle. Although, under ideal conditions of low weed densities and plants less than 15 cm in height there was no reduction herbicide deposition onto target plants or reduction in efficacy. In treatments with less than ideal conditions of high weed densities and exceedingly tall target plants there was a reduction in herbicide effectiveness. Results from this work further reinforce the utility of two stage and air induction nozzles in reducing off-target movement of dicamba to sensitive crops such as tobacco while maintaining herbicide effectiveness when used in proper field conditions.

## AP 21

### Quantifying dicamba residue in contaminated sprayers

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The deregulation of dicamba-tolerant cotton and soybean has increased the potential for dicamba exposure to tobacco through drift or tank contamination. Improper cleaning of sprayer equipment and off-target dicamba exposure can have detrimental consequences to the crop and future marketing opportunities. The objectives of this study are to document the contamination potential of dicamba residues in a spray tank after using a



standard rinsing procedure and to evaluate response of flue-cured tobacco when treated with contamination rates.

Two and a half gallon polyethylene vessels, similar to a commercial spray tank, were used to simulate various tank cleaning scenarios. A “triple rinse” cleanout method was used as a standard cleaning procedure. Water only, a commercial cleaner, and ammonia were evaluated, along with a no cleanout treatment. Each formulation was replicated three times across two separate runs.

Spray vessels were contaminated with 1X rate of dicamba; simulating a use rate to that applied to dicamba-tolerant soybean or cotton. The tanks then underwent a triple rinse cleanout, adding the cleaner on the second rinse cycle. Rinse volumes were 10 % of the 1X mix size (2 gallon). A 20 mL sample of each rinseate was collected from each rinse within each cleaner and analyzed via HPLC to determine herbicide concentrations. Once the triple rinse procedure was completed, the vessel was again filled with water representing follow-up tank use and another sample was collected.

No difference was observed when a three-rinse method was used; regardless of cleaning agent (water, tank cleaner, and ammonia). Measurable differences were observed across the number of rinses. Recovered amounts after triple rinsing procedure could still cause visual injury and yield reduction.

## **AP 22**

### **Field screenings of S-metolachlor for weed suppression in flue-cured tobacco**

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With limited herbicide options and a growing concern to herbicide-resistant weeds, there is a strong need for additional chemical weed control materials in tobacco. S-metolachlor is labeled for use in a variety of agronomic and horticultural crops in the United States, and for use in tobacco internationally. S-metolachlor has been shown to be effective in providing residual weed control of common weed species in tobacco production, such as yellow nutsedge (*Cyperus esculentus*), Palmer amaranth (*Amaranthus palmeri*), and various annual grasses. The objectives of this research are to, i) evaluate tobacco tolerance to S-metolachlor at different rates (1,069 g ai/ha and 2,138 g ai/ha) and application methods (pre-transplant incorporated (PTI) and pre-transplant (PRE-T), ii) evaluate crop and weed response to herbicide programs currently recommended by Cooperative Extension, and iii) to generate efficacy and pesticide residue data that will support a federal label for U.S. tobacco production. Visual estimates of percent weed control and crop injury were recorded at two, six, and nine weeks after transplanting. Cured leaf yield, quality, and value were also documented. At one location, severe stunting and plant death was observed with PTI applications, regardless of application rate (50-75 % of non-treated check). Stunting was minimal (<10 %) in all PRE-T treatments across all locations. Treatments comprised of S-metolachlor and other herbicides resulted in better weed suppression than treatments with S-metolachlor alone. Preliminary results indicate that S-metolachlor may be a suitable candidate for use in tobacco production when applied PRE-T or post-directed after transplanting.

## AP 23

### Engineering tobacco plants to degrade the potential NNK precursor pseudooxynicotine

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Tobacco-specific nitrosamines (TSNAs) form when nitrous oxide species react with tobacco alkaloids. Although most published literature simplistically credits nicotine as being the alkaloid precursor for the potent TSNA NNK [4-(methyl nitrosoamino)-1-(3-pyridyl)-1-butanone], the protective methyl group on the pyrrolidine ring of nicotine makes this compound a poor substrate for nitrosation. Instead, it is likely that an oxidized derivative(s) of nicotine, rather than nicotine itself, serves as the direct alkaloid precursor to NNK. Of the several known oxidized nicotine derivatives that have been characterized, pseudooxynicotine (PON) is arguably the best candidate due to its structural similarity to NNK. To explore the role of PON as an intermediate in NNK biosynthesis, we expressed the PON-degrading enzyme pseudooxynicotine amine oxidase (PAO) from *Pseudomonas* strain HZN6 in transgenic flue-cured (K326) and Burley (TN90) tobaccos. Proper function of the microbial PAO enzyme in tobacco was demonstrated by observing the degradation of deuterated PON (d<sub>3</sub>-PON) that was supplied through the transpirational stream using a detached leaf assay. Under field conditions, transgenic tobacco plants expressing the PAO gene accumulated significantly less PON at harvest than the non-transgenic controls. PAO-mediated reductions in PON were also associated with reductions in NNK in the cured leaf, particularly in the flue-cured K326 background. The results from this study not only reveal new insights into the role of PON as a precursor for NNK formation, but also represent a new strategy for reducing NNK levels, especially in flue-cured tobaccos.

## AP 24

### Downregulation of a putative nitrate transporter gene substantially reduces the accumulation of TSNAs in air-cured tobaccos

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Air-cured Burley tobacco leaves typically exhibit high levels of nicotine-derived nitrosamine ketone (NNK) and N'-nitrososornicotine (NNN). It has been previously demonstrated that leaf nitrate accumulation in air-cured tobaccos serves as the source of nitrosating agents that contributes toward the production of both NNK and NNN. Within this context, the reduction of leaf nitrate stores might represent a viable strategy for efficiently reducing NNK and NNN in tobacco products. In *Arabidopsis*, several genes encoding nitrate transporters have been identified, including *AtCLCa*, whose tonoplast-

localized gene product mediates sequestration of nitrate into the vacuole. The best *AtCLCa* orthologue gene candidate in *Nicotiana tabacum* was named *CLC-Nt2* and is present as two copies within the genome, with one originating from *N. sylvestris* (*CLC-Nt2-S*) and the other from *N. tomentosiformis* (*CLC-Nt2-T*). For proof of concept, we generated anti-*CLC-Nt2* constructs to silence both gene copies in transgenic plants also lacking the functional nicotine demethylase genes, *CYP82E4* and *CYP82E5*. *CLC-Nt2*-RNAi plants and control plants were grown in the field. Interestingly, downregulation of *CLC-Nt2* reduced nitrate storage in cured Burley leaves by 60-70 % without impacting yield. Consequently, NNK was reduced by around 40 % and NNN by 40-50 % in the leaf lamina. Smoke analysis using prototype cigarettes manufactured using the low-nitrate leaves demonstrated a 35 % reduction in NNK and a 47 % reduction in NNN. Our data confirm that decreasing nitrate levels in air-cured tobacco leaves contributes to decreased NNK and NNN in both lamina and smoke by impacting the nitrosation process. Inducing, selecting, and pyramiding knockout mutations within *CLC-Nt2* genes likely represents an efficacious means of generating non-transgenic, reduced-tobacco-specific nitrosamine Burley varieties. Finally, maximal reductions in NNN content would be predicted by combining knockout *CLC-Nt2* technologies with the Zyvert trait.

## AP 25

### 2017 stable reduced converter (SRC) dark tobacco crop

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On January 23, 2017, FDA published “Tobacco Product Standard for N-nitrosornicotine Level in Finished Smokeless Tobacco Products” in which they proposed an NNN ceiling of 1.0 ppm (DWB) through the end of product shelf life. During the past several decades substantial efforts have been made by the tobacco industry and academic institutions to reduce NNN levels and its precursor nornicotine in tobacco products. Research on the mechanism of nornicotine formation led to the identification of three tobacco genes (*CYP82E4*, *CYP82E5* and *CYP82E10*) encoding for cytochrome P450 nicotine demethylases that convert nicotine to nornicotine. Through conventional breeding, we developed dark tobacco varieties (Stable Reduced Converter/SRC varieties) containing the three non-functional nicotine demethylase genes. Tobacco varieties containing this new technology, named ZYVERT® technology, were grown in different locations for on-farm research tests for 3 years and showed an averaged NNN reduction of 74 %. In 2017 Altria Client Services contracted with growers in Kentucky and Tennessee for production of about 3.5 million pounds total of dark air-cured and dark fire-cured SRC variety incorporating ZYVERT® Technology. Tobacco bales from the SRC tobacco variety and commercial low converter (LC) varieties were sampled at delivery and analysed for TSNAs and alkaloids. NNN reductions in the SRC crop averaged 53 % and 68 % in dark air-cured and dark fire-cured, respectively, compared to the LC crop.

## AP 26

### Dark air-cured, dark fire-cured and Burley tobacco TSNA levels, yield and quality in response to potassium rate and source

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Field trials were conducted at the University of Kentucky Research and Education Center in Princeton, Kentucky, in 2016, using KTD14LC in both dark air-cured (DAC) and dark fire-cured (DFC) trials. The trial was repeated in 2017 at Princeton KY, Lexington KY, and Murray KY and included additional varieties of NL MadoleHC in the dark trials, and TN90LC, and TN90HC in the Burley trials. Potassium chloride (KCl) and potassium sulfate ( $K_2SO_4$ ) were used as potassium sources. Treatments were arranged in a randomized complete block design with four replications, including an untreated check. Potassium sources were broadcast applied prior to transplanting at 112, 224, and 336 kg  $K_2O\ ha^{-1}$ , respectively. In 2016, the DAC trial had significantly lower TSNA levels than the DFC trial. In the DAC trial, KCl showed to have significantly lower TSNA levels than those of  $K_2SO_4$  however, KCl had a significantly higher yield than  $K_2SO_4$ . In 2017, both potassium sources yielded higher than the untreated check. KTD14LC had higher yields than the NL MadoleHC. In three of the four dark trials, KCl showed a higher-grade index (GI). In the DFC Princeton trial, GI increased as potassium levels increased. In the Burley trial there was a rate response, the untreated check yielded less than the other treatments except for the KCl treatment at 112 kg  $ha^{-1}$ . All three locations showed that KCl treatments had lower TSNA compared to  $K_2SO_4$ . In 2017 at Princeton, KCl had a 42 % reduction of TSNA in the DAC trial, and a 32 % reduction in the DFC trial as compared to  $K_2SO_4$ . At Murray, KCl treatments had a reduction of 7 % in TSNA in the DAC trial, and a 24 % reduction in the DFC trial compared to  $K_2SO_4$ . The Burley trial KCl treatments had a 31 % reduction in TSNA compared to  $K_2SO_4$  treatments.

## AP 27

### The research and application of systematic technologies for reducing tobacco specific nitrosamines in tobacco and cigarette smoke

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Reduction of tobacco specific nitrosamines (TSNAs) in tobacco and cigarette smoke is a research hotspot. The systematic technologies for reducing TSNAs in both agricultural and industrial areas were developed and applied. The TSNA levels for the upper and middle leaves of Burley tobacco could be reduced by 23.0 % and 23.9 %, respectively, by the foliage spray of purslane extract during tobacco cultivation. Through breeding improvement of Maryland tobacco Wufeng 1# to reduce nicotine conversion rate, TSNA levels for the upper and middle leaves of Maryland tobacco could be reduced by 66.3 % and 70.1 %, respectively. By spraying 4.5 % (W/W) of purslane extract and 0.4 (W/W) nanometer silica dispersion liquid during threshing and redrying, the TSNA level of tobacco could be reduced by 36.0 % and 20.5 %, respectively, compared with that of the control. During tobacco storage, the TSNA level of tobacco using vacuum packaging could be 45.98 % lower than using ordinary packaging and the TSNA level of Burley and Maryland tobacco stored at 20 °C could be 49.5 % and 49.6 % lower than that of the tobacco stored at ambient temperature. The level of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in the cigarette smoke could be selectively reduced by 34.6 % when 5 % (W/W) of cytochrome P450 recombinase was added to Burley tobacco during processing. Using 10 % of reconstituted tobacco processed with 10 % (W/W) of nanometer silica and 4 % of cytochrome P450 recombinase could result in a 20.0 % decrease of TSNAs in cigarette smoke. The TSNA level in cigarette smoke could be reduced by 24.1 % by using complex cigarette filter containing 0.6 mg of modified nanometer silica and 16.8 mg of macroporous silica gel instead of cellulose acetate filter with the similar parameters. All the above TSNA reduction technologies combined and applied in cigarette manufacture could approach a selective reduction of 58 % of TSNAs in cigarette smoke and a 45 % decrease of the cigarette hazard index.

## AP 28

### Effect of vacuum packaging on the formation of TSNAs in tobacco leaves during storage

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Experiments were carried out to further clarify the effect of NO<sub>x</sub> on the formation of TSNAs during tobacco storage. Vacuum treatment was used to change the packing method of tobacco leaves, after which the changes of TSNAs after high temperature treatment and one year of natural storage were studied. Results showed that the TSNA content increased after leaves were treated at 45 °C for 15 days. Compared with the non-vacuum treatment, the increment of four individual and total TSNAs in Burley/flue-cured tobacco leaves and midrib after vacuum packaging were significantly decreased. The TSNA content of Burley tobacco midrib decreased the most, reaching 6.79 µg/g, and the TSNA content of flue-cured tobacco leaves and midrib decreased by 36.2 % and 58.7 %, respectively. After one year natural storage of Burley tobacco with different packing methods, it was found that the increment of TSNAs in the vacuum treated tobacco was least, only increasing by 31 % compared with pre-storage, whereas the TSNA content in samples packaged with plastic bag and newspaper increased by 142.8 % and 140.2 %, respectively. Meanwhile, due to the samples being effectively isolated from the air, the neutral aroma components were significantly higher than samples in other treatments (P<0.05), reaching 632.9 µg/g. Therefore, controlling the storage environment and scavenging NO<sub>x</sub> could be crucial to reduce or inhibit TSNA formation during leaf storage.

## AP 29

### Changes in content of TSNAs and other compounds of different tobacco types during 6-year long-term storage

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Samples of flue-cured, Burley and sun-cured tobaccos from different production areas were collected to investigate the changes in content of tobacco specific nitrosamines (TSNAs) by the SPE-LC-MS/MS method during 6-year long term storage under natural

conditions. Alkaloids and nitrate content and other compounds were also measured. Research showed that NNN, NNK, NAB, NAT and total TSNA content of all tested tobacco samples increased continuously throughout the whole storage period following the quadratic curve model. TSNA content of Burley tobacco was the highest and increased most significantly followed by sun-cured tobacco. Alkaloid and nitrate content decreased during storage. The nicotine content of sun-cured tobacco and the nor nicotine content of Burley tobacco were the highest, and decreased the most, respectively. Nitrate content of different tobacco types varied greatly within the tobacco types, with Burley tobacco being 109.7 times higher than that of flue-cured tobacco. Volatile ketones, the important aromatic compounds, changed differently with tobacco types and compounds, although all could be fitted into a quadratic curve model. Total amino acid and aspartic acid content in Burley tobacco were the highest, followed by sun-cured tobacco. Flue-cured tobacco had the lowest total amino acid content, although the proline content was the most abundant.

## **AP 30**

### **Identification of sucker control genes**

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In tobacco, depending on strong shoot apical dominance, suckers grow vigorously after topping. A sucker can develop sequentially at least three times at each axil during the cultivation period. Suckers negatively affect tobacco yield and quality. Therefore, sucker control is a key process to achieve high yields and good quality in leaf tobacco production. Many genes that control axillary meristem have been reported in many plants. Furthermore, some orthologues of those genes have been isolated in tobacco. However, the function of those genes has not been well identified in tobacco. To gain sucker control genes, we first performed screening of candidate genes that might regulate sucker growth. We selected 10 genes via BLAST analysis and 24 genes via tissue-specific gene expression profiles. Then, transgenic plants with reduced expression of total 34 genes by RNAi-mediated gene silencing were evaluated for the effects of sucker growth after topping. Among the 34 candidate genes, seven genes were shown to be involved in sucker growth. Particularly, five of the seven genes were shown to be involved in the second sucker growth. RNAi-transgenic tobaccos of these five genes showed normal growth of the first sucker, but the second sucker was suppressed. These data indicate that these genes are useful to develop low sucker tobacco varieties.

## AP 31

### Genetic strategy for reducing sucker pressure in tobacco

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Tobacco plants exhibit strong apical dominance because molecular signals from the shoot apical meristem (SAM) mediate hormonal regulation effectively inhibiting axillary bud growth. Upon SAM removal, however, hormonal signals are lost enabling axillary buds to grow into new shoots (or “suckers”). In traditional tobacco production practices, manual removal or chemicals are used for sucker management. Chemical use not only increases leaf production costs but can also leave undesired residues on cured leaf. Therefore, development of tobacco varieties with reduced or inhibited sucker pressure would have a positive impact on tobacco production.

Expression of a cell death gene under control of axillary bud specific promoters resulted in reduction of sucker growth. However, due to promoter leakage, next generation viable seed was not obtained. To achieve viable seed production, promoter analyses were conducted. Upon promoter modification and improvement, sucker control plants produced viable seed and the phenotype was successfully passed to the next generation. In this presentation, we will discuss promoter modifications and T1 plant performance in both greenhouse and field.

## AP 32

### Genome-wide identification and functional characterization of senescence-associated genes of the NAC transcription factor family in tobacco

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The NAC family is one of the largest families of plant-specific transcription factors. NAC proteins play important regulatory roles in a variety of developmental and stress response processes in plants. Members of the NAC family transcription factors have been shown to be important regulators of leaf senescence in a number of plant species. Here, we report the identification of the NAC family in tobacco (*Nicotiana tabacum*) and the characterization of the potential roles of some of the tobacco NAC transcription factors in regulating leaf senescence. A total of 154 NAC genes (*NtNACs*) were identified and clustered together with the *Arabidopsis* NAC family into fifteen groups (a-o). Transcriptome data analysis followed qRT-PCR validation and showed that the majority of the senescence-upregulated *NtNAC* genes fall into subgroups b and f. A number of known senescence regulators from *Arabidopsis* also belong to these two subgroups. Among these senescence-upregulated *NtNACs*, *NtNAC080*, a close homolog of *AtNAP*, is



a master regulator of leaf senescence in *Arabidopsis*. Overexpression of *NtNAC080* caused early senescence in *Arabidopsis* leaves and *NtNAC080* mutation induced by Cas9/gRNA in tobacco led to delayed senescence.

## **AP 33**

### **Comparative research of gene expression profiles on exposure to high and low level nitrate signals**

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Nitrogen is one of the important nutrients for plants. Nitrate is also one of the important signal molecules for plant growth. In order to elucidate how the tobacco root responds to outside nitrate-nitrogen signals, a transcriptome sequencing technique was used to study the profiles of gene expression in tobacco roots at 0 h, 6 h, 12 h, and 24 h after exposure to high and low nitrate treatments. KEGG pathway analysis indicated that the gene expression profiles in tobacco roots 6 h after being exposed to high and low nitrogen nutrients were pivotal and included many common as well as different expressed genes. Some genes in the roots were simultaneously up-regulated after the 6 h treatment at high and low levels of nitrogen nutrition and then decreased in both groups. These genes were mainly involved in phenylpropanoid biosynthesis, alanine, aspartate and glutamate metabolism pathways. Some important genes such as glutamate dehydrogenase, glutamic acid decarboxylase and asparagine synthetase which were involved in the carbon and nitrogen metabolism were up-regulated. Results also indicated that amino acids and carbon/nitrogen metabolism pathways were vital metabolic pathways that responded to outside nitrogen nutrition and may be the common signal pathways in root physiological activities no matter what the nitrate level. However, some genes involved in tropane, piperidine and pyridine alkaloid biosynthesis, pyrimidine metabolism, purine metabolism, fructose and mannose metabolism, and starch and sucrose metabolism pathways were down-regulated at low-nitrogen levels and were up-regulated when nitrogen levels were high. Results suggested that low levels of nitrate nitrogen could lower the alkaloid synthesis and energy metabolism in the root of flue-cured tobacco while high levels of nitrate nitrogen nutrition could promote these metabolic pathways.

## **AP 34**

### **An alternative strategy of risk reduction for tobacco smokers**

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Over the past decade, regulatory bodies such as the World Health Organisation (WHO) and the U.S. Food and Drug Administration (FDA) have been working towards regulation to support harm reduction for smokers. It was proposed to introduce ceilings on specific

compounds in tobacco smoke which are believed to be harmful or potentially harmful. Different lists of compounds have been proposed. Unfortunately, many of these compounds are negatively correlated and to reach these ceilings no real solution exists by breeding or by blending.

In recent years, WHO and FDA have worked towards developing regulation to lower nicotine in tobacco products. While WHO proposes to reach the limit of feasibility, the FDA is working on the non-addictive limit. Lowering nicotine content in tobacco products may prove counterproductive with the increase of illicit trade and the composition of products escaping all control.

However, with the development of new genetic tools, another risk reduction strategy can be considered in a comprehensive way. Toxicogenomics and transcriptomics by high-throughput sequencing (RNAseq) enable the assessment of cigarette smoke toxicity on human cells. This type of analysis could be integrated into an association mapping approach in the tobacco plant. It can be expected to identify molecular makers related to global mutagenicity of cigarette smoke. New tobacco cultivars could be developed taking into consideration the reduction of risk for the smoker.

## AP 35

### Development of THA mutant line having markedly increased threonine in cured leaves

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Threonine (Thr), an amino acid, is known to be related to tobacco smoking flavour and aroma. Obtaining Thr-rich cured tobacco leaves is difficult because Thr contents in fresh tobacco leaves are decreased drastically during curing processes. To increase Thr contents in plants, the modification of aspartate kinase (AK), the key enzyme of Thr biosynthesis, is a major approach, but growth inhibition occurs in the AK modified mutant. As another approach, we specifically emphasized the inactivation of threonine aldorase (THA), which is a key enzyme regulating Thr degradation. It is known for its high expression during the senescence stage rather than the growth stage.

This study investigated how the knockout mutation of the THA gene affects Thr accumulation in cured tobacco leaves and tobacco growth. First, each mutant tobacco plant having nonsense mutation of *NtTHA1-S* encoded in the S-genome derived from *N. sylvestris*, and *NtTHA1-T* encoded in the T-genome derived from *N. tomentosiformis* was screened from the ethyl ethanesulfonate-mutagenized tobacco library (var. Tsukuba 1: flue-cured tobacco). Subsequently, double mutant lines (sstt) and control lines (SSTT) were obtained by crossing between single mutants. Double mutant lines and control lines were cultivated in the field. Then leaves (leaf position) were harvested 50 days after topping. The Thr content in the harvested fresh leaves showed no clear difference between the double mutant and the control. In contrast, after flue-curing, the Thr content in double mutant leaves was higher than that of the control. Furthermore, agronomic performance of double mutant (sstt) was not different from that of control.

Double mutant line (sstt) backcrossed with Burley (var. TN 90) was also evaluated. The result was identical to that found for the flue-cured tobacco mutant. In conclusion, THA mutation is a good measure to increase Thr levels in cured tobacco leaves without affecting tobacco growth or yield.

## AP 36

### Use of reverse genetic approach to study the role of tobacco trichome-produced cembratriene-diols in pest/insect interaction

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Diterpenes cembratriene-diols (CBT-diols), sucrose esters, labdanoids and hydrocarbons are prominent tobacco trichome exudate constituents. They are considered to be the first line of defense barriers against pests/insects through chemical toxicity, alteration of ovipositional behavior, allergic and irritant responses to herbivores. The particular role of CBT-diols in tobacco pest defense has not been clearly elucidated. Some data indicate their role in budworm oviposition stimulation and larvae toxicity, resistance to aphid colonization, aphid attraction, susceptibility to Japanese and flea beetles, and fungitoxic properties. The goal of this study was to test the impact of the absence of cembratriene-diols (CBT-diols absent due to cyclase knockdown) on the resistance/susceptibility of field-grown plants to infestation by aphids, budworms, and hornworms. Field experiments involved *Nicotiana tabacum* lines TKF 2002LC and DHK 960 and their RNAi knock down counterparts that had no or low levels of CBT-diols. The composition of trichome exudate was verified by GC-MS. The field design was a randomized complete block with 12 replications. No treatment with pesticides, insecticides or fungicides was made in the experimental field. Plants were evaluated for infestation and level of damage during the season, and the data was analyzed statistically. In line TKF 2002, removal of CBT-diols caused significant ( $P < 0.001$ ) prevention of aphid infestation in the entire knockdown population. This result is direct evidence that CBT-diols play an important role in aphid attraction and colonization.

In line DHK 960 the role of CBT-diols in aphid infestation was inconclusive due to a different response during the two-year experiment. Under low hornworm pressure there was a significant reduction ( $p < 0.01$ ) of percent damaged and infested DHK 960 knockdown plants. Results suggest that CBT-diols are favored by hornworms.

## AP 37

### Leaf surface chemicals improvement through molecular regulation of glandular trichome formation of tobacco

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Tobacco (*Nicotiana tabacum*) plant is covered with dense trichomes distinguished in morphology structure and secretion ability. Long-stem glandular trichome is the most important type of tobacco trichome. It could specifically synthesize and secrete diterpene and sucrose esters, composing the main constituents of tobacco leaf chemicals, and contribute greatly to plant resistance and leaf quality. B-type cyclins, which play important roles in the transition of G2-to-M have been reported to participate in reproductive organ development and trichome formation. In this study, tobacco B-type cyclin gene, *NtCycB2*, was cloned and transferred to the cultivated variety K326. Transgene plants analysis showed that *NtCycB2* overexpression caused the obvious decrease of glandular trichome density and abnormal development of lateral roots. On the contrary, knockout of *NtCycB2* through CRISPR-Cas9 technique could promote the formation of glandular trichomes, most of them branched with more than one glandular head. *NtCycB2* gene knockout plants proved to have many advantages, compared to *NtCycB2* gene overexpression plants, with resistance to aphid attack and drought, cold and UVB stress. This suggested their great potential use in tobacco variety improvement. Therefore, several homozygous gRNA knockout mutant strains with expression vector deleted were selected and cultivated in the field and the agronomy and leaf chemistry characteristics were comparatively analyzed. Except for trichome density changes, no other morphological differences were discovered between the *NtCycB2* knockout plants and the wild control. Detection of leaf surface chemical components by GC/MS indicated that the production of diterpene and sucrose ester compounds were significantly increased in *NtCycB2* knockout plants, which are supposed to benefit leaf aroma quality and plant resistance. These results indicate that *NtCycB2* plays a critical role in glandular trichome initiation, leaf surface chemical accumulation and defense in the tobacco plant.

## AP 38

### Multi-omics data revealed *NtWRKY75* involved in trichome development of *Nicotiana tabacum*

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Tobacco leaf is densely covered with various types of trichomes, which play important roles in stress responses and aroma components. In order to detect important regulatory components involved in trichome development, we first tried to identify the variations using resequencing datasets of 254 different cultivars. Then, the potential candidates

were identified by genome wide association analysis. The transcriptome level of these candidates was further checked between two cultivars TI1068 and i35 (TI1068 was high-secretory, whereas i35 was low-secretory). Finally, *NtWRKY75* was regarded as one of the important potential candidates that may regulate trichome development. RNAi result of *NtWRKY75* in K326 indicated that the trichome was much reduced compared with the wild-type. Using the gene regulatory network of tobacco, our study revealed that *NtWRKY75* may regulate trichome development by specific LRR receptor-like kinase.

## AP 39

### Effects of tobacco curing with flower buds in the same bulk-barn on the quality of flue-cured tobacco leaves

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In order to explore new technologies of tobacco curing and flavouring, tobacco leaves were flue-cured with different amounts of fresh tobacco flower buds placed in the curing barn and the results were compared with those of conventional tobacco curing. The economic characteristics, appearance quality, sensory quality, conventional chemical content and aroma content of tobacco leaves after curing were analyzed and compared. The results showed that tobacco flower buds showed less effect on the economic characteristics, appearance quality and the content of conventional chemical composition, but had great influence on sensory quality and the content of aroma substances. Compared with conventional tobacco curing, the ratio of tobacco high grade leaves after curing with flower buds increased significantly by 3.26 percentage points. The score of sensory quality increased significantly by 2.90 points. The aroma and aroma content increased, while the offensive odour and aftertaste decreased. There is a certain improvement in the flavour style of tobacco leaves. The contents of the aroma substances, including beta acetone, two hydrogen gooseberry, solanone, furfural, 5-methyl furfural, beta cyclocitral and 3,4-two methyl-2,5-uran two ketones, linalool, geraniacetone, mega three enenone, and 3-hydroxyl-beta two hydrogen damascone, increased significantly. Meanwhile the contents of these aroma substances in tobacco buds after curing were significantly lower than those before curing. These results suggested that the flower buds added during curing caused the increase of aroma substances in cured tobacco leaves. This will open up a new method for tobacco curing and flavoring.

## AP 40

### Plant metabolism studies with <sup>14</sup>C-maleic hydrazide (MH) in tobacco and evaluation of analytical methods

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Maleic hydrazide (MH) is used as a sucker control agent for tobacco. Reportedly, absorbed MH in tobacco plants is metabolized to its glucoside conjugates and is converted to the bound form. However, how much original MH can be converted to those forms in tobacco plants has not been reported. Regarding analytical methods, present analytical methods including the ISO method and in-house methods exist to determine MH and, allegedly, its metabolites. Nevertheless, it remains uncertain whether all forms can be ascertained, or not, using the respective analytical methods. This study was conducted 1) to clarify the metabolite types and amounts and 2) to evaluate present MH analytical methods. <sup>14</sup>C-labeled MH with inert ingredients was applied to flue-cured tobacco. Tobacco leaves were harvested 28 days after the application. Then, leaves were cured in a small curing chamber. After cured leaves were ground and extracted with MeOH/water, the radioactivity in these extracts/post-extraction solids (PES) was measured using a liquid scintillation counter. The respective radioactive compounds were characterized using HPLC radiochromatography and thin-layer co-chromatography. 1) Based on the results, the percentage of total radioactive residue (%TRR) was calculated for each compound. In the extract with MeOH/water of cured tobacco, MH-O-glucoside (MH-O-Glc) and MH-N-glucoside (MH-N-Glc) were observed as major metabolites with the TRR 13.6 % and 3.3 %, respectively. 2) When <sup>14</sup>C-MH treated tobacco was extracted under present analytical conditions, these conjugates were not observed in each extract. Furthermore, the remaining radioactivity in PES differed under respective analytical methods. These results identify the precise %TRR of major metabolites and suggest that glycoside conjugates could be hydrolyzed or not extracted during the analytical process of the present MH analytical methods. %TRR of the parent compound, metabolites and/or non-extractable fraction should be examined when different analytical methods are applied.

## AP 42

### **Evaluation of cured leaf residues of several common pesticides used in dark air-cured and dark fire-cured tobacco**

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Pesticide residue trials were conducted from 2011 to 2017 to evaluate cured leaf residues of eight commonly used pesticides registered for use on dark tobacco in the United States. Pesticides tested included azoxystrobin, acephate, lambda-cyhalothrin, and maleic hydrazide in 2011-2014, and spinosad, butralin, flumetralin, and sethoxydim in 2015-2017. Each pesticide was applied using a maximum allowable dose philosophy, where maximum rates and number of applications according to product labels were used. Timing of applications were similar to standard timings used by growers. However, final applications were made at the minimum preharvest interval allowed on the product labels. These field experiments were conducted as two adjacent experiments each year. Both trials were transplanted at the same time with the same dark tobacco variety. All applications were made at the same time for both trials, and both trials were stalk-harvested at the same time. The only difference was that one trial was air-cured while the other trial was fire-cured. Following curing, two samples of 0.2 kg of cured leaf were collected from each plot, one sample from the upper stalk area and the other from the lower stalk area. Average residues in air-cured tobacco ranged from 3.22 to 12.97 ppm for azoxystrobin, 1.58 to 4.1 ppm for acephate (methamidophos), 0.49 to 0.92 ppm for lambda-cyhalothrin, 53 to 83 ppm for maleic hydrazide, 0.51 to 5.40 ppm for spinosad, 1.29 to 2.12 ppm for butralin, and 1.32 to 11.77 ppm for flumetralin. Average residues in fire-cured tobacco ranged from 2.2 to 12.95 ppm for azoxystrobin, 0.69 to 1.0 ppm for acephate (methamidophos), 0.46 to 0.78 ppm for lambda-cyhalothrin, 48 to 70 ppm for maleic hydrazide, 1.17 to 6.27 ppm for spinosad, 1.35 to 2.13 ppm for butralin, and 0.89 to 7.73 ppm for flumetralin. No sethoxydim residues were found in any sample.

## AP 43

### **Pyrethroid resistance in the cigarette beetle *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae)**

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Spraying application of contact insecticides onto building surfaces (walls and floors) is a key measure to control the cigarette beetle *Lasioderma serricorne* (F.) population in tobacco warehouses. Pyrethroids have been used widely as effective surface-spray agents since the 1970s. Recently however, resistance to deltamethrin (a popular pyrethroid) was found in a *L. serricorne* population collected in Germany. This study was

conducted to ascertain if the pyrethroid resistance has spread among field populations of *L. serricornis*. Specifically, we examined the efficacy of two pyrethroids (permethrin and bifenthrin) for seven strains that were collected originally from tobacco warehouses at different locations. The treatment was applied by dipping adults for 10 s in the insecticide solution. Their viability was assessed 48 h after the treatment. Then LC<sub>99</sub> was determined. Results show that both insecticides exhibited high efficacy against four of seven strains at practical dose levels (235-384 ppm in LC<sub>99</sub> for permethrin and ≤25 ppm in LC<sub>99</sub> for bifenthrin). By contrast, high resistance was observed in the other three strains: most insects survived exposure even at an over-label dose (10,000 ppm). These results suggest that pyrethroids lose their effectiveness against many *L. serricornis* field populations through resistance development. Therefore, pyrethroids cannot be recommended today as insecticides of primary choice for *L. serricornis*.

Considering the development of pyrethroid resistance, we evaluated three insecticides as potential alternatives (fenitrothion, pirimiphos-methyl, and spinosad) with respectively different modes of action from that of pyrethroids. The efficacy of insecticides to pyrethroid-susceptible and pyrethroid-resistant strains was assessed in the manner described above. Susceptibility of pyrethroid-susceptible and pyrethroid-resistant strains to those insecticides was not significantly different (within 2.3-fold resistance ratio). The results indicate that insecticides such as organophosphates (fenitrothion and pirimiphos-methyl) and spinosad have potential for use as surface-spray agent alternatives to pyrethroids.

## AP 44

### Resistance mechanism of *Phytophthora nicotianae* to dimethomorph

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Tobacco black shank is an important oomycete disease that can cause serious tobacco yield loss in China. As a new fungicide, dimethomorph, has been used more and more widely to replace metalaxyl to control tobacco black shank. As the emergence of fungicide resistance will reduce the control effect and duration of the fungicide, it is of great importance to study the resistance mechanism of *Phytophthora nicotianae* to dimethomorph. In this study, four stable high resistant mutants with a resistance level 250 times greater, were obtained in the laboratory using a dimethomorph-amended media method. No significant difference was observed between the resistant mutants and the sensitive isolates, which indicates that the resistance risk of *P. nicotianae* to dimethomorph is moderate. The comparison of cellulose synthase gene sequences between resistant mutants and sensitive strains suggested that two different base mutations in the *CesA3* gene resulted in Type I high resistant mutant G3325C (V1109L) and Type II high resistant mutant G3231T (Q1077H), respectively. Thus, we speculated that the point mutants of V1109L and Q1077H on *CesA3* were responsible for the



molecular resistance of *P. nicotianae* to dimethomorph. Two AS-PCR methods corresponding to the above point mutations were also established to detect the high resistant mutants. This study will guide the scientific use of dimethomorph and provide a reference for dimethomorph-resistance management strategies.

## AP 45

### **New genetic source for black shank resistance and genomic loci associated with its resistance**

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Black shank causes severe tobacco yield loss in Japan because of root and leaf infection by *Phytophthora nicotianae*, the causal agent of this disease. Several cultivated varieties in Japan have partial resistance derived from Florida 301, which is known as the most popular resistance source for black shank, but these varieties do not exhibit adequate resistance. This study was conducted to find new genetic sources with high resistance to black shank and to identify the genomic loci associated with its resistance. We first evaluated the levels of black shank resistance of several varieties. As a result of root and leaf inoculation tests, a tobacco variety Black Shank Resistant (BSR) exhibited high levels of stable resistance. Further experiments were therefore conducted using BSR. A doubled haploid (DH) population was generated from a cross between BSR and Bright Yellow 4 (susceptible to black shank). Then a linkage map was constructed from genotyping data by next generation sequencer. Subsequently, resistant and susceptible DH lines were selected by inoculation tests. A few genomic loci were determined from associations between genotype and phenotype in the resistant and susceptible DH lines. We excluded the loci that seemed to be already introgressed into the several commercial varieties from Florida 301 and investigated the effects of the remaining loci on black shank resistance. An F<sub>3</sub> population from a cross between a resistant DH line and a breeding line was generated. The F<sub>3</sub> individuals that possessed the loci exhibited high resistance to black shank. Our results suggest that the genomic loci we found in BSR can improve black shank resistance of the current commercial varieties.

## AP 46

### **New strategies for early detection of blue mold in asymptomatic tobacco: a mixed fluorescence and LAMP based-approach under preliminary test at Fattoria Autonoma Tabacchi**

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One of the fundamental criteria of integrated pest management (IPM) is that crops should be treated only when monitored pest and disease exceed their critical thresholds. Also, zero-residue tobacco represents not only a target of social responsibility, but also a sustainability issue for tobacco growers, in a context of increasing attention to toxicology, residue problems, and environmental impact. Recent advancements in genomics identified Loop mediated isothermal AMPLification (LAMP) as a technique that is able to test the presence of a pathogen in plant tissues directly in the field, on asymptomatic plant material, in a short time (30 min), at room temperature, and by non-specialized manpower. A parallel testing technique is represented by non-destructive fluorescence spectroscopy to identify phenolic compounds produced by tobacco in response to a pathogen. The deep blue UV excited autofluorescence can identify the presence of a pathogen before outbreak. To calibrate LAMP and fluorescence pre-symptomatic detection on tobacco for *Peronospora hyoscyami* f.sp. *tabacina* (BM), FAT promoted two research projects in 2017-2018: SM@rt-Meteo, and PhoTO, to identify the presence of BM in greenhouse and field tobacco, and drive crop treatments accordingly. Lab and greenhouse calibration was preliminarily carried out, and ten monitoring stations, including varietal tests, and commercial crops in areas particularly subject to BM, were identified since 2017. Greenhouse artificially inoculated plants were tested with both techniques at various stages after infection with good calibration results. The same was done in the field, also to identify pros and cons of each method in a relevant environment, including costs and time response efficiency. These two predictive tests were challenged on tobacco, to mutually calibrate their results, for the first time. So far, this activity is still at a research stage, but it is a pivotal one to plan a responsible zero-residue and/or organic tobacco.

## AP 47

### Enhanced resistance to potato virus Y (PVY) and resistance-breaking PVY in tobacco *eIF4E-S* and *eIF(iso)4E-T* double mutant

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Eukaryotic translation-initiation factors in plants, *eIF4E* and *eIF(iso)4E*, play key roles in infection by potyviruses and other plant RNA viruses. Mutations in the genes encoding these factors might reduce susceptibility to viruses. They are the basis of several recessive virus-resistance genes that are used widely in plant breeding. In tobacco (*Nicotiana tabacum* L.), deletion mutants of *eIF4E-S* have been used as sources of resistance to Potato virus Y (PVY; the type member of the genus Potyvirus). However, emergence of resistance-breaking strains of PVY (RB-PVY) has been reported worldwide. In an earlier study, we demonstrated that loss-of-function of a tobacco *eIF(iso)4E-T* gene reduces susceptibility to a RB-PVY. Here, we demonstrated that knock out of both *eIF4E-S* and *eIF(iso)4E-T* confer enhanced resistance to both PVY and RB-PVY. By crossing an *eIF(iso)4E* mutant with a variety TN90, which lost *eIF4E-S*, plants without functional *eIF4E-S* and *eIF(iso)4E-T* were obtained. When PVY and RB-PVY were inoculated, TN90 and *eIF(iso)4E-T* mutant respectively showed resistance to PVY and RB-PVY. They showed no necrotic symptoms seven days after the inoculation (DAI), but showed symptoms 14 DAI. However, the *eIF4E-S* and *eIF(iso)4E-T* double mutant showed enhanced resistance to both viruses: necrotic symptoms were not observed, even at 28 DAI. Consequently, the effect of simultaneous lack of functional *eIF4E-S* and *eIF(iso)4E-T* genes on virus resistance was synergistic. The *eIF4E-S* and *eIF(iso)4E-T* double mutant is expected to be useful for breeding of PVY-resistant and RB-PVY-resistant tobacco.

## AP 48

### The *NSm* of tomato spotted wilt virus is an elicitor of *RTSW*-mediated virus resistance in tobacco and its ability in HR induction is dissociated of movement function

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Tomato spotted wilt virus (TSWV, order *Bunyvirales*, family *Tospoviridae* and genus *Orthotospovirus*) is one of the most destructive viral pathogens of plants. Recently, a single dominant gene conferring complete resistance to TSWV (*RTSW*) was found in a wild tobacco, *Nicotiana glauca*, and has been introgressed into cultivar tobacco. However, whether there is a TSWV avirulence (*Avr*) factor against *RTSW* remains obscure. To identify the *Avr* factor corresponding to *RTSW* in TSWV, Agrobacterium-mediated

transient expression of TSWV open reading frames (ORFs) in TSWV-resistant (*RTSW*) and -susceptible (*rtsw*) tobacco plants was conducted. Hypersensitive response (HR)-type cell death was observed only when *NSm* of TSWV was expressed in *RTSW*-bearing tobacco leaves in a genotype-specific manner. To clarify whether the movement function of *NSm* is coupled with its function in HR elicitation, a series of movement-defective mutants were generated. Amino acids (aa) substitution mutagenesis indicated that the *NSm* mutants defective in targeting plasmodesmata and cell-to-cell movement were still capable of inducing *RTSW*-mediated HR. Collectively, our results clearly demonstrated that *RTSW*-mediated resistance is triggered by the TSWV movement protein but is independent of its movement function.

## AP 49

### The influence of *Nicotiana alata*-derived introgression on plant malformations of tobacco breeding lines resistant to tomato spotted wilt virus

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Tomato spotted wilt virus (TSWV) is one of the most destructive viruses for tobacco cultivation. Cultivar “Polalta” carries a resistance gene introgressed from a wild species, *Nicotiana alata*, but the use of this cultivar in breeding is hampered by morphological deformations of hybrids resulting from crosses with other cultivars. Over the last decade, a few breeding lines (e.g. DH3 and DH6) were derived at IUNG from cultivar “Polalta” through androgenesis and selection of TSWV-resistant genotypes. The aim of this study was to characterize genomic location and phenotypic effects of the *N. alata* introgression in these breeding lines. “Polalta”, *N. alata*, and *N. tabacum* were subjected to whole genome sequencing, and comparison of the obtained sequences allowed for locating *N. alata* introgression in cultivar “Polalta” on linkage group 7 in a region between 0-40 cM. Then, *N. alata* and *N. tabacum* sequences from this region were used to design species-specific primers in order to detect the presence of this introgression in DH3 and DH6 lines and F<sub>2</sub> plants derived from crosses between these two lines and a high-quality flue-cured cultivar “WAC 121D7”. The above-mentioned species-specific primers were used to genotype a segregating F<sub>2</sub> population grown in field conditions. Among the 1,543 F<sub>2</sub> plants, 15.3 % were homozygotes with introgression (ALA/ALA), 51.3 % were heterozygotes, and 33.2 % were homozygotes without introgression (“tobacco type”, TOB/TOB). Only three recombinant plants (0.3 %) were detected in the F<sub>2</sub> studied population. Morphological deformations, such as thick, irregular leaf veins and sometimes also abnormally narrow leaves, were observed in approximately half of the ALA/ALA homozygotes and heterozygotes (in 55.1 % and 47.7 % of plants, respectively). In contrast, only 29.5 % of TOB/TOB homozygotes showed such deformations. Therefore, *N. alata* introgression is likely to carry a genetic factor that has a negative impact on morphology of the hybrids.

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**POSTERS**

**APPOST 01**

**Identification of transcriptional factors of nitrate reductase gene promoters and NRE2 *cis*-element in *Nicotiana tabacum***

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This research aimed to identify the transcriptional factors of nitrate reductase gene (*NIA1* and *NIA2*) promoters and hypothetical *cis*-element NRE2 from flue-cured tobacco. Yeast one-hybrid experiments of NR gene (*NIA1* and *NIA2*) promoters and NRE2 *cis*-element were successfully carried and several potential TF genes were screened, such as CRM-domain containing factor CFM3, sulfite oxidase-like protein, pepsin-like aspartic protease domain, and RING finger protein. CRM-domain containing factor CFM3 was identified from the *NIA1* promoter which was annotated as chloroplastic/mitochondrial-like of *Nicotiana*, having homology with predicted protein CFM3 in tobacco. The CRM domain is an RNA-binding domain identified in three group II intron splicing factors in chloroplasts, in a family of uncharacterized proteins in plants. CFM3 is a CRM-domain protein related to chloroplast splicing factor CRS1, which dually functions in chloroplast group II intron splicing and mitochondrial gene expression. Another gene identified from the *NIA1* promoter was annotated as a sulfite oxidase-like protein in *Nicotiana tabacum*. Sulfite oxidase is the smallest eukaryotic molybdenum enzyme that utilizes a molybdopterin cofactor and a heme group. From the *NIA2* promoter one binding protein was found to have a pepsin-like aspartic protease domain. Eukaryotic pepsin-like proteases have two domains with similar topological characteristics: C- and N-terminal domains. However, they have limited sequence homology except for the sequences near the active site, indicating that this enzyme may have evolved from ancient copying activity. The active site motif (Asp-Thr/Ser-Gly-Ser) is conserved between retroviruses and eukaryotic proteases, as well as eukaryotic N- and C-terminal pepsin-like proteins. RING finger protein containing RING finger motifs was identified from 4 tandem NRE2 *cis*-elements. RING finger proteins contain zinc finger domains consisting of one or several Cys and His residues. Zinc fingers can serve as DNA-binding domains to bind DNA, meeting the structural requirements of TFs. In conclusion, the identified TF genes from *NIA1* and *NIA2* promoters and the NRE2 *cis*-element may help us to understand the regulatory pathway of nitrate signal response in tobacco.

## **APPOST 02**

### **Development and application of tobacco SSR markers based on genome re-sequencing of different tobacco types**

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The development of molecular markers based on genome re-sequencing is a new method and trend. In this research three tobacco types, including two flue-cured tobacco cultivars (LY1306 line, Qinyan96), one sun-cured tobacco cultivar (Wanmao 3) as well as one Maryland tobacco cultivar (Wufeng 1), were re-sequenced on genomic nucleotide sequences. Combined with the genomic sequence of cultivars K326 and TN90, which have been sequenced and released in the National Center for Biotechnology Information (NCBI), the insertion and deletion (InDel) sites and single nucleotide polymorphisms (SNP) sites of these four re-sequenced tobacco cultivars were analysed. Seven simple sequence repeats (SSR) candidate alleles were identified from the genomic analysis that were supposed to be polymorphic among these cultivars. Five of them were confirmed to be applicable after amplifying the fragments from the SSR allele sites, respectively. Verification PCR tests carried out on ten tobacco cultivars, including different tobacco types, revealed that they can be used to classify the tobacco cultivars or the tobacco types. Three of the SSR sites, including NW-015889872.1, NW-015854676.1 and NW-015890969.1, were shown to be very effective for the classification of flue-cured, Burley and Maryland tobacco types, as well as sun-cured tobacco types.

## **APPOST 03**

### **Using fPAR as a modelling parameter for tobacco yield estimation in regional cropping areas**

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Remote sensing has shown its usefulness in many applications for tobacco crop evaluation: using aerial or satellite collected data, mathematical models could be developed to investigate the status of a crop or to prognosticate its yield. Remote sensing is especially useful when dealing with extensive growing areas. The aim of the present paper is to evaluate the efficiency of the fraction of absorbed photosynthetically active radiation (fPAR) as a tobacco yield modelling parameter. fPAR covers the spectral range of 400 to 700 nm that a canopy absorbs and it is a value often supplied by observation satellites. The study area covers 17 municipalities in the State of Rio Grande do Sul, Brazil, with a total area of 7200 square km. The method uses fPAR data collected weekly by NASA's Modis satellite during the whole tobacco growing period. The comparative analysis of the fPAR profiles during such periods allows the municipalities to be classified

into five groups according to evolution of behaviour. The final calibration to obtain the tobacco yield models for every group was done using data from nine consecutive years (2003-2012). Through the regression of fPAR values over the corresponding “Earth true” yield data from the Instituto Brasileiro Geral de Estadisticas (IBGE), linear calibration models were obtained. The evaluation of the method was done by applying the obtained models to the 2013 and 2014 crops of the five municipality groups. The absolute errors relative to the IBGE terrestrial registers resulted in all the cases being in the range of 4 % to 7 %. The results obtained show that fPAR is a satisfactory modelling parameter for tobacco yield evaluation especially for its application to extensive cropping areas.

## **APPOST 04**

### **Pest and disease surveillance in flue-cured tobacco growing zones of India**

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Pest and disease are major yield limiting factors in tobacco production and these biotic factors reduce the productivity besides affecting the quality of leaf. The control of these pest and diseases warrant more pesticide usage resulting in pesticide residues in cured leaf. A pest and disease surveillance study was conducted for three years from 2014-2016 on tobacco in three flue-cured Virginia (FCV) growing zones (Northern Light Soils [NLS], Traditional and Mysore) of India covering 72 villages and 144 farms in NLS, 206 villages and 412 farms in Traditional and 154 villages and 308 farms in Mysore, with an objective to identify the pest endemic areas and in turn to adopt residue free technical interventions in pest management.

The cumulative data for surveillance from the three seasons showed that the leaf eating caterpillar incidence was the highest in Mysore and Traditional (8.74 % and 8.64 %). The aphid infestation was lowest (0.52 %) in the Traditional area. The leaf curl incidence was high (3.49 %) in NLS, followed by Mysore (2.73 %). The tobacco mosaic virus incidence was >6 % across the three years both in Mysore and NLS regions. Fusarium wilt incidence was noticed only in the Mysore area with a range of 1.15 % to 3.98 %, and was not significant in the NLS and Traditional areas.

Orobanche, a complete root parasite, has been observed consistently for the last three years and its incidence was very high (8.45-9 %) in NLS and Mysore during 2014 and high in Traditional during 2015-2016. This study helped in identifying the most endemic areas of pest and diseases for guiding the crop development teams on possible risk elements.

## APPOST 05

### Effect of Qianhefu water-soluble foliar fertilizer on the quality of flue-cured tobacco leaf

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A field experiment was carried out to investigate the effect of Qianhefu water-soluble foliar fertilizer on the quality of tobacco leaf. The results showed that the foliar fertilizer played an important role in improving agronomic traits of tobacco growth, promoting the physical indexes such as equilibrium moisture content and leaf weight, coordinating the intrinsic chemical composition of the tobacco leaf and increasing the total aroma. The amounts of chromoplast pigments and their degraded products were the highest (215.81 µg/g and 535.85 µg/g, respectively) after the fertilizer treatment. The contents of chlorophyll, carotene, neophytadiene and degraded products of carotene increased by 280 %, 54.7 %, 26.4 % and 57.3 %, respectively, compared with the control (i.e. treated with water). The iron, zinc and potassium content in tobacco leaves increased after treatment with the higher fertilizer concentrations, and was the highest after spraying during the tobacco fast growing period. However, the fertilizer did not affect the magnesium content in tobacco leaves because there was no magnesium in the fertilizer. Therefore, the use of the leaf-surface fertilizer Qianhefu can greatly improve the quality of tobacco leaves and the economic benefit.

## APPOST 06

### Effect of biochar fertilizer on microbial functional diversity of tobacco-planting soil

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The structure and function of soil microbial communities have been widely used as indicators of soil quality and fertility. The effect of biochar application on carbon sequestration has been studied, but the effect of biochar fertilizer on soil microbial functional diversity has received little attention. To identify the effect of biochar fertilizer on soil abiotic and biotic properties and provide evidence for the soil improvement with biochar fertilizer input, soil microbial activities and related processes in the 0-20 cm soil



layer were determined in a tobacco field after biochar fertilizer addition at rates of 0, 0.9, 1.5, and 2.1 t ha<sup>-1</sup>. The results showed that the biochar fertilizer significantly increased the amounts of soil organic carbon (SOC), microbial biomass carbon (MBC) and microbial biomass nitrogen (MBN), and decreased the amount of mineral nitrogen. The biochar fertilizer increased average well colour development (AWCD) values in Biolog EcoPlates. Biochar fertilizer addition significantly increased Shannon-Wiener index (H), Simpson's dominance (D) and McIntosh diversity index (U), and decreased the evenness index (E). Principal component analysis and heatmap analysis clearly differentiated the treatments, and microbial use of six categories of substrates significantly increased after biochar fertilizer addition. Our results indicated that the addition rate of 1.5 t ha<sup>-1</sup> biochar fertilizer has the potential to improve soil fertility and soil microbial activity.

## **APPOST 07**

### **Rotation and manure amendment increase soil macro-aggregates and associated carbon and nitrogen stocks in flue-cured tobacco production**

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Flue-cured tobacco production in China is typically over-fertilized and mono-cropped. To understand how this agronomic management affects soil structure and organic matter, this study investigated the effect of rotation, fertilizer rate and manure amendment on the proportion of water stable aggregates and aggregate-associated soil organic carbon (SOC) and total soil nitrogen (TSN) concentrations and stocks in tobacco production. Two tobacco rotation systems (tobacco monocropping and tobacco-rice) with four fertilizer managements (0, 75, and 112 kg N/ha, and 60 kg N/ha + Manure) were established in 1998. Eighteen years after treatments were implemented, soil aggregation, and aggregate-associated SOC and TSN were significantly affected by rotation and fertilizer management. Compared to tobacco monoculture and current fertilizer management, rotation and manure amendment increased macroaggregate (>250 µm) proportion and geometric mean diameter, but decreased the proportion of microaggregates and silt-clay sized fractions (<250 µm). Rotation and manure amendment simultaneously increased the percentage of macroaggregate fractions and associated SOC and TSN stocks at the expense of the microaggregate and silt-clay size class and associated SOC and TSN stocks. Using rotation and/or manure amendment in tobacco production appears to maintain desirable soil physical and chemical properties via macroaggregates stabilization, which leads to the conservation of SOC and TSN stocks.

## APPOST 08

### **Study on absorption and transport of potassium (K) by foliar application in tobacco leaves**

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Effects of the foliar fertilizer on the uptake of potassium (K), as well as translocation and distribution among different position of tobacco leaf were studied under lack of K conditions. In order to study the suitable contents and the critical value of nutrient deficiency of K on tobacco under different levels of K, hydroponic experiments were used. After treatments of 30 g·L<sup>-1</sup> KNO<sub>3</sub> to the median leaves of tobacco lacking in K, leaf samples were collected during different periods, respectively. Dry weight and nutrient contents were measured. In addition, the relative contents of K in different tissues of veins or petiole were determined by scanning electron microscopy (SEM) and energy dispersive spectrometer (EDS). The results showed that the growth of tobacco was significantly affected by K content (< 4 mmol·L<sup>-1</sup>) and that the critical value of tobacco K deficiency was 11.5 % mg·kg<sup>-1</sup>. 0.04 day after the foliar fertilizer treatments, the K was transported to different parts of the leaves with high speed. The concentration of K in the superior leaves was significantly higher than that in the inferior leaves, indicating that K was preferentially transported to superior leaves. EDS analysis showed that K tended to be transported to tender meristems, and could also be transported through phloem and xylem to superior leaves and inferior leaves simultaneously. K was distributed mainly in the parenchyma in the tobacco leaves. The relative contents of K in the adaxial epidermis, adaxial parenchyma, vascular tissue, abaxial parenchyma and abaxial epidermis of the superior leaves, median leaves and inferior leaves were increased significantly, with the vascular tissue increasing most.

## APPOST 09

### **Degradation property of degradable film and its effect on flue-cured tobacco development and soil ecological environment**

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Aiming at decreasing agricultural pollution caused by plastic film, degradation of photo-oxygen degraded and bio-degradable films and their effects on flue-cured tobacco development, soil temperature, moisture, enzyme activities and fungi community were studied. The results between plastic film covering and open field were compared. The results indicated that the degradation performance of bio-degradable film was better

than that of photo-oxygen degradation film. The degradation degree of the two films applied to flue-cured tobacco in 245 days was degradation level 5 and 4, and the residual amount of the films was 40.1 % and 64.0 %, respectively. The development and biomass of flue-cured tobacco increased markedly with film covering compared to open field, and the effects of plastic film were best, followed by bio-degradable film, and lastly by photo-oxygen degradation film. In the early stage of tobacco plant growth, the temperature and moisture content of soil treated with two kinds of degradable film were higher than that of the open field, which was significantly lower than that of the plastic film. In the middle and later stage of the tobacco plant growth, it was similar to that of the plastic film, slightly lower than the open field. Film covering significantly increased the soil acid phosphatase activity, and soil enzyme activity of plastic film treatment was best, followed by open field and bio-degradable film. In addition, the number of fungi operational taxonomic units (OTU) in soil with bio-degradable film treatment increased by 12.7 % more than plastic film. The microbial abundance of *Ascomycota* and *Basidiomycota* in the soil promoted and decreased severely with the application of the two degradable films. The OTU species abundance of soil fungi with the treatment of open field was better than film-covered, and the soil fungi OTU uniformity of plastic film mulch was superior to other treatments. This illustrated that the microbial fungi community structure was more uniform and stable because the soil environment was less affected by the outside world with plastic film mulching. Bio-degradable film had excellent degradability and no significant difference in the development of flue-cured tobacco with plastic film, which showed that it could be applied to tobacco production.

## APPOST 10

### Determination of maleic hydrazide and its two glucosides in tobacco leaves using hydrophilic interaction liquid chromatography tandem mass spectrometry

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A hydrophilic interaction liquid chromatography tandem mass spectrometry-based method for the simultaneous determination of maleic hydrazide (MH) and its two glucosides in tobacco leaves was developed. The method was based on the synthesis of MH-O- $\beta$ -D-glucoside and the identification of MH-O- $\beta$ -D-glucoside and MH-N- $\beta$ -D-glucoside in tobacco leaves. The developed method was compared with the reference methods (including the tobacco industry standard YCT405.5-2011) and the sample preparation of this method was found to be simpler, more efficient, safer, and with higher throughput than those of the reference methods. This method was also found to have shorter instrument analysis time and higher sensitivity than that of the reference methods. The distribution of MH and its glucosides in tobacco leaves can also be determined with this method. Method validation was performed and correlation coefficients ( $r^2$ ) of MH and MH-O- $\beta$ -D-glucoside were in the range of 0.9971-0.9972. Recoveries were in the range of 83-112 %, with intra-day repeatability 2.7-3.8 %, inter-day repeatability 7.1-8.3 %, limits of quantitation 0.8~1.0 mg/kg, and limits of detection 0.3~0.5 mg/kg. The developed

method was used for the study of the metabolism of MH in tobacco leaves. At day 28 after MH spraying, content of MH in tobacco leaves decreased by 80.8 %, of which only 7.6 % was transformed to MH-glucosides. The transformation of the remainder of the MH needs to be studied.

## APPOST 11

### Field trials with low nicotine tobacco varieties developed by conventional breeding technique: first results

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In 2015, the World Health Organisation (WHO) Study Group on Tobacco Product Regulation (“TobReg”) issued an advisory note recommending a strategy of reducing nicotine in tobacco to substantially lower levels of 0.4 mg/g. In the U.S., the Food and Drug Administration (FDA) is also actively considering this option.

Nicotine levels in the plant cannot be reduced without the development and availability of relevant new cultivars and massive changes in agronomic practices as it is a naturally synthesized alkaloid. Furthermore, to achieve viable “Very Low Nicotine” or VLN levels an extensive and lengthy research programme into targeted genetic manipulations would be necessary.

Following the publication of the WHO advisory note, a collaboration between breeders, leaf suppliers, farmers, and manufacturers was initiated in 2016. The aim was to assess low nicotine tobacco varieties in natural growing environments when both normal and extreme agricultural practices were applied. Both non-commercial and commercial Burley and flue-cured varieties were tested. Low nicotine varieties and controls were developed with conventional breeding techniques; GMOs were excluded due to regulatory restrictions on their use. The assessment focused on resistance to disease, leaf quality and taste, yields and farmer livelihood. Field trials were conducted in Zimbabwe and Malawi with the support of the Tobacco Research Board (TRB) and Agricultural Research and Extension Trust (ARET).

The first results showed low yields (around a 50 % reduction), poor aromatic characteristics, low quality and commercial value, with levels of nicotine still higher (>0.5 %dwb) than the WHO published target. These results raise a number of concerns from both a technical agronomy perspective and, more significantly, from the perspective of sustainable farming economics.

Additional investigations and field trials are planned to explore to what extent and duration improvements can be achieved. This is clearly a long-term project, as the development of viable new cultivars with reduced nicotine could take a decade, and commercialisation on a larger scale still longer.

## **APPOST 12**

### **Degradable residues characterization and possible MRL setting for albendazole, flusilazole and imazalil in tobacco**

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A modified QuEChERS method combined liquid chromatography-tandem triple quadrupole mass spectrometry (HPLC-MS/MS) was developed for three novel pesticides, albendazole, flusilazole and imazalil, which are used for the control of tobacco leaf spot diseases. The established analytical method was applied for the study of dynamics and residue characteristics of the three fungicides under field conditions, the results of which could provide references for the establishment of related maximum residue limits (MRLs). The recoveries of albendazole, flusilazole, and imazalil in tobacco leaves were 84.9 %-104.4 %, 75.8 %-103.0 %, and 83.7 %-92.5 %, respectively, with relative standard deviations (RSDs) 1.2 %-8.1 %, 1.0 %-7.8 %, 2.8 %-8.8 %. The sensitivity, accuracy and reproducibility met the method requirements for pesticide residue analysis. The half-lives of albendazole, flusilazole, and imazalil in fresh tobacco leaves were 5.4-16.1, 4.3-7.7, and 4.4-6.9 days. When the fungicide was applied three times at the recommended maximum dose and 1.5-fold dose, the terminal residues of albendazole, flusilazole, and imazalil in cured tobacco leaves were 0.33-1.33 mg/kg, 0.17-1.00 mg/kg, and 0.76-2.00 mg/kg with a pre-harvest interval (PHI) of 14 days. Based on these test results, possible MRLs in tobacco for albendazole, flusilazole, and imazalil, derived on the same principles used for setting MRLs for food and feed, are 2 mg/kg, 2 mg/kg, and 5 mg/kg, respectively.

## **APPOST 13**

### **Genetic diversity of potato virus Y infecting tobacco in major tobacco growing fields in Northern Vietnam**

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Potato virus Y (PVY) is the type member of genus Potyvirus and one of the most common species and most pernicious of Potyvirus. PVY has a wide host range and infects several important *solanaceous* crops, including potato, tomato and tobacco. In Vietnam, potato

virus Y is a severe disease in almost all tobacco growing areas. The disease had a high incidence and caused severe damage especially in Bac Son, Lang Son Province, in 2015. The objective of this study was to determine the genetic diversity of PVY infecting tobacco in the major growing tobacco region of Northern Vietnam which is essential for disease prevention. In this study, tobacco leaves with typical symptoms of PVY were collected from major growing tobacco fields in three provinces in Northern Vietnam, including Cao Bang, Lang Son and Bac Giang. CP gene fragments encoding the capsid protein of PVY were amplified by Reverse Transcript PCR and sequenced by ABI 3500 system. The sequences obtained were analyzed and classification trees were built by using MEGA6 software. From the results, we classified four groups of PVY, including PVY<sup>0</sup>, PVY<sup>c</sup>, PVY<sup>NTN</sup> and a new group that is similar to group KC296833 on potato in China but we did not determine the strain of the new PVY. PVY<sup>0</sup> is the most common group and PVY isolated in Lang Son is the most diverse. Two strains PVY<sup>c</sup>, PVY<sup>NTN</sup> were only found in tobacco cultivation in Lang Son Province, and the four strains were found in Bac Son. The determination of genetic diversity is important in the tobacco growing areas in Northern Vietnam and is essential for PVY control in the field.

## APPOST 14

### **Study on the detection and automatic diagnosis of tobacco diseases based on machine vision and machine learning**

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Tobacco disease diagnosis in China at present mostly remains in the artificial stage, with such problems as poor objectivity and low efficiency. A novel tobacco disease detection and automatic diagnosis system based on machine vision and machine learning is described in this paper. The main work is shown as follows: (1) in the process of disease image acquisition, under the influence of environmental factors such as illumination, weeds and soil, disease images usually have a complicated background and the existing automatic disease segmentation algorithms are often not ideal. To solve the above problem, a novel image enhancement and segmentation method based on Retinex and Grabcut theory is proposed. The method does not need to place the samples on a white or specific background for easy processing and can complete the enhancement and segmentation work of all leaf images regardless the type of background; (2) the use of a semi-supervised machine learning algorithm to optimize the feature extraction model based on limitation Boltzmann net, so as to realize the self-optimization of the feature extractor under the guidance of the target task; (3) a user-friendly interface and easy extension of tobacco disease diagnosis system based on SVM algorithm has been developed. The system can read the local disease image recognition and the users only need a simple interaction to obtain the disease species and the methods of prevention and cure. The users are thus guided to rationally use pesticides to prevent and control disease effectively. The study has widened the range of the application of machine vision and machine learning and provides a good reference for the development design of an intelligent agriculture system.

## APPOST 15

### **Effect of potato virus Y on growth, yield and chemical composition of flue-cured tobacco in Northern Vietnam**

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Potato virus Y (PVY) is a plant virus that causes significant losses to tobacco. PVY damage is severe in the tobacco growing provinces Lang Son and Bac Giang with disease rates from 10-45 %, with some areas infected 100 %. The objective of this study was to evaluate the effect of PVY on growth, yield and chemical composition to form the basis for managing disease in the field. An experiment was carried out using mechanical inoculation on tobacco cultivars C9-1 and GL7 in 2017. Treatments were replicated three times in a randomized block design. Only plants in the center two rows of each plot were inoculated. The infected leaves were collected and ground in a potassium phosphate buffer with 1 g infected leaf/2 ml buffer. Before inoculation, leaves of experimental plants were dusted with Carburundum 600 mesh. Each treatment was inoculated 15, 35, 50 and 60 days after transplanting. Control plants were not inoculated. The result of the experiment showed that early inoculation of C9-1 (15, 25 and 35 days after transplanting) caused height reduction of 47,4 %-62,0 % and yield reduction of 45,9 %-58,6 %. Similarly, to C9-1, GL7 experienced height reductions of 57,0 %-73,8 % and yield reductions of 49,4 %-58,9 % when inoculated 15-35 days after transplanting. PVY also modified the chemical composition of flue-cured leaves. Nicotine content was always lower in cured leaves from diseased plants than in cured leaves from healthy controls. This occurred especially when inoculation was done 15-35 days after transplanting, with nicotine reduced by 21,8 %-42,9 % and sugar by 6,1 %-56,5 %. Inoculation done 50 and 60 days after transplanting lightly affected yield and chemical composition but did not affect the total number of leaves and the height of the tobacco plant. From the results, efforts should be made to prevent the spread of PVY during the first month after transplanting by destroying infected plants and treating aphids.

## APPOST 16

### **Monitoring of agricultural drought in tobacco growing areas in Poland in 2009-2017**

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Occurrence of unfavourable weather conditions (drought) causes significant losses in crop yields in the world. McKeown et al. [2006], in their work on long-term climate patterns related to yields, stated that by 2030 the periodic occurrence of agricultural drought in crops will contribute to a 30 % reduction in yield. In Poland, the evaluation of

agricultural drought has been implemented using the Agricultural Drought Monitoring System (ADMS), conducted by IUNG-PIB [Doroszewski et al. 2008, 2012]. ADMS uses the climatic water balance index (CWB) to monitor drought conditions in Poland.

In Poland, tobacco is grown mainly in the central-eastern and northern parts of the country. These are areas with a long tradition of growing this plant. In many cases, tobacco is grown on soils of the first category, which are highly exposed to water scarcity due to their internal structure and composition (they quickly dry out). The influence of unfavourable weather conditions (drought) undoubtedly contributed to the reduction of tobacco yields. The low CWB values recorded in reports showing a threat of drought have indicated the possibility of yield reduction in tobacco crops. At the same time, in the years when the CWB values did not reach the limit values and did not exceed them, an increase in the yield was demonstrated.

On the national scale, the yield of tobacco increased from 23 dt/ha in 2009 to 25.3 dt/ha in 2016. The high yield levels recorded in 2016 were undoubtedly largely due to meteorological conditions, in particular due to large atmospheric precipitation in August, which resulted in an increased tobacco biomass. Some of the yield increases could also be indirectly influenced by varietal progress, associated with increased resistance of plants to diseases and the development of a tighter plant canopy resulting in suppressed weed growth.

## **APPOST 17**

### **Comparison of the degree of malformations in different breeding materials carrying *Nicotiana alata*-introgression conferring resistance to tomato spotted wilt virus**

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Tomato spotted wilt virus (TSWV) has been an important problem in tobacco cultivated in a moderate climate zone. The transfer of *RTSW-al* (TSWV resistance gene) from *Nicotiana alata* to the *N. tabacum* genome and the development of Virginia-type cultivars is difficult due to the coupling of the resistance factor with the occurrence of plant morphological deformations. Androgenesis technique and selection of resistant genotypes using sequence characterized amplified region markers linked to TSWV resistance allowed the development of introgression lines DH3 and DH6 resistant to TSWV. A further breeding process involving crossing with the high-quality cultivar "WAC 121D7" resulted in hybrids F<sub>1</sub> (DH3 x WAC 121D7, DH6 x WAC 121D7) and then F<sub>2</sub> hybrids. Primers specific for *N. alata* and *N. tabacum* sequences, located in the chromosomal region in which *N. alata* introgression may occur in the experimental plants, were then used to distinguish two types of homozygotes (ALA/ALA-with introgression, TOB/TOB-tobacco type) and heterozygotes among F<sub>2</sub> individuals. The aim of the study was to compare the degree of deformations in both DH lines and in F<sub>1</sub> and F<sub>2</sub> hybrids. In the case of parental line DH3 and F<sub>1</sub> and F<sub>2</sub> hybrids derived from this line, the percentage of plants



with malformations equalled 53.3 %, 90.0 %, and 55.0 %, respectively. In turn, in the DH6 line and its F<sub>1</sub> and F<sub>2</sub> hybrid generations, the percentage of individuals that exhibited morphological changes accounted for 43.3 %, 73.3 %, and 32.9 %, respectively. Plants with malformations were less common among the putative-resistant F<sub>2</sub> plants (ALA/ALA homozygotes) derived from DH6 compared with those plants derived from DH3. The respective percentages of ALA/ALA homozygotes with malformations equalled 30.1 % and 59.5 % for (DH6 x WAC 121D7)F<sub>2</sub> and (DH3 x WAC 121D7)F<sub>2</sub>. These results indicate that the F<sub>2</sub> progeny derived from the DH6 line was a better breeding material than F<sub>2</sub> plants derived from the DH3 line.

## APPOST 18

### Characteristics of bacterial community structure in tobacco-planting soils under different fertilization practices

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To evaluate the fertilization practices on soil fertility and the microbial ecosystem, a tobacco pot experiment with different fertilization treatments was conducted in a completely randomized design. The five treatments were: no fertilizer (CK); N fertilizer (T1); NPK fertilizer (T2); NPK fertilizer plus decomposed straw (T3); biochar (T4). Compared to the T2 treatment, application of manure significantly increased the concentrations of soil organic carbon, mineral N and the dry matter accumulation of tobacco plants. The tobacco rhizosphere bacterial community was investigated using 16S rDNA high-throughput sequencing. The results indicated that the predominant bacterial phyla were Proteobacteria, Bacteroidetes and Firmicutes. At the genus level, the bacterial community was significantly altered by the presence of tobacco plants and the fertilizer treatments. The relative abundance of *Aeromonas* were significantly decreased in the fertilized soil. Compared with the T1 treatments, the relative abundance of *Bacillus* and *Paenibacillus* in the T2 treatment and *Georgfuchsia* and *Pseudorhodoferax* in the T4 treatment were significantly decreased and increased, respectively. The results indicate that application of chemical fertilizer and manure in the tobacco-planting soil could improve soil nutrients and influence the bacterial community structure and thus plant growth.

## APPOST 19

### **Influence of biochar on the composition of a soil microbial community under tobacco (*Nicotiana tabacum*) cultivation**

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Biochar is a solid material with an extremely high carbon content and highly porous structure. It has been recognized as a suitable soil amendment and is used to improve soils by increasing the organic carbon content, water and fertilizer retention capacities, density and activity of soil microorganisms, and by reducing nutrient loss. We further investigated the variation of populations of bacteria, fungi and actinomycetes during tobacco development under different fertilisation treatments. We then performed high-throughput sequencing of the 16S rRNA gene and ITS. The results showed that application of biochar in the soil altered the composition of the soil microbial community; the number of operational taxonomic units (OTUs) increased for bacteria and decreased for fungus. Furthermore, biochar did not alter the dominant phyla of soils and dominant genera for bacteria, but dominant genera for fungus changed significantly.

## APPOST 21

### **Possibility of estimation of on-site population density of tobacco beetle**

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The tobacco beetle (*Lasioderma serricornis*) is usually monitored by pheromone trap. Their infestation in a factory and a warehouse can be indirectly understood through monitoring data. It is impossible to directly obtain the number of tobacco beetles (population density) and seasonal prevalence of occurrence in a site. We are able to presume on-site population density from monitoring data, i.e. the number of catches. We therefore conducted a series of experiments in a warehouse to investigate relationships between the number of beetles released and the number of catches and to confirm whether fluctuation of the number of catches by pheromone trap coincided with fluctuation of population density.

Twelve NEW SERRICO traps, pheromone traps for the tobacco beetle, were attached to walls and wood boards in place of a pillar in a warehouse (roughly 24 × 36 m). The traps were put at about 10 m intervals and 1.5 m height. Fifty, 100, 200, 300 or 400 male beetles were released into the warehouse every week. Only a sex pheromone lure was used. The catches were counted a week after release. The traps were replaced by new ones every four weeks. Experiments were carried out from July to October in 2013, 2014 and 2015. Average temperature was from 20 to 34 °C and average relative humidity was from 50 to 75 %.

The two trends, the number of catches by pheromone trap and the number of individuals released, almost completely concurred in all three years. The relationship showed a highly significant positive correlation. The result suggested that fluctuation of the number of catches coincided with fluctuation of on-site population density. Moreover, because a constant capturing efficacy was obtained from experiments in the warehouse, it suggested the possibility of estimation of on-site population density based on the number of catches.

## APPOST 22

### **Bacterial wilt (*Ralstonia solanacearum*) resistant flue-cured hybrids - current Malawi situation**

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In Malawi, flue-cured tobacco production is negatively affected by both aerial and soil borne diseases. Bacterial wilt (*Ralstonia solanacearum*) is one of the major soil borne diseases that attack the crop, reducing cured leaf yield and quality. Currently the country has no flue-cured tobacco varieties resistant to bacterial wilt. ARET started a breeding program to develop bacterial wilt resistant flue-cured tobacco varieties with high cured leaf yield and acceptable quality. From 2014 to 2017 ARET evaluated eight advanced bacterial wilt resistant flue-cured tobacco lines alongside one popular variety, KRK 26 and a local check, AFH 4. The trials were laid out in randomized complete blocks and replicated three times. Data was collected on leaf yield, disease reaction and cured leaf quality (colour and grade outturn) and analysed using analysis of variance (ANOVA). Means were separated using the least significant difference (LSD). Three year results on bacterial wilt incidence (%) did not show any significant differences among the test lines. GRH11-9 with 21.4 % and GRH with 30.7 % gave values less than the acceptable threshold level. Nematode reaction showed significant differences ( $P \leq 0.05$ ) among the lines. GRH11-9 and GRH11-10 gave the same mean score, 3.3 better than AFH 4 showing moderate resistance. Yield performance was similar between the test lines and the two checks, KRK 26 and AFH 4. GRH11-2 and GRH11-31 gave yields >2000 kg/ha just like AFH 4. Yield ranged from 1594 kg/ha to 2166 kg/ha. Results on colour distribution (%) of cured leaf also showed no significant differences between the test lines and the checks, demonstrating that the test lines had the same leaf quality as the checks. In terms of leaf grade distribution GRH11-5 and GRH11-31 produced significantly higher proportions ( $P \leq 0.001$ ) of first quality leaf, together with the two checks, 34.0 % and 32.3 %, respectively.

## APPOST 23

### Overexpression of tobacco GCN2 induces plant response to various stresses

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The study of the mechanism of GCN2 involved in the plant response to various stresses could provide a theoretical basis for crop variety breeding. General control non-derepressible-2 (GCN2) could phosphorylate the  $\alpha$  subunit of the eukaryotic initiation factor eIF2 to down-regulate the initiation of protein synthesis, subsequently reducing the global protein biosynthesis. GCN2 could also respond to biotic and abiotic stresses. To investigate the function of GCN2 and explore its roles in plant stress response, we cloned *NtGCN2* from *Nicotiana tabacum* by RACE-PCR. The expression of *NtGCN2* and the phosphorylation of *NteIF2 $\alpha$*  were then analyzed with plant hormones, including salicylic acid (SA), azelaic acid (AZA), methyl jasmonate (MeJA). Analysis of different stresses (*Bemisia tabaci* infection, drought, and cold) was done with Real-time quantitative RT-PCR and west-blotting, respectively. The results indicated that *NtGCN2* is involved in the response of plants to multiple biotic and abiotic stresses. The content of total soluble sugars and reducing sugars decreased, whereas that of chlorophyll a and b increased in the OE plants. We also observed that the overexpression of *NtGCN2-1* significantly influenced different physiological processes, promoting seed germination and root elongation. The content of total soluble sugars and reducing sugars decreased, whereas that of chlorophyll a and b increased in the GCN2 overexpressing plants. In addition, the overexpressing plants had a lower content of reactive oxygen species and exhibited higher antioxidant activities. These physiological alterations could be attributed to the changes in endogenous phytohormones, decrease in the SA and abscisic acid content, and accumulation of MeJA and AZA. It indicated that the overexpression of *NtGCN2* in tobacco stimulated the plant defense responses via phosphorylation of *NteIF2 $\alpha$*  and the regulation of plant hormones, and via changes in the antioxidant ability and plant nutrient status.

## APPOST 24

### Development and evaluation of tobaccos engineered for high leaf-oil accumulation

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Demand for plant-derived oil (triacylglycerols, TAG) commodities is expected to more than double in coming decades due to increasing food oil requirements. Furthermore, the emerging shift to use renewable feedstock for fuels and industrial chemicals has the

potential to create additional demand for renewable oils. Currently, plant oils are predominantly derived from dedicated oilseed crops (soybean, canola, sunflower, etc.) and fruits (palm, olive, coconut). Their supply is presently matched to food demand and cannot sustainably meet both the food and industrial demands of the future. To overcome supply limitations, CSIRO Plant Oil Engineering group has developed and patented a revolutionary technology for engineering high levels of TAG in leaves of high biomass crops including tobacco. Preliminary studies were conducted in the glasshouse to quantify leaf oil accumulation in this engineered tobacco. Engineered and empty vector control plants were grown in a replicated study in glasshouse conditions. Leaf oil was quantified by subsampling leaf tissue from experimental plants and analysing total oil per leaf dry weight (LDW). Initial reports were successful, having >35 % oil LDW in leaves of engineered plants compared to the control with <1 % oil LDW. If these levels can be achieved in field grown tobacco and other high biomass crops, productivity of oil crops could exceed that of palm and enable cost-competitive production of oil for renewable fuels and industrial chemicals with the long-range expectation for petroleum feedstock. To further explore this potential, these lines were evaluated in the field in 2017 at Kentucky to produce materials for biomass examination and oil development. Larger trials are currently underway in 2018. This presentation will outline the basis of the technology, present information on leaf-oil development from glasshouse-grown and field-grown plants, and outline the potential for developing high-oil tobacco into a feedstock for oil-based industrial products, renewable fuel, and oleochemicals.

## APPOST 26

### ***Nicotiana* species as sources of cytoplasmic male sterility in tobacco breeding**

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The majority of cytoplasmically male sterile (cms) forms in tobacco, *Nicotiana tabacum*, have been produced by moving the whole nuclear genome from one species and re-installing it in the cytoplasmic milieu of another species thereby disrupting the complementarity between the nucleus and the cytoplasm. At present, the genus *Nicotiana* is estimated to comprise over 80 different species. The cytoplasmic factors from ca. 30 of these species have been reported to be transferred to the cultivated tobacco *N. tabacum*. All known alloplasmics of tobacco are at least partially male sterile, with the exception of those involving *N. sylvestris*, the ancestral donor of the cytoplasm to *N. tabacum*. The majority of *N. tabacum* alloplasmics represent the staminal type of CMS. A few belong to the postmeiotic type (cms *knightiana*, cms *raimondii*, cms *paniculata* and cms *rustica*). In some lineages of the latter alloplasmics and in cms *glauca*, occasional production of vestigial pollen was observed. Of the 28 alloplasmic cms forms of *N. tabacum* studied for agronomic performance, more than half were found to be unsuitable, mostly due to the depressing effect on yield and crop quality. Seven species - *N. longiflora*, *N. paniculata*, *N. amplexicaulis*, *N. benthamiana*, *N. maritima*, and *N. velutina* - were indicated as potentially usable sources of cms. Cms *glauca* and cms *undulata* were

repeatedly confirmed as having agronomic potential but neither has a record of commercial deployment. Three cms sources have actually been deployed in commercial tobacco hybrids: cms *suaveolens* and its suspected cytoplasmic variant (cms “bigelovii”) and cms “tabacum-mutant”, a suspected cytoplasmic variant of cms *glauca*. Of these three, cms *suaveolens* is at present probably the most widely used cms in tobacco breeding. However, some of its faults, notably in hybrid seed production, are making breeders seek for new, improved cms sources.

## **APPOST 27**

### **Critical period for weed control in flue-cured tobacco**

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Field experiments were conducted to determine the critical period for weed control (CPWC) in flue-cured tobacco. This period consists of two separately measured timings; a critical weed-free period and a critical timing for the weed removal period. Treatments consisted of weed removal at 2, 4, 6, 8, and 10 weeks after planting (WAP) and weed-free

periods of 2, 4, 6, 8, and 10 WAP. In addition, a season long weedy and season long weed-free treatment were included. The crop was subjected to standard management practices; aside from herbicides and cultivation. The experimental design was a randomized complete block design with four replications.

Results showed that the critical timing of removal was 4.4 weeks after transplanting (WAT) and the critical weed free period was 6.8 WAT; therefore, the critical period of weed control in flue-cured tobacco was between 4.4 and 6.8 WAT in mixed populations of both grass and broadleaf weed species. No difference in yield was observed when flue-cured tobacco was kept weed-free for the first 6 WAT or when weeds were removed at 6 WAT and kept clean for the remainder of the season. Yields for treatments where weeds were not removed until 8 and 10 WAT were similar to the season-long weedy treatment.

Trends with crop value and quality are consistent with trends associated with yield. In general, trends with total alkaloids and reducing sugars were similar to that of yield; the higher the yield, the higher the nicotine content with lower reducing sugars. The lower the yield, the lower the nicotine and higher reducing sugars. This is expected as treatments with lower yield were subjected to greater weed competition; potentially reducing light and nutrient availability to the tobacco plant.

## APPOST 30

### **Nicotine reduction and consumer perception: a scientific approach for quantifying possible impact of low nicotine cigarettes on consumer behaviour**

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In 2015, the WHO Study Group on Tobacco Product Regulation (TobReg) issued an advisory note recommending a strategy to reduce nicotine in tobacco to levels which would not be sufficient to lead to the development and/or maintenance of addiction. In the US, the FDA is also considering regulating nicotine content in cigarettes.

Nicotine is naturally synthesised by the tobacco plant, so any lowering of nicotine levels in tobacco cannot be achieved without a careful genetic selection of crop varieties and substantial changes to existing agronomic practices. Otherwise, field trials with low nicotine tobacco varieties developed by conventional breeding techniques (reported separately in a poster) shows that crop yield and leaf quality are reduced in field trials when nicotine is reduced.

Additionally, nicotine plays a role in the smoking experience (sensory effect, taste, aroma, etc.). Several publications report that reduced nicotine cigarettes are unsatisfactory for consumers. The implementation of a low nicotine regulatory agenda may encourage consumer to switch to products that will provide a more familiar experience. Considering the consumer dissatisfaction to low nicotine products, we developed dynamic population modelling to quantify the possible changes of consumer behaviour in sense of acceptance, cessation or switching to other trade products, including illicit ones. Baseline status transitions were derived from published data. Several scenarios were considered to cover a range of probabilities for smokers to switch to illicit products. The impact on smoking prevalence and illicit trade over time was then simulated. Our poster will present the various possible impacts of the implementation of reduced nicotine regulation, if consumers have limited access to acceptable alternative products.

# CORESTA CONGRESS 2018

## SMOKE SCIENCE and PRODUCT TECHNOLOGY

### ORAL PRESENTATIONS

#### ST 01

##### ***In vitro* biological safety evaluation of cigarette smoke**

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Traditionally, toxicological evaluation of cigarette smoke has focused on the harmful chemical content of smoke. Harm-reduced cigarettes should not only refer to these chemical compounds, but also to the degree of the effect of whole smoke on human cells. Therefore, the biological and toxicological assessment of the whole smoke is urgent and important. The impact of smoking on the human body is mainly on the bronchus and lungs. It is of great significance to construct an evaluation system that can simulate the physiological state of the human bronchial (lung) in a smoking state. BEAS-2B cells were exposed *in vitro* to cigarette smoke (1R5F reference cigarettes and e-vapor) which was diluted by smoking machine Borgwaldt RM20S. Diverse platforms were used to examine the transcriptomes and proteomes of human bronchial epithelial (HBE) cells. Air treatment was used as control. A method we developed was used for toxicological and biological assessment based on the gene expression levels within different metabolism pathways in the cells. We calculated the score for measuring the effect of cigarettes on the HBE cells (termed as effect score, ES). A case study showed that the ES of MSS was 52, and the ES of e-vapor was 40, which suggested the e-vapor might have less effect on the HBE cells. Our data suggested that alterations in cellular glycerophospholipid biosynthesis are important consequences of e-vapor exposure and the presence of nicotine in e-vapor elicits a cellular response distinct from e-vapor alone including alterations of cytochrome P450 function, retinoid metabolism, and nicotine catabolism. Our method could generate informatic data and establish a baseline for *in vitro* toxicological and biological evaluation of cigarettes. The ES value could be used to inform the cigarette industries or governmental bodies in discussions of the risks and future regulation of these products.



## ST 02

### Effect of heating temperature on chemical characterisation and biological activity of tobacco smoke emission

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Cigarette smoke is mainly produced by distillation, pyrolysis and combustion reactions when the tobacco is burnt. Heating type cigarettes have been reported to have the potential to reduce or eliminate some toxicants found in cigarette smoke because heating temperatures are below pyrolysis and combustion.

In this study we investigated the effect of heating temperatures on smoke chemistry and *in vitro* biological activity of tobacco smoke. We designed a bench-top furnace that heated tobacco between 200~700 °C and evaluated smoke compounds and biological activities from the gas phase and the aerosol phase. For the smoke chemistry study, tar, nicotine, water, glycerin, eight kinds of carbonyl compounds, six kinds of phenol compounds, four kinds of TSNA (tobacco specific nitrosamines) and benzo[a]pyrene were analyzed. The cytotoxic potencies of both the total particulate matter (TPM) and gas vapour phase (GVP) were assessed using neutral red uptake assay and GSH consumption assay, respectively. In addition, mutagenicity of the TPMs were assessed in *Salmonella typhimurium* strains with and without S9 metabolic activation.

In this presentation, the effect of heating temperatures between 200 to 400 °C on smoke chemistry and cytotoxicity and mutagenicity of particulate and vapor phase smoke will be explained. Also, the content of risk constituents will be compared with the biological activity from heated tobacco smoke.

## ST 03

### Solvent-free extraction method for *in vitro* testing with tobacco vapor products

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Since extracts made with emissions from ENDS and tobacco vapor products (TVP) generally exhibit weak biological responses in commonly used *in vitro* tests compared to those from conventional cigarettes, it is essential to generate very high concentration extracts. However, concentration of extracts made with the historical method using Cambridge filter pad (CFP) with dimethyl sulfoxides (DMSO) is limited from a technical perspective. We therefore developed an extraction method without using an extraction solvent. The vapor of a TVP was trapped on multiple CFPs, and all the CFPs were compressed together without any solvents using an originally designed device, and then shaken with a shaker. Although pressure was applied to the CFPs, the temperature of the

extract remained unchanged. The extract was then collected by centrifuge. The recovery rate of the condensate and the ratio of the major constituents were comparable to those obtained by the historical method with DMSO. The maximum concentration of the extract was approximately 1,000 mg/mL; it was at least 10 times higher compared to the maximum concentration obtained by the historical method for conventional products. This new method prevents interaction between the solvent and test systems and enables preparation of high concentration extracts. The new method can be useful for various *in vitro* tests with products exhibiting very weak toxicities.

## ST 04

### Assessing the cytotoxicity of e-liquids without the use of the neutral red uptake assay

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As part of the pre-clinical assessment performed for consumer products in the tobacco industry, an *in vitro* assessment of a tobacco product's cytotoxicity was performed using the neutral red uptake (NRU) assay. Since 2004, the CORESTA *In Vitro* Task Force has recommended the performance of the NRU for cigarette smoke condensate testing. The objective of this study was to assess the cytotoxicity of (-)-nicotine and (-)-nicotine-containing e-liquids in the NRU assay. Balb/c 3T3 cells were exposed to (-)-nicotine for 24 hours according to the INVITTOX Protocol 3a (1990). Following this exposure, the viability of the cells was determined spectroscopically by measuring the absorbance of the dye Neutral Red, which had been taken up into the lysosomes of the cells. The test was performed as part of a Good Laboratory Practice study. It was found that the robust relationship between absolute cell number (assessed by an electronic cell counter) and the amount of Neutral Red stored by a given population of treated cells was lost when (-)-nicotine was the test item. Disruption of lysosomal volume by (-)-nicotine appeared to underlie the loss of data integrity in the NRU assay. In the course of a search for an orthologous high-throughput method to assess the cytotoxicity of (-)-nicotine and e-liquids, several alternative *in vitro* approaches were evaluated. The most consistent and robust approach that emerged was the use of the tetrazolium salt WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt], which produces a water-soluble formazan dye, in direct proportion to the number of living cells, upon reduction in the presence of an electron. A validation study was performed to characterise the performance of the WST-8 assay over several days and with different laboratory technicians performing the test.

## ST 05

### Structure-activity relationships of propylene glycol, glycerin, and select analogs for carbonyl thermal degradation products

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In 2016, the Food and Drug Administration (FDA) issued draft Guidance to Industry regarding the submission of Premarket Tobacco Applications for electronic nicotine delivery systems (ENDS). In this draft guidance, the FDA recommends reporting several carbonyl compounds, including formaldehyde, acetaldehyde, and acrolein, whose presence in electronic cigarette aerosols is typically attributed to thermal degradation of propylene glycol (PG) and/or glycerin (Gly). Studies were conducted using carbon-13 labeled PG and Gly to elucidate the mechanisms and sources of the specific carbonyl compounds. These studies utilized a model reaction system based on microwave heating. Gly was found to be the primary source of formaldehyde while PG was found to be the primary source of acetaldehyde and acrolein. Formaldehyde and acetaldehyde generation from Gly is typically attributed to the retro-aldol condensation of 3-hydroxypropanal. This reaction was studied in further detail utilizing the microwave model system. These results were used to design PG and Gly analogs containing functional group substitutions to block reactive sites on the PG and Gly analogs. Blocking the reactive sites was anticipated to reduce the thermal decomposition of PG and Gly and thus provide information on the critical reaction centers required for the formation of the observed products. The results of these studies will be reported as well as the carbonyl yields from these PG and Gly analogs.

## ST 06

### E-cigarette flavour transfer screening method by GC/MS

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The increasing popularity of e-cigarettes has led to a diverse array of flavoured e-liquid products that continue to increase in chemical complexity. Whilst many of the flavour ingredients used in e-liquids can be assessed and deemed to be safe when inhaled, others may be toxic or respiratory sensitizers when the user is exposed above a threshold of toxicological concern (TTC). Consequently, measuring the transfer of target compounds from e-liquid to aerosol is of increasing importance in the risk assessment process.

Historically, a standardised approach to assess flavour transfer would be to measure the concentration of the target compound in the e-liquid as well as in the collected aerosol and subsequently calculating the relative transfer. However, developing and validating a

quantitative assay for many flavour compounds in the fast-moving world of e-liquid flavours is time consuming and expensive.

We will present a flavour transfer screening method for compounds amenable to GC/MS. We will demonstrate how it is possible to obtain practicable information on flavour transfer by comparing instrument responses for e-liquid and e-aerosol without the need for calibration curves for each of the individual flavour compounds and how this approach compares to quantitative analysis by more established approaches. The use of a “Limit Test” (pass, fail, more information required) is described to show whether the TTC for target compounds in the aerosol has been exceeded and how this information can be used to inform product development.

## ST 07

### **Evaluation of the formaldehyde hemiacetals and acetals relevant to electronic cigarettes**

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In 2016, the Food and Drug Administration (FDA) issued draft Guidance to Industry regarding the submission of Premarket Tobacco Applications for Electronic Nicotine Delivery Systems (ENDS). In this draft guidance, the FDA recommends reporting toxicants in aerosols, including formaldehyde. Recent publications suggested that 1) formaldehyde levels in ENDS products were underreported because formaldehyde may react with propylene glycol (PG) and glycerin (Gly) in the aerosol to form hemiacetals; 2) the equilibrium would shift from the hemiacetals to the acetals in the acidic DNPH trapping solution. In both cases, neither the hemiacetal nor the acetal would react to form the target formaldehyde hydrazone due to the lack of the carbonyl functional group; thus, underreporting formaldehyde. These reports were studied in our laboratory. Our results showed that the aerosol generated from formaldehyde spiked e-liquids gave near quantitative recovery of formaldehyde in the aerosol suggesting that if any hemiacetal was formed in the aerosol it would readily hydrolyze to free formaldehyde in the acidic DNPH trapping solution. We demonstrated that a custom synthesized Gly-hemiacetal added to DNPH trapping solution would readily hydrolyze to form the formaldehyde hydrazone. We also demonstrated that acetals of PG and Gly added to DNPH trapping solution would not hydrolyze to form the hydrazone. In order to further investigate the reported presence of acetals in e-liquids and aerosols, we developed a qualitative GC-MS method for the identification of the PG and Gly acetals of formaldehyde. No acetals of PG/Gly were detected in the e-liquids or the aerosols. These results demonstrate that formaldehyde levels in e-cigarette aerosols are not underreported due to hemiacetal and acetal formation.

## ST 08

### **FTIR chemometrics applied to e-liquids quantitative analysis**

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As the electronic cigarette industry evolves, the need for new rapid analytical techniques also grows. New methods are needed, both to monitor quality in a high throughput manufacturing environment and in a fast-paced product development space. We report on the development and validation of a quantitative Fourier Transform Infrared spectroscopy (FTIR) method for rapid (two minute), simultaneous analysis of all the major components of e-liquids. E-liquids are commonly analyzed by Karl Fischer analysis for water, and gas chromatography for components such as nicotine, propylene glycol, vegetable glycerin, and menthol. Compared to the traditional techniques, the FTIR method is faster, less expensive, requires no sample preparation and eliminates the need for consumables, hazardous solvents, laboratory gases, and reduces instrument maintenance. Data analysis using chemometrics is automated and simple to implement. The pre-processing algorithms, such as mean centering, derivatization, and cross validation are powerful tools in chemometrics, which allow accurate calibration of analytes with multiple infrared absorbance bands. Our method is well suited for high throughput routine analysis, including quality control, shelf life studies, and research involving water uptake in e-liquids. We will present the results of a full method validation and compare the results to validated Karl Fisher and gas chromatography methods.

## ST 09

### **Puff-by-puff analysis of nicotine, menthol and other volatile components in menthol flavoured heat-not-burn tobacco product**

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Heat-not-burn (HnB) tobacco products have rapidly become an accepted alternative to cigarettes worldwide in recent years. Since the main additives in tobacco materials of HnB products, such as nicotine, menthol and other volatile components, are released mainly through distillation and evaporation, consistent and efficient release of these compounds among puffs may greatly affect HnB product puffing quality. Puff-by-puff analysis of nicotine, menthol and volatile components conducted using a linear smoking machine, traditional trapping methods and chromatographic instruments provides an evaluation for consistent release of compounds from HnB products.

Two commercial menthol HnB products (Product A and B) were used for puff-by-puff analysis of nicotine, menthol and volatile components. Aerosol generation of each tobacco stick was conducted from puff no. 1 to 6 by smoking machine under HCI mode.

Since the amount of compounds in a single puff is normally below LOQ (Limit of detection), several numbers of tobacco sticks were used for puffing for each compound analysis. The same puff no. of different tobacco sticks was collected in the same Cambridge filter pad (CFP) or impinged with the extraction solution. The total amount of nicotine and menthol in each CFP was determined by GC-FID and the average amount in each different puffing no. was then calculated. Volatile components of each puff no. were collected by CFP followed by a single impinge with extract solution and qualitatively analysed by GC-MS.

The levels of nicotine and menthol in the aerosol of each HnB tobacco product differed from puff to puff. Product A showed higher variation between puffs than Product B for two compounds, a higher total release amount in aerosol was observed, and the patterns of each compound in six puffs were different between the two HnB products. The numbers of volatile components released puff-by-puff also showed some differences among puffs and between products.

## ST 10

### **Quantitative determination of selected carbonyls in tobacco, tobacco materials, e-liquids and emissions of conventional cigarettes and novel tobacco products (NTPs)**

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**Background/aims:** Carbonyls play an important role in the toxicological assessment of tobacco and nicotine containing products. Four commercially available tobacco and nicotine containing products (Conventional Cigarette/CC, E-cigarette/EC and two Heat-not-Burn Tobacco products/HnBT1 and HnBT2) were analysed with respect to the occurrence of eight carbonyls (formaldehyde, acetaldehyde, acetone, acrolein, propylaldehyde, crotonaldehyde, 2-butanone, butaldehyde).

**Methods:** The concentrations of the respective carbonyls were determined by HPLC/UV in tobacco, tobacco materials, e-liquids and emissions, i.e. mainstream smoke/aerosol and gas phase sampled in phosphate buffered saline (PBS) solution (as used for *in vitro* testing). Emissions generation was done with a linear smoking/puffing machine and carbonyls were trapped on a dinitrophenylhydrazine (DNPH) pre-treated Cambridge Filter Pad (CFP) as well as in impingers containing a PBS solution with DNPH.

**Results:** Six carbonyls were found in the CC, two in the e-liquid and four on two HnBT sticks. The CC exhibited the highest levels of all investigated materials. In the mainstream smoke/aerosol of the CC, 2 to 1,000 times higher carbonyl concentrations compared to the other three NTPs were measured. All eight carbonyls were determined in the smoke of the CC, whilst for the other three NTPs only six or five carbonyls were found. Quantitative analysis showed that for all four products, the major amount of carbonyls were formed during burning/heating (~90 %). The ratio of carbonyls trapped into PBS was between 0 % and 83 % and dependent from the analyte as well as product.

**Conclusion:** All three NTP materials exhibited lower levels of carbonyls compared to the CC. The concentration of carbonyls in the emissions of the CC were by far the highest of all products. This indicates that even for NTPs, carbonyls might not only be released from the material by a simple distillation process, but also formed during product usage.

## ST 11

### **Determination of the transfer of nicotine and selected ingredients into heat-not-burn tobacco product mainstream aerosol by using gas chromatography**

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Recently, tobacco manufacturing companies launched heat-not-burn tobacco products (HNBs) such as iQOS, Glo and lil. The aerosol of HNBs is generated by the heating of a tobacco type material below 350 °C. In conventional cigarettes the aerosol is formed through distillation and pyrolysis at temperatures from 200 °C up to 600 °C as well as through combustion at 900 °C or higher.

The construction of the consumable used to generate the aerosol of HNBs is usually similar to a conventional combustible cigarette, i.e. it consists of a tobacco/tobacco material rod and a filter section. In the tobacco material of HNBs, nicotine as well as ingredients such as menthol and humectants are typically found. The factors driving the aerosol generation as well as aerosol chemistry of HNB products are dependent on the device type (inter alia heating profile, operating time) and the consumable (inter alia blend type).

The analysis of the transfer of ingredients applied onto the tobacco/tobacco material of the HNB consumable into the aerosol is important to characterize HNBs. In our study we analyzed the aerosol constituents nicotine, menthol and humectants of HNBs by using gas chromatography. The transfer of the three constituents was determined by analyzing the respective concentration of the individual constituent in mainstream aerosol during puffing, in the tobacco material and filter section of the HNB consumable. In addition, we carried out puff-by-puff analysis to follow up the release dynamics of the three individual constituents. The results of our study are important to understand the release dynamics of nicotine as well as ingredients added to HNBs and can be used in the field of product development.

## ST 12

### **Puff resolved determination of selected constituents in the aerosol of commercially available tobacco heating products using photoionisation mass spectrometry**

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Commercially available tobacco heating products (THPs) are somehow hybrids between conventional cigarettes and e-cigarettes, as tobacco material is not burnt, but heated. Although the first THPs were coal heated and launched decades ago, currently available THPs are working mainly electrically.

Vacuum photoionisation (PI) is a reliable tool performing on-line, puff resolved analysis of smoking products. However, SPI (Single-Photon-Ionization) ionises a wide range of organic molecules and REMPI (Resonance-Enhanced-Multi-Photon-Ionization) focuses primarily on aromatic structures. Adapting the used REMPI wavelength supports spectroscopic approaches for analysis and can also allow the separation of isobaric compounds. Especially the comprehensive use of SPI and REMPI with several wavelengths enables a sophisticated analytical view into the release mechanism of a smoking product.

Within this study two commercially available THP devices were compared using 118 nm SPI as well as 248 nm and 266 nm REMPI. The devices show significant differences in puffing duration, total vapour amount and puff-by-puff nicotine release. Furthermore, the release dynamics and composition of other compounds and HPHCs (Harmful or potentially harmful compounds) varies between both devices. Furthermore, the puff-by-puff release profiles are highly related to the heating/temperature programme used in the THP devices. One device seems to exhibit an additional heating impulse for the second half of the puffs. Even if the substance release spectrum is more pronounced, the main organic compounds being released are glycerol and nicotine. All other compounds appear at a much lower concentration level. Although the absolute amount of individual compounds in aerosol are significantly different between both THPs, the results change if individual compound concentrations are normalized to total amount of gas phase/vapour or total amount of nicotine as reference values.

Overall, photoionisation MS, especially if SPI and REMPI is combined, is a well-suited tool for the investigation of THPs and other smoking/puffing products on a puff by puff on-line basis.



## ST 13

### Release mechanism of nicotine in heat-not-burn tobacco products

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In this paper, the release of nicotine from reconstituted tobacco, on which different amounts of glycerol and water were applied, was investigated at different temperatures. The thermal release behaviour of nicotine from reconstituted tobacco was verified by thermogravimetry-gas chromatography/mass spectrometry. The apparent activation energies of nicotine and glycerol released from reconstituted tobacco were calculated by classical physicochemical methods.

The release of nicotine from reconstituted tobacco below and above 180 °C was investigated to get insights into the mechanism of nicotine release.

It was found that the release of nicotine from reconstituted tobacco treated with glycerol became constant at heating temperatures above 180 °C. On the basis of the calculated results, the release path and mechanism of nicotine from reconstituted tobacco particles is postulated. The results showed that in heat-non-burn tobacco products, glycerol was not only a common aerosol forming agent, but could also promote the release of nicotine. The major reason for this finding is that glycerol and water in tobacco products would form a water-glycerol system and the bubbling point of this system is higher than that of pure water. In conclusion, nicotine mainly releases its non-protonated and monoprotonated species from reconstituted tobacco containing glycerol at a low temperature range, whilst nicotine releases its diprotonated species in reconstituted tobacco without glycerol at a high temperature range.

## ST 14

### Characterization of nicotine pharmacokinetic profile and subjective effect measures for e-vapor products relative to cigarette and nicotine gum

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The rate and extent of nicotine uptake along with subjective measures are commonly used to assess dependence potential of new tobacco products. We conducted two clinical studies with ~70 adult smokers (AS) each to evaluate the dependence potential of three mentholated e-vapors (mEVP, nicotine: 3.5-4.0 %) and three non-mentholated EVPs (nmEVP, nicotine: 2.5-4.0 %) relative to subject's usual brand cigarette (CIG) and Nicorette® Fresh Mint™ nicotine gum (4 mg, NG) by measuring nicotine pharmacokinetics, subjective effects, and product use behavior. AS used assigned products *ad libitum* for 4 hours followed by controlled (CTRL: 10-puff EVP/CIG, or one NG for 10-min) and uncontrolled use (UCTRL: *ad libitum* use of one unit for 10-min). Questionnaires on

Smoking Urges-Brief, Modified Cigarette Evaluation and Use the Product Again were administered in *ad libitum* use session. Blood samples and responses to Tobacco/Nicotine Withdrawal and Direct Effects of Product questionnaires were collected during CTRL and UCTRL. On average, during *ad libitum* session, subjects used 0.29-0.31 g (mEVP) and 0.27-0.32 g (nmEVP) e-liquid, 7-8 CIGs, and 2-3 NGs. The proportions of subjects who indicated they would use the product again ranged from 49 to 53 % (mEVP), 44-53 % (nmEVP), 72-75 % (CIG) and 33-34 % (NG). The plasma nicotine  $C_{max}$  (range, ng/mL) for mEVP (CTRL: 5.13-5.73; UCTRL: 7.66-7.93) and nmEVP (CTRL: 3.67-6.17; UCTRL: 6.94-10.97) were significantly lower ( $p < 0.05$ ) than CIG (CTRL: 11.62-12.39; UCTRL: 14.02-14.07) but higher ( $p < 0.05$ ) than NG (CTRL: 1.97-2.60; UCTRL: 1.85-2.59). The maximum reductions in "Urges to Smoke" score were significantly lower ( $p < 0.05$ ) for all EVPs under UCTRL and 3 EVPs under CTRL than CIG. The maximum scores for "Is the Product Pleasant Right Now" for all EVPs were statistically lower than CIG and greater (except 1 nmEVP under CTRL) than NG. We conclude that under the study conditions the dependence potential of the six EVPs is generally lower than cigarettes and higher than nicotine gum.

## ST 17

### **A clinical study investigating changes in exposure to cigarette smoke chemicals in U.K. smokers who switch to using a tobacco heating product for a five-day period**

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Smoking is a leading cause of numerous diseases, particularly lung cancer, chronic obstructive pulmonary disease and cardiovascular diseases. Consequently, there is a recognized need to provide smokers with alternative nicotine-delivery devices that have less chemical toxicants compared to conventional cigarette smoke. Tobacco heating products (THPs) hold great potential for reducing the harms associated with tobacco use. Therefore, a comprehensive scientific assessment is important to fully characterise the reduced exposure and/or reduced risk THPs offer to cigarette smokers.

A clinical study was carried out to test the hypothesis that biomarkers of cigarette smoke exposure are reduced when smokers switch from smoking commercial cigarettes to using THPs.

This clinical study, conducted in Belfast, U.K. (ISRCTN80651909), was approved by a local Research Ethics Committee and run in accordance with ICH-GCP. 150 healthy smokers smoked combustible cigarettes during a two-day baseline period, after which they were randomized to either continue smoking cigarettes, switch to using a THP or completely quit any nicotine or tobacco product use, for five days. Both baseline and post-randomisation 24-h urine samples were collected for biomarker of exposure (BoE) analysis and exhaled carbon monoxide was also measured daily.

Interim results from the urinary BoE and exhaled CO showed reductions in levels in subjects who switched to the glo™ device for five days, as well as reductions in the levels in subjects who abstained from any tobacco use for five days.

This study demonstrated that when smokers switched from smoking combustible cigarettes to using a THP, their exposure to smoke toxicants decreased, in many cases, to similar levels as cessation. These results suggest that THPs have the potential to be reduced exposure and/or reduced risk tobacco products. Further clinical studies are required to confirm the sustainability of these exposure reductions and the translation to reductions in smoking-related health risks.

## **ST 18**

### **Infrared thermal imaging applied to e-cigarette heater core temperature measurement**

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The core of most electronic nicotine delivery systems (ENDS) is a heater designed to aerosolize a nicotine containing liquid formulation. Accurate temperature measurement of the heater core can be difficult. Traditional thermocouple measurements are limited by their single point nature, and their tendency to act as a heat sink. Infrared thermal imaging (thermography) overcomes these limitations and can provide more information than a single point temperature measurement. We present a thermography method that can be used to measure the temperature of the entire heater core area of an ENDS in real time during puffing. Temperature measurements using our method have a spatial resolution of  $\sim 45 \mu\text{m}^2$ . We can record the temperature profiles during a puff as the hottest pixel versus time, or an average of all of the pixels within a set temperature range or spatial area. The technique allows us to visualize the heater core, and determine the homogeneity of heat delivered. We will present applications of this technique including measuring the rate of temperature rise, the temperature profile during use, the maximum and average temperature during a puff, and temperature versus liquid content in the device. Thermography can be coupled to other analytical techniques such as FTIR spectroscopy to monitor thermal degradation of the e-liquid during use. Our results show the formation of formaldehyde increases when the heater core maximum temperature approaches 350 °C.

## ST 19

### **Characterization of a temperature regulated e-cigarette for potential use as a reference device**

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The development and adoption of a well characterized reference e-cigarette would be useful during the routine analysis of e-cigarette emissions. One possible class of devices that might be suitable for use as a reference e-cigarette are temperature regulated devices. Temperature regulated devices control heating of the coil system by measuring changes in coil resistance during use. Temperature control may limit the production of thermal degradation products (TDPs) and dry puffing by the user.

In this study, we used 20 commercial devices fitted with a nickel coil inside a refillable tank. Devices were analyzed for total aerosol yield, and the formations of aldehydes. Devices were tested over a coil temperature range of 201 °C to 316 °C. A temperature of 300 °C was chosen for evaluation of device reproducibility and repeatability. Devices

were found to have a reproducibility of 20-37 % RSD in aerosol mass generation and 8-70 % RSD for formaldehyde/gram. Devices produced 20-300, 5-100, and 2-10 ug/gram of formaldehyde, acetaldehyde, and acrolein respectively. Furthermore, only 50 % of devices used were found to be reproducible with a RSD within 20 %. Devices were found to have a repeatability of 33 % RSD in aerosol mass generation and 84 % RSD for formaldehyde/gram. Devices produced 30-600, 10-300, and 2-40 ug/gram of formaldehyde, acetaldehyde, and acrolein respectively. Transfer efficacy studies were performed at a lower temperature (231 °C) with a 50:50 PG:VG 2 % nicotine e-liquid, spiked with formaldehyde and acetaldehyde. Devices showed good reproducibility with RSDs of 8.9 % and 9.1 % on a per gram basis for formaldehyde and acetaldehyde transfer. Results from this study showed that temperature regulated devices cannot be utilized to precisely produce thermal degradation products, but can be used as an analytical reference to accurately assess the delivery of aldehydes from liquids that contain known amounts of these compounds.

## ST 20

### **Evaluation of cigalike products with novel mouth-end configurations and novel e-liquids**

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Recently, cigalike e-vapor products have come on the market with novel mouth-end configurations and/or novel e-liquids. Moreover, mentholated e-liquids have been reported to increase pH-values and attractiveness. Consequently, we used the same glassmouth experimental setup as reported previously to study the effects of changes in design or e-liquid. Designs used included a 2-hole rectangular shaped device with prefilled cartomizers (5 % nicotine, menthol-like flavor, benzoic acid), 4-hole cylindrical device with prefilled cartomizers (4 % nicotine, menthol), and V2 EX blank cartomizers filled with custom-made e-liquids based on NicVape 50 mg/mL (5 %) nicotine in PG with additives (menthol, vanillin, for example at 1 % and benzoic acid at 2 %). For each sample, 50 puffs were taken using the square-wave puffing regimen specified in CRM 81. The glassmouth contained 10 mL 1700-0304 artificial saliva (pH ≈6.7). Puff-by-puff pH-values were obtained with a Hanna Instruments HI-1053B electrode connected to a Hach H260G meter with Data Logger software. The same instrumentation was used to determine the pH-values of the artificial saliva before and after exposure to the aerosols generated from the e-liquids. Final saliva pH-values were 2-hole rectangular, 6.69; 4-hole cylindrical, 7.11; menthol, 7.75; vanilla, 7.60; and benzoic acid, 7.08. Corresponding aerosol pH-values at 50 puffs were: 5.71, 7.40, 8.27, 7.64, and 7.12. Based on estimation of the nicotine content of the saliva (LC), the lower the saliva pH, the lower the nicotine content. The aerosol from the 2-hole rectangular device and the sample with 2 % benzoic acid (in V2 EX blank cartomizer) did not fill the glassmouth and tended to layer above the saliva. The aerosol from the 2-hole rectangular device was more translucent than the aerosols from the other samples.

## ST 21

### **Rapid determination of important indexes of e-cigarette liquid based on near-infrared spectroscopy and PSO-SVR algorithm**

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As e-cigarettes deliver nicotine without burning tobacco, they are a safer and less toxic alternative to conventional cigarettes and have quickly become a popular substitute for traditional tobacco cigarettes. Relative density and refractive index are two fundamental physical properties of the e-cigarette liquid and they are often used to confirm the uniformity and batch stability of the liquid. The values of relative density and refractive index are currently mainly determined by means of a density meter and refractometer respectively, which is time consuming and labour intensive. Therefore, the rapid determination of the values of the two parameters is important for the determination of the quality of e-cigarette liquids. In order to improve the detection efficiency of the two quality parameters, a novel near-infrared spectroscopy (NIR) combined with a particle swarm optimization-support vector regression (PSO-SVR) algorithm was applied to build the prediction models. Compared with the traditional partial least squares (PLS) models and principal component regression (PCR) models, the experimental results showed that the PSO-SVR models could obtain the best results with the determination coefficient ( $r^2 = 0.99495, 0.99375$ ) and RMSEP = 0.00204, 0.00151 for prediction sets. It indicated that the models could be applied for the rapid and accurate determination of the values of the two important quality parameters of e-cigarette liquid. The proposed method lays a foundation for the further implementation of online analysis of the relative density and refractive index and rapid determination of other quality parameters.

## ST 23

### **A statistical analysis of variability in polyaromatic hydrocarbons (PAHs) from reference and commercial cigarettes**

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This study was conducted to characterize the variability of mainstream PAH emissions data and to investigate the ability to detect differences amongst products. The mainstream yields of a reference cigarette (Kentucky Reference 3R4F) were obtained for a period of approximately one year in order to characterize variability in analysis results across 19 PAH compounds using estimates of repeatability and reproducibility.

For the compounds measured, eight had yields that were below the limit of quantification. Averaged quantitative yields ranged from 324 ng/cig for naphthalene, to less than 0.4 ng/cigarette for 5-methylchrysene and Dibenz(a,h)anthracene. Repeatability

ranged from 7.6 % for benzo[a]pyrene to 21.5 % for benzo[c]phenanthrene. The corresponding reproducibility estimates ranged from 43 % to 121 %.

Precision is largely dependent on the value of the number of replicates (n), since the standard error decreases as the number of replicates increases. To illustrate this, B[a]P emissions from the 3R4F, with a relative standard deviation of 8 % (most precise) and Cyclopenta(c,d)pyrene with a relative standard deviation of 21 % (least precise) were chosen. Given 5 replicate observations, the minimum detectable difference between the sample mean and the true population mean for B[a]P would be 7 % and 19 % for Cyclopenta(c,d)pyrene. However, if the sample size was increased to 20, the minimum detectable difference for B[a]P would be 3 % and 9 % for Cyclopenta(c,d)pyrene.

Additionally, potential year-to-year variability of mainstream PAH yields from four Canadian flue-cured cigarette brands, manufactured over a 30-year period, were tested. The year-to-year consistency of the yields per unit 'tar' of the four commercial flue-cured brands ranged in yield from 0.3 to 0.6 ng per mg tar. The yields were quite stable over the 30-year time frame. Thus, year-to-year product variation may not be a significant source of variability in mainstream PAH yields.

## ST 24

### **Temporal variability of analytical testing for e-vapor products and impact on number of replicates**

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In May 2016, the U.S. Food and Drug Administration (FDA) published a guide for industry "Pre-market Tobacco Product Applications for Electronic Nicotine Delivery Systems." Among other things the guidance document provided testing recommendations to support Pre-market Tobacco Application submissions for ENDS products, including a recommendation to generate data sets from a minimum of ten replicates per batch and from a minimum of three batches for product analysis.

To determine an appropriate number of replicates, it is important to consider the effect of the number of replicates on the uncertainty associated with the analytical result. For example, when all testing is conducted within a single laboratory, a component of the uncertainty of the analytical result is attributed to the temporal variability of the analytical method within that laboratory. That is, if two different samples are tested at two different times, temporal variability of the analytical method limits the precision of the comparison between the two samples. A consequence of temporal variability is that repeated testing in the short term has a rapidly diminishing effect on the uncertainty of the associated analytical measurement. Furthermore, if the standard errors of the analytical results are calculated without including temporal variability, the resulting standard errors can dramatically underestimate the actual uncertainty of the corresponding analytical results.

Estimating the temporal variability of some analytical methods indicates that conducting ten replicates in some analytes may be excessive.

## ST 25

### **Misperception of e-cigarette harm growing among American adults, 2013-2015**

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Electronic cigarettes (e-cigarettes) have been characterised as significantly less harmful than smoked tobacco by an increasing number of public health authorities. However, the proportion of American adults who perceive e-cigarettes to be equally or more harmful than traditional cigarettes has increased over the last few years.

To quantify this, an analysis of the U.S. Population Assessment of Tobacco and Health (PATH) study was conducted to examine changes in how adults in the United States perceived the relative harm from wave 1 (September 2013 to December 2014) to wave 2 (October 2014 to October 2015).

According to the survey, 65 % of adult smokers perceived e-cigarettes to be equally or more harmful than combustible cigarettes in 2015. This is a significant increase over the 54 % who reported the same perception in 2013.

The proportion of adult current and everyday smokers who believed e-cigarettes were just as, or more, harmful than smoking increased substantially from 43 % in 2013 to 57 % in 2015 (+14 %). This is despite the growing independent scientific evidence base reporting the relative safety of e-cigarettes compared to combustible tobacco during that time.

Misperceptions of the relative harm of e-cigarettes compared with conventional cigarettes need to be urgently addressed, particularly among smokers who may benefit from switching to e-cigarettes. Of the smokers that switched to e-cigarettes between 2013 (wave 1) and 2015 (wave 2), 95 % correctly identified e-cigarettes as being less harmful than conventional cigarettes.

If nothing is done to change the misperceptions of the relative harm of e-cigarettes compared with conventional cigarettes, based on current trends, it is estimated that in five years, 70 % of the U.S. smokers will perceive e-cigarettes as harmful as, or more harmful than, cigarettes.

## ST 26

### **Non-addictive factors in the use of tobacco and nicotine-containing consumer products**

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One explanation for the use of tobacco and nicotine containing consumer products is that various sensorimotor elements (e.g. taste, feel, ceremony) maintain a pleasurable, long-term habit. Recent experiences with nicotine-free consumer products and very low



nicotine content cigarettes do demonstrate that sensorimotor elements can be reinforcing but are, in general, insufficient to maintain long-term behaviour. Rather the presence and moreover the bio-availability of nicotine in sufficient quantities is generally acknowledged to be the main predictor of continued product use.

At the exposure levels associated with the use of tobacco and nicotine-containing products, nicotine exerts biological effects on several body systems leading to changes in cardiovascular, muscular, endocrine, and nervous system function. Accumulating evidence suggests that nicotine induced changes in central nervous system function - psychoactive effects - are central to the use of such products.

The commonly accepted hypothesis is that these psychoactive effects simply serve to mitigate the negative withdrawal experiences associated with nicotine dependence. Yet many users of tobacco and nicotine-containing consumer products fail to meet a dependence classification. This implies that product use is also motivated by factors other than those related to an underlying "addiction" and further suggests that the psychoactive effects of nicotine may be deliberately employed to achieve desired behavioural outcomes.

Cognitive enhancement and mood regulation have been identified as two key elements in decisions to use tobacco and (more recently) nicotine containing consumer products. This presentation will examine the existing scientific evidence to establish whether cognition can in fact be enhanced and mood regulated by nicotine and whether these effects serve as non-addictive factors in the use of these products.

## ST 27

### **Characterization of puff topography of adult conventional cigarette smokers and exclusive e-vapor users during *ad libitum* use of MarkTen® e-vapor products**

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**Introduction:** We characterized puff topography in adult exclusive e-vapor users [EV] and cigarette smokers [CS] on MarkTen® e-vapor products: (3 non-menthol [2.5-4.0 % nicotine] and 3 menthol [3.5-4.0 % nicotine]).

**Method:** CS (n=61, 52 % male) and EV (n=60, 52 % male) enrolled in the study self-selected into the non-menthol (n=30CS, n=30EV) or menthol (n=31CS, n=30 EV) group. On Day 1, subjects were randomized to use three products in-clinic for 90 minutes each. Subjects then used one product exclusively for one week and returned for in-clinic topography measurements on Day 8.

**Results:** Non-menthol group: The average on Day 1 in CS was 91.7-99.3 puffs across the three products; puff durations (PD) 2.1-2.2 seconds and puff volumes (PV) of 50.4-51.7 ml. The average on Day 8 in CS was 79.3-82.3 puffs; PD 2.3-2.4 seconds and PV 50.6-51.2 ml. The average on Day 1 in EV was 78.4-89.4 puffs; PD 3.2-3.6 seconds and PV 63.2-67.4 ml. On Day 8, the average in EV was 71.3-75.6 puffs; PD 3.7 seconds and PV 63.9-66.5 ml.

Menthol group: The average on Day 1 in CS was 57.9-68.5 puffs across the three products; PD 2.0-2.2 seconds and PV 43.0-46.8 ml. On Day 8, the average in CS was 53.6-61.5 puffs; PD 2.2-2.5 seconds and PV 46.1-51.2 ml. On Day 1, the average in EV was 68.2-80.0 puffs; PD 2.4-2.8 seconds and PV 48.4-53.5 ml. The average on Day 8 in EV was 59.3-63.2 puffs; PD 2.7-2.9 seconds and PV 53.1-55.7 ml. Statistically significant differences were observed between EV and CS for PD and between Day 8 and Day 1 for puff counts and PD in both groups. The product effect within the menthol group for PD and PV was significant.

**Conclusions:** 1) Our findings align with the current literature, EV took longer puffs than CS, and 2) both EV and CS took fewer puffs with longer durations after one week of product use.

## ST 28

### Measurement of puffing topography and mouth level exposure: Japan

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Background: BAT has developed the Tobacco Heating Product THP (1.0) which comprises an electrical heating device (glo™ device) and consumable tobacco rods (Neostiks™). A use behaviour study was conducted to demonstrate that consumers use this product in a way that exposes them to reduced levels of the substances from this product compared with a conventional cigarette.

Objectives: The primary objectives of this study were to (1) measure the puffing topography and (2) estimate the mouth level exposure (MLE) to NFDPM, nicotine and menthol to smokers of 7-8 mg (ISO) tar menthol and non-menthol cigarettes in Japan. The secondary objective was to (3) evaluate the potential blocking of the air inlet zone in the tobacco heated consumable (THP) whilst in use by the consumer.

Methodology: A four-arm study was undertaken in Japan to determine the puffing topography, mouth level exposure and average daily consumption by consumers of the tobacco heating product THP (1.0)X variants: the non-mentholated THP (1.0)T and the mentholated THP (1.0)M. The extent of lip blocking of air inlet holes while using THP (1.0)T was also assessed. Arms 1, 2, and 4 included smokers, and arm 3 included regular THP users.

Results: Smokers of 7-8 mg ISO nicotine free dry particulate matter (NFDPM) non-mentholated cigarettes took on average larger mean puff volumes from THP (1.0)T than from conventional cigarettes, but puff numbers and durations were similar. Mouth level exposure to NFDPM and nicotine levels were significantly lower when using THP (1.0)T than conventional cigarettes. Similar trends were observed among smokers of 7-8 mg ISO NFDPM mentholated cigarettes who used mentholated cigarettes and THP (1.0)M. Regular users of THP exhibited similar puffing behaviours as conventional smokers when using THP (1.0)T. None of the smokers blocked the air inlet holes when using THP (1.0)T.

## ST 29

### **Modification of standardized methods for the measurement of nicotine in very low nicotine content cigarettes**

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In March 2018, the U.S. Food and Drug Administration (FDA) issued an advanced notice of proposed rulemaking (ANPRM) for a nicotine product standard that would limit the amount of nicotine in combusted tobacco products. FDA requested comments regarding setting maximum nicotine levels in tobacco filler at 0.3 to 0.5 mg/g. FDA is also considering if this proposed product standard should specify a method for manufacturers to use for the determination of nicotine in very low nicotine content (VLNC) tobacco fillers. The ANPRM states that CORESTA developed a standardized method for the analysis of nicotine in unburned tobacco and tobacco products (CRM 62). However, CRM 62 has not been demonstrated to be fit-for-purpose for the analysis of tobacco fillers with nicotine levels in the range of 0.3 to 0.5 mg/g. Similarly, the ANPRM mentions that ISO 10315 is a standardized method that was developed for the analysis of nicotine in mainstream smoke, but as is the case with CRM 62, ISO 10315 has not been demonstrated to be fit-for-purpose for the analysis of VLNC smoke condensates. Our efforts were focused on lowering the limit of quantitation (LOQ) for these two methods. We will present the method modifications and validation results for CRM 62 and ISO 10315 for the determination of nicotine in VLNC tobacco filler and smoke condensate. The limit of quantitation for the modified methods was determined to be 0.02 mg/g and 0.01 mg/cigarette for CRM 62 and ISO 10315, respectively.

## ST 30

### **Analysis of small alkylamines in mainstream cigarette smoke**

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Small alkylamines, such as monomethylamine (MMA) and trimethylamine (TMA), are present in cigarette smoke at low levels as known combustion by-products of plant materials and are used in analytical determinations for primary aromatic amines. Analysis of these constituents may be of interest for tobacco-related product characterization. Due to characteristics such as high volatility, hydrophilicity, and low molecular weight, these constituents present a significant challenge for quantitation; methodology in the literature for these analytes in cigarette smoke as a matrix is limited. In order to develop a robust quantitative method, several platforms were explored including IC, GC-NPD,

GC-MS, GC-MS/MS and LC-MS/MS. Each platform presented challenges with regard to selectivity and/or sensitivity and complexity of sample preparation needed. We found that both analytes were present at 2-20 times lower levels than ammonia for both ISO and Health Canada intense smoking regimes and that mass spectroscopy detection provided the highest advantages for sensitivity and selectivity. MMA was found to require derivatization due to its low molecular weight and sensitivity issues by MS when measured directly. Stable calibration curves and good recoveries were obtained for 3R4F and 1R6F cigarettes. We would like to present our novel approaches to analysis to achieve higher sensitivity and selectivity, and to discuss platform limitations and comparisons.

## ST 31

### **Study on nicotine release from snus products with fiber-optic sensing detection technology**

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In order to strengthen the quality and safety control of snus products, the development and application of an optical fiber sensing drug dissolution analyzer were investigated to study nicotine release. An automatic testing method for the *in situ* release of nicotine from pouched snus was developed to evaluate the dynamic of nicotine release, and samples of four commercial products were characterised. Samples were placed in the settlement basket of a modified YR-1A optical fiber nicotine dissolution meter and then were analyzed following the operating process, finally the nicotine dissolution diagram was obtained and the level of nicotine release rate ( $\text{min}^{-1}$ ),  $\lambda$ , was calculated. Experimental results showed that after 45 minutes, the nicotine dissolution from samples became stable. The nicotine concentration/response relationship curve was linear within 10-80  $\mu\text{g/ml}$  and the correlation coefficient was 0.999. The coefficients of variation of nicotine release ranged from 3.4 % to 7.4 %. Approximately 62 %-94 % of nicotine in the samples was released, and the range of  $\lambda$  was between 0.02  $\text{min}^{-1}$  and 0.06  $\text{min}^{-1}$ . The design of the instrument integrated optical fiber technology, spectrum technology, computer technology and analysis technology as a whole. The input spectral signal of nicotine concentration was processed by computer automatically, and the sampling frequency and measurement time were accurately controlled at the second level. The designed optical fiber nicotine dissolution meter allows the *in situ* continuous, real-time and on-line analysis of nicotine release from snus products.

## ST 32

### **Characterization and certification of smokeless tobacco reference products**

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The constituents of raw tobacco products are naturally variable, and product design, the manufacturing process and the evolving science of tobacco analytics may increase this variability. The call for certified tobacco reference materials and their potential application in product research and regulation indicates a need to understand this variability. The United States Food and Drug Administration (FDA) awarded a Cooperative Agreement to the Center for Tobacco Reference Products at the University of Kentucky to produce and characterize four smokeless tobacco reference products including two snus, a moist snuff and a loose-leaf chewing tobacco. The statistical method for certification highlights the impact of measurement variability and laboratory differences on the certification process and therefore ultimately on the Certificate of Analysis. Characterization data from four ISO-accredited laboratories were used for the certification. Of the 14 parameters examined, acetaldehyde, formaldehyde, free nicotine, total nicotine, arsenic and benzo[a]pyrene exhibited larger than expected measurement variability, and crotonaldehyde was below the limit of quantitation for all samples. Graphics of the data and Cochran and Grubbs outlier analyses illustrate the types of variability experienced for the six constituents with large measurement variability, including consistent laboratory means with large between-lab variance and large repeatability standard deviations within a specific lab.

## ST 33

### **Comparison of potential health risks of combustible, heat-not-burn, and electronic cigarettes**

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Given U.S. Food and Drug Administration (FDA) regulation of tobacco products via the Family Smoking Prevention and Tobacco Control Act, there is a growing recognition of the need for evidence to assist both applicants and the FDA in making determinations of whether certain products may raise different questions of public health or are appropriate for the protection of public health. Quantitative risk assessment (QRA) is a useful tool to quantify relative public health impact. QRA was used to estimate cancer risk and cardiovascular, respiratory and reproductive or developmental toxic effects for conventional combustible cigarettes, heat-not-burn (HNB) cigarettes, and electronic cigarettes (e-cigarettes). Machine-generated yields of several harmful and potentially harmful constituents (HPHCs) specified by FDA in mainstream smoke or aerosol were obtained from the literature for representative products from the three different categories of tobacco products. Potential health risks were evaluated following the

standard QRA process, utilizing toxicity values from regulatory sources and standard default exposure factors from the U.S. Environmental Protection Agency and FDA, supplemented with assumptions to develop estimates of lifetime exposures. Emissions of HPHCs from HNB and e-cigarettes were considerably lower than those from cigarette smoke. Compared with conventional cigarettes and assuming comparable usage patterns, reductions in cancer risk were estimated to be 69 % for HNB and 99 % for e-cigarettes. In addition, reductions of 20 % to 99 % of cardiovascular, respiratory, and reproductive or developmental effects were estimated for HNB and e-cigarettes. These results demonstrate that estimated lifetime health risks are lower for HNB and much lower for e-cigarettes than those associated with smoking conventional cigarettes. Based on these findings, substituting conventional cigarette smoking with the use of HNB products or e-cigarettes will substantially reduce exposure to tobacco-specific toxicants and may reduce risk of cancer and non-cancer health effects for the individual user.

## **ST 34**

### **Potential health risks of exposure to toxic metals in e-cigarettes**

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As the use of e-cigarettes continues to grow worldwide, research on the potential health risks associated with these devices is also increasing. The National Academies of Sciences, Engineering, and Medicine recently concluded that while e-cigarettes are not without health risks, they are likely to be far less harmful than conventional cigarettes. Because e-cigarettes generate an aerosol by heating an e-liquid with a metallic coil, a possibility exists for transfer of metals from the coil to the aerosol. There is a growing concern that e-cigarettes may be a potential source of exposure to metals. The objective of this study is to evaluate potential health risks associated with exposure to metals in e-cigarette-generated aerosol in comparison with conventional cigarette mainstream smoke using quantitative risk assessment (QRA). Metal concentrations in machine-generated e-cigarette aerosol and conventional cigarette mainstream smoke were obtained from the literature. Potential health risks were evaluated following a standard QRA process, utilizing toxicity values from regulatory sources and standard default exposure factors from the U.S. Environmental Protection Agency (USEPA) and U.S. Food and Drug Administration (FDA), supplemented with assumptions to estimate lifetime exposures. Cadmium accounts for over 80 % of the health risk attributable to metals in conventional cigarette smoke. However, cadmium was rarely detected in e-cigarette aerosol, and other metals present in e-cigarette aerosol were reported at much lower levels than in conventional cigarette smoke. Estimated cancer risks of metals in e-cigarette aerosols did not exceed the USEPA's acceptable level of 1 in 1,000,000 and non-cancer hazards are 100 to 10,000 times below the USEPA acceptable level. These risk estimates for metals in e-cigarette aerosols represent greater than 99 % reduction compared with conventional cigarette smoke. Based on these findings, the potential health risk from exposure to metals from e-cigarettes is orders of magnitude below estimated risk from conventional cigarettes.

## ST 35

### **Different population modeling approaches lead to directionally similar conclusions about the potential for tobacco harm reduction**

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There are a number of dynamic population models that can be used to quantify the overall population health effects of beneficial and/or harmful changes in tobacco use patterns. These models can be broadly categorized as either following a single birth cohort - consisting of individuals who are initially the same age and who begin as non-users of tobacco products - to a specific age; or, following a mixed cohort - consisting of individuals of different ages and with different tobacco use histories - to a specific calendar year. The dissimilar approaches employed by these models can lead to different estimates for population effects, and thus present challenges to policymakers wanting to make informed decisions regarding the introduction and/or promotion of less harmful alternatives to cigarettes. Comparisons between modeling estimates are further complicated by incompatible terminology and definitions. We have explored methodological differences between models, including the choice of outcome measures (smoking prevalence, survivors, premature deaths or smoking-related mortality), the choice of exposure measures (tobacco use status, duration or amount), and the estimation of mortality rates. Given similar input data, underlying assumptions and outcomes of interest, the single cohort and mixed cohort models provide different estimates for population health effects. However, these differences are unlikely to be extreme enough to lead to directionally dissimilar conclusions regarding the potential for tobacco harm reduction. Findings from this research show that different population modeling approaches using similar input data lead to directionally similar conclusions about the potential for tobacco harm reduction.

## ST 36

### **Multi-dimensional tipping point analyses: Assessing simultaneous shifts in tobacco use patterns from a higher to a lower risk product**

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The Dynamic Population Modeler (DPM(+1)) employs a birth cohort framework to estimate effects on population mortality if tobacco use patterns shift from a higher- to lower-risk product. DPM(+1) allows for evaluation of changes in use patterns within the context of 'tipping point' analyses, which estimate the magnitude of a beneficial use pattern needed to offset the population effect of harmful use patterns. In a birth cohort

followed from age 13 to age 72, in 5-year intervals, we specified transition probabilities for a counterfactual scenario whereby 3 % of individuals who would have never used tobacco instead initiate modified-risk tobacco product (MRTP) use (up to age 27; *additional initiation*), and 25 % of those MRTP initiators transition to cigarette use (next age interval; *gateway effect*). Assuming a 92 % reduction in all-cause mortality risk for the MRTP (relative to cigarettes), population harm was offset if at least 1.6 % of smokers who would have continued to smoke instead switched to MRTP use (each age interval, for ages 18+; *switching*). In addition, ranges of probabilities for up to three transitions can be assessed simultaneously. Assuming 1-10 %, 0-50 % and 0-10 % for *additional initiation*, *gateway effect* and *switching*, respectively, 2 % *switching* offset the population harm resulting from, for example, 4 % *additional initiation* with 20 % *gateway effect*, 5 % *additional initiation* with 10 % *gateway effect*, and 6 % *additional initiation* with 4 % *gateway effect*. Tipping point analyses allow regulators to assess the magnitude of simultaneous changes in use patterns likely to result in an overall population benefit or harm. Such analyses may reduce the immediate need for empirical projections of beneficial and/or harmful changes in use patterns during regulatory decision making.

## ST 37

### **Assessing the population health impact of authorizing the marketing of a smokeless tobacco product with a proposed modified risk claim**

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Computational models can be used to predict population effects following the market authorization of a tobacco product with a modified risk claim. Using best modeling practices we developed and validated a population model. Since moist smokeless tobacco (MST) is primarily utilized by males, the Markov compartmental model is based on a theoretical cohort of one million males starting at age 13 and followed to age 73, accounting for various transition states with defined transition probabilities. To determine the survival probabilities from using a MST product (candidate product), mortality models were coupled with excess relative risk. Results are presented as the difference in number of survivors and years of additional life expected by comparing a Base Case (where cigarettes and MST products are available under the existing scenario) and Master Case (where the candidate product is available with a modified risk claim authorized by FDA). Nationally representative transition probabilities were used for the Base Case. A Master Case scenario was estimated from a study involving 3,290 male participants, where we measured the percent difference between the relevant responses of a test group of users and non-users of tobacco products (exposed to the modified risk claim associated with the candidate product) and control group (exposed to the candidate product without the modified risk claim), and then applied the percent difference to the Base Case transition probabilities. The estimated outcome of



authorization of a modified risk claim for MST is 1120 premature deaths prevented with 32,856 additional years of expected life. Extending inferences from a single-cohort to multi-cohorts, leads to ~93,000 premature deaths prevented over a 60-year period. Our results suggest that a net benefit to the population can be expected upon market authorization of the candidate product. Limitations of model predictions should be taken into consideration when drawing inferences from these results.

## ST 38

### **Impact of cigarette paper parameters on smoke yields of cigarettes with different circumferences**

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Cigarettes with reduced circumference are very popular in several markets. Over the last decade a significant growth rate has been observed worldwide.

Changing the circumference has an influence on the physical properties of the unlit cigarette and therefore the burning characteristics and smoke chemistry of the lit cigarette. Following this, it is important to understand the effects of a reduction in circumference as well as changes in physical paper properties on the burning characteristics and smoke chemistry. The aim of this study is to investigate the influence of physical cigarette paper parameters on the smoke yields, combustion temperature and ash appearance of cigarettes having different formats.

In a first step, 11 cigarette papers were produced varying the basis weight (25 g/m<sup>2</sup> - 35 g/m<sup>2</sup>), air permeability (30 CU-70 CU) and filler content (30 %-40 %). The burn additive type and concentration were kept constant. Afterwards filterless cigarettes were produced in three different formats (King Size (KS), Demi Slim (DS), Super Slim (SS)) using the same tobacco blend and the same tobacco density.

The influence of the physical cigarette paper parameters on smoke yields and burning characteristics is qualitatively similar for all cigarette formats. The absolute yields are significantly different, e.g. when increasing air permeability CO yields for KS cigarettes are decreasing from approximately 12 mg/cig to 10 mg/cig and for SS cigarettes from approximately 8 mg/cig to 6 mg/cig. The CO to CO<sub>2</sub> ratio is higher for KS cigarettes compared to SS cigarettes. As expected the ash appearance of SS cigarettes is significantly better than that of KS cigarettes.

The results show that physical cigarette paper parameters are a relevant factor for the design of cigarettes of all formats.

## ST 39

### Impact of cigarette paper and blend on super slim, slim and king-size cigarette designs

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Superslim (SS) cigarettes account for 5 % of total cigarette sales on the Chinese market, with a year to year increase of 2.1 %. Similar to this fast-growing trend, many more brands of slim (SL) cigarettes have been offered on the market since 2017. To further understand the impact of various cigarette components, a cigarette matrix was designed with tobacco blends used on the domestic market. Cigarette papers with 50 CU and 75 CU and ash conditioner of 0.75 % and 1.5 % mixed citrate were used. Cigarettes in three sizes SS, SL, and King-size (KS) were manufactured with inclusion levels of 3.3 %, 8.8 % and 17.2 % of reconstituted tobacco. Cigarette samples were analysed for their physical properties, tobacco blend, and smoke deliveries using the ISO smoking regime. The results showed that: a) encapsulated cigarette pressure drop followed the order: SS > SL > KS designs; b) tar and nicotine deliveries were lower as reconstituted tobacco inclusion level increased, total deliveries of tar and nicotine were proportional to the weight loss during puff and decreased with decreasing circumference; c) carbon monoxide (CO) deliveries were significantly lower with higher citrate inclusion, higher paper porosity (75 CU vs. 50 CU) may also reduce CO deliveries; d) overall deliveries of SS cigarettes were lower than SL which were lower than KS cigarettes. In conclusion this study provides more insights into the selection and use of components for the designs of cigarettes with reduced circumference.

## ST 40

### Transfer of cigarette paper ingredients into mainstream smoke

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To study the transfer of ingredients added onto cigarette paper into mainstream smoke, three ingredients, levulinic acid (LA), ethylvanillin glucoside (EVG) and encapsulated ethylvanillin (EnEV), were added to cigarette paper. King-size (KS) and superslim (SS)

cigarettes with specifically flavoured cigarette paper were manufactured. The smoke deliveries of the two (KS and SS) cigarettes were determined under the ISO smoking regime. The amount of nicotine and the ingredients, LA and ethylvanillin (EV), were measured in mainstream smoke and filter tips. The results indicate that: 1) the transfer rates of the ingredients from paper into mainstream smoke are  $LA > EnEV > EVG$ ; 2) the ratio of the total amount of ingredients to nicotine ( $LA_{\text{smoke+filter}}/\text{nicotine}_{\text{smoke+filter}}$ ,  $EV_{\text{smoke+filter}}/\text{nicotine}_{\text{smoke+filter}}$ ) from the SS cigarettes are significantly higher than those from KS cigarettes; 3) the ratio of the ingredients between mainstream smoke and filter tips ( $LA_{\text{smoke}}/LA_{\text{filter}}$  and  $EV_{\text{smoke}}/EV_{\text{filter}}$ ) is much lower than the ratio of nicotine between mainstream smoke and filter tips ( $\text{Nicotine}_{\text{smoke}}/\text{Nicotine}_{\text{filter}}$ ).

Our results provide some evidence that ingredients on cigarette paper are released differently into mainstream smoke when compared to constituents in tobacco filler. The chemical/physical properties of the ingredients as well as their spatial position on the cigarette paper during combustion are the main drivers for the observed release behavior of ingredients from cigarette paper. Furthermore, our results clearly indicate that the impact of the cigarette paper on emissions is significantly higher for cigarettes with reduced circumference (SS) than for KS cigarettes.

## ST 41

### **Functional filter wrapping materials and their impacts on specific properties of factory-made cigarettes and heated tobacco products**

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Filter wrapping materials are a synonym for mouthpiece covering sheets that include tipping and filter plug wrap paper. Both sheets play an essential role for the manufacturing of conventional filter cigarettes and heated tobacco products (HTP) as they connect the filter plug with the tobacco rod, control the level of smoke constituents through permeability and shape the geometrical dimensions of smoking articles. In the present study, additional functionalities of filter wrapping materials will be demonstrated together with their potential application for factory made cigarettes (FMC) and HTP and their respective influence on selected product features. The individual functionalities are based on tactile, haptic, sensory and physical effects, in which the latter are focused on smoke yields and temperature reducing material properties. In order to enhance the haptic interaction between the consumer and the FMC/HTP as well as the tactile comfort during the smoking process, mechanical embossing of tipping paper, a velvety surface coating and a hydrophobic lip-release varnish will be discussed. Since tipping paper represents a non-burnt component of FMC/HTP, it will be shown that flavours can be applied for the stimulation of the primary tastes inside the oral cavity without toxicological concerns from pyrolytic side products. Finally, the application of active substances on filter plug wrap paper will be introduced to reduce gaseous deliveries and the thermal energy of the HTP smoke. The efficiency of these active agents will be investigated under pilot plant test conditions and underlined by quantitative results.

## ST 43

### On-line puff resolved analysis of cigarette smoke, e-cigarette vapour and vapour of tobacco heating products

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Photo ionisation-time of flight mass spectrometry (PI-TOFMS) has been established for on-line analysis of complex gas mixtures. Cigarette smoke, e-cigarette vapour and the vapour of tobacco heating products provide good examples for such complex gas mixtures. Many toxicants, such as butadiene, acetaldehyde, naphthalene, phenol or polycyclic aromatic hydrocarbons (PAH), can be detected with single puff-resolution in the smoke or vapour of smoking/vapour products.

Vacuum PI-MS can be divided into SPI (Single-Photon-Ionization) ionizing a wide range of organic molecules and REMPI (Resonance-Enhanced-Multi-Photon-Ionization) focusing primarily on aromatic structures. Especially the more sophisticated complementary use of SPI and REMPI can access a new information range.

By focusing the sampling on the origins of substance release or formation it is possible to achieve temporal and spatial resolved analysis of released gas mixtures. This allows, for example, to obtain access into the burning zone of a cigarette with a temporal resolution of 0.1 seconds and a spatial resolution of 0.5 mm.

If a sum information of a whole puff is sufficient, the substance identification and quantification can be improved by using a fast GC approach. A single puff can be collected on a column to analyse it in the delay between two puffs. Depending on the used puff regime, the GC parameters and its separation capabilities can be optimized.

Various smoking/vaping products, ranging from conventional cigarettes to tobacco heating products and e-cigarettes, were investigated using PI based MS approaches. Also, semi-legal THC containing smoking products such as joints were investigated on a puff resolved basis.

In summary, the release of desired active compounds (e.g. nicotine or  $\Delta^9$ -THC) and undesirable HPHCs (harmful or potentially harmful compounds) is related beyond the product category to a wide set of parameters such as puff regime, environmental conditions and technical parameters. The puff resolved release profiles enable a reliable understanding of release and reaction pathways.

## ST 44

### **Time and spatially resolved *in situ* determination of temperature, pressure and gas phase concentration of selected smoke constituents inside a burning superslim cigarette. Part I – Thermophysical mapping**

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Superslim cigarettes in China are a relevant product category on the market. Accurate determination of key thermo-physical and -chemical parameters in superslim cigarettes are essential for the mechanistic understanding of combustion as well as pyrolysis processes. The objective of this study is to combine *in situ* temperature and pressure measurement techniques with a fast sampling technique to obtain detailed physical and chemical information within a burning superslim cigarette. The first part of this comprehensive mechanistic study on superslim cigarettes presents the results of the temperature and pressure profiles inside the burning cigarette. A reference superslim format test piece (circumference: 17 mm) was mounted on a micrometre test bench to ensure precise insertion of an array of 0.254 mm thermocouples for gas-phase temperature and another multiple 0.35 mm diameter quartz tubes for pressure measurements. For chemical characterization, a single heated 0.5 mm sampling microprobe, coupled to a single-photon soft ionisation (SPI) mass spectrometer, was also inserted at different sampling points. Synchronisation among the different measurement techniques was achieved by mapping two probes at the same time (i.e. temperature and pressure or temperature and chemistry). Dedicated software was developed to integrate the data sets from multiple sampling points for visualization of the synchronized physical and chemical data. The highly heterogeneous combustion system in a superslim cigarette was mapped for the first time to obtain a fundamental thermophysical understanding. The data from temperature and pressure measurements were used to calculate the axial gas velocity, which is a key parameter in understanding the complex toxicant formation processes.

## ST 45

### **Time and spatially resolved *in situ* temperature and pressure measurements with soft ionisation mass spectrometry inside a burning superslim cigarette. Part II – thermochemical mapping**

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Superslim cigarettes are an increasingly popular category of cigarettes in China. Accurate measurements of key thermophysical and thermochemical parameters in superslim cigarettes is essential for mechanistic understanding of pyrolysis processes, thermal desorption, combustion properties and quality control. The second part of a comprehensive mechanistic study on superslim cigarettes investigated the thermochemical reactions reflected by selected key combustion and pyrolysis marker compounds, all mapped using a fast *in situ* chemical sampling technique. In addition to the integrated temperature and pressure sensors presented in the first part of this work, a fast *in situ* chemical sampling technique was also synchronised with these sensors and used to obtain detailed chemical information from this dynamic combustion system in response to the ISO airflow protocol. The single heated 0.5 mm chemical sampling microprobe, coupled to a single-photon soft ionisation (SPI) mass spectrometer through a heated transfer line, was also inserted to extract highly time and spatial resolved data from the superslim combustion system. The novel approach allowed the comprehensive description as a synthesis of complex and dynamic varying data of the main thermophysical and thermochemical phenomena. Time-resolved mapping of e.g. benzene, nicotine and NO was used to illustrate thermal desorption, combustion and pyrolysis as formation-led chemical reactions. Furthermore, degradation pathways of e.g. nicotine to indole and ammonia can be spatially correlated to temperature and flow distribution conditions in the cigarette.

## ST 46

### Numerical simulation of cigarette smoking process

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A comprehensive two-dimensional (2D) mathematical model was developed to simulate the process of cigarette smoking. The developed model features four characteristics: (1) adopting kinetic models for water evaporation, tobacco pyrolysis and char oxidation; (2) applying the mathematical relationships between the release amounts of certain products (i.e. tar and CO) and different reaction variables (i.e. temperatures and oxygen concentrations); (3) introducing the transfer of mass, energy and momentum; (4) considering the filtration effect of cigarette filter to tar. The said characteristics are expressed through a set of simultaneous equations that can be numerically solved by Fluent software. The temperature field, char density field, flow velocity field in cigarettes during smoking, the CO and tar density fields in mainstream smoke and the filtration efficiency of filter to tar were simulated via the model. The model was validated by comparing the predicted values of site-specific temperature in cigarettes, puff number, filtration efficiency, CO and tar release with the corresponding experimental data. The results showed that the predicted values correspond well with the experimental data, for example, the predicted puff number was 7 and the experimental one was 6.2. The relative deviations between the predicted and experimental values of the site-specific temperature, CO release, tar release and filtration efficiency are <18 %, 6.4 %, 1.8 % and 3.4 %, respectively.

## ST 47

### **Study on reducing the fragmentation rate of paper-making reconstituted tobacco during the cutting process in the cigarette factory**

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It is known that reconstituted tobacco leaf tends to generate dust and particles during the cutting process in the cigarette factory. To solve the issue, analysis of the composition structure of manufacturing waste was carried out. Effects on the fragmentation rate of reconstituted tobacco base sheet were studied with the following factors: 1) beating degree; 2) use of additives in the pulping process; and 3) use of calcium carbonate. The analysis showed that the composition of the manufacturing waste included detached fibers from the edge of reconstituted tobacco, ash from the raw tobacco materials, particles and powder produced by the tobacco pellets, calcium carbonate, and a small quantity of short and small fibers lost during the beating of tobacco stem and wood pulp. There is a negative correlation between the fragmentation rate of reconstituted tobacco base sheet and the beating degree. When the beating degree increases, the fragmentation rate during cutting decreases. The use of guar gum in the pulping process can effectively reduce the fragmentation rate. In conclusion, this study provides potential solutions for reducing the impact of the stated problem, and improving the efficiency of utilizing paper-making reconstituted tobacco.

## ST 48

### **Research on colour improvement of paper-making reconstituted tobacco**

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Paper-making reconstituted tobacco (PRT) is an important raw material for the tobacco industry, with various benefits including reducing cost, smoke tar delivery, and impact on the environment. The different appearance of PRT compared to regular cigarette tobacco blend has been observed by the consumer. In this study, a method with a colorimeter was developed for the measurement of colour difference. Some key technical methods were taken to reduce the colour difference, such as modifying the coating ratio and the



level of calcium carbonate and using concentrated liquid after Maillard reaction. The effect of storing environment on the colour of PRT was also studied. The results showed that: a) with the increase of coating rate, the colour of PRT grew darker and became closer to the colour of cigarette tobacco blend; b) the increase of calcium carbonate makes PRT lighter; c) adding concentrated liquid after Maillard reaction is a very important and useful method to narrow the colour difference between PRT and cigarette tobacco blend without adding any other additives; and d) the colour of PRT is influenced by such factors as storage time, temperature and humidity. The impact of storage conditions is small, therefore, conventional storage conditions can satisfy the requirements of PRT. In conclusion, this study provides a science-based understanding of the colour differences between PRT and cigarette tobacco blends, and practical approaches to resolve the issue.

## **ST 49**

### **Digital characterization of atomization characteristics of nozzle for tobacco casing**

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The atomizing process at the nozzle during application of casing materials to tobacco was investigated in order to more uniformly spray casing on tobacco and to further improve the efficiency of tobacco casing. The atomization characteristics, such as atomization cone angle of the nozzle, atomized particle size and particle size distribution, were studied by digital characterization with a high-speed camera and image recognition processing technology on a 30 kg/h atomization test platform. Moreover, the division of the atomization zone at the nozzle in various sections was studied on the basis of characteristic droplets that could be absorbed by tobacco more easily. The results showed that: (1) the atomization cone angle, the atomized particle size and particle size distribution could be visualized in digital form by a high speed camera and image recognition processing technology; (2) the particle size range for characteristic droplets was determined as being 0.05 mm to 0.8 mm; (3) on the basis of characteristic droplets, the atomization zone of the nozzle was divided into three sections, namely an injection section, an atomization section and a diffusion section; (4) the results of this study provide a new method and technology for improving the efficiency of tobacco casing.

## ST 50

### **The development and application of near-infrared spectroscopy analysis of the network system of the tobacco leaf**

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Based on the basic mode of "Internet + near-infrared fast detection technology + tobacco", the near-infrared spectroscopy analysis of the network system of tobacco leaf raw materials was constructed with the "trinity" of near infrared fast analytical technology, network technology and chemometrics multidimensional data mining technology. A new model of "digitized support for rapid analysis and evaluation of tobacco quality" was developed. The unified management and sharing application of tobacco raw material data and information in the main producing areas of Yunnan Province of China has been achieved.

First of all, a standardized network laboratory of near-infrared spectroscopy analysis was established according to China Metrology Accreditation (CMA) requirements and near-infrared analysis related standards, so as to ensure the reliability and accuracy of near-infrared analysis data.

Secondly, near-infrared analysis data and information from each near-infrared spectroscopy laboratory were integrated. The near-infrared spectroscopy network platform was established based on the Web 2.0 mode of technology of the Hadoop Cluster/Oracle Database, which is supported by massive information (big data). Up to now, the Cloud Database consists of nearly 0.7 million tobacco leaf data collected in the main tobacco planting areas of Yunnan Province, and has been used in the various functional departments of the Technology Center of the China Tobacco Yunnan Industrial Co., Ltd.

Thirdly, a data mining analysis system was established by integrating the advanced spectrum processing method of chemometrics, the multivariate quantitative analysis method and the pattern recognition qualitative analysis method. It provided technical support for explaining the attribution to tobacco raw material quality and discovering the inherent quality law.

## ST 51

### Flavour migration through capsule shell

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A capsule is a widely used flavour carrier in cigarettes. It is a very effective tool to deliver menthol and other flavours to mainstream tobacco smoke. According to observations from our previous studies, capsules tend to develop hairline cracks after a certain period of time in an open cigarette pack. This study measured and compared flavour migration through different capsule shells with various flavours. All capsules used in this research were commercially available and widely used in the industry: animal and non-animal based.

Three impingers were used to evaluate flavour migration through capsule shells. The first one was filled with 50 g of the capsule being analysed, the second one with methanol as solvent and the third was used to capture solvent vapours. Fixed air flow was adjusted through impingers for a certain period of time. The volume of aliquot was measured and checked for the presence of flavours by Agilent 5973N GC-MS each day. Flavour migration rate was calculated for each product. The study showed that the type of capsule shell and flavour had a significant influence on flavour migration through capsule shells.

This study provides very practical and insightful data about shelf life and flavour migration from capsules.

## ST 52

### Consumers' perceptions of disease-specific modified-risk claims are best evaluated in a disease-specific manner

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Manufacturers seeking to make claims about modified-risk tobacco products (MRTPs) must assess consumers' perceptions of risk reduction for these products. Claims made for the MRTP and assessment of perceived risk reduction can be global (e.g. "reduced harm" and assessment of a broad spectrum of risks) or disease-specific (e.g. "reduces oral cancer" and assessment of oral cancer risk). We assess whether consumers respond to disease-specific modified-risk claims in a disease-specific manner. U.S. adult current, former and never tobacco users (n = 9,830) recruited from online research panels viewed advertisements stating that smokers who switch completely to Camel Snus (CS) could reduce their risk of lung cancer; a random half of respondents also viewed a claim for reduction of oral cancer risk. Respondents then rated the risk for lung cancer and oral cancer on a 1-7 scale, separately, for CS and cigarettes; the difference in the respective tobacco product types indicated perceived risk reduction. Advertisements also included four government-mandated smokeless tobacco warnings, randomly rotated. We

compared those who viewed a warning about mouth cancer with those who viewed a warning about addiction. Findings indicated that the presence or absence of an oral cancer claim had no effect on perceived risk reduction for lung cancer (both groups,  $-1.8 \pm 0.02$ ). With regard to warnings, respondents who viewed the mouth cancer warning reported no risk reduction for oral cancer ( $0.0 \pm 0.03$ ), whereas those who viewed the addiction warning perceived lower risk for oral cancer ( $-0.4 \pm 0.03$ ), especially if they viewed the reduced-oral-cancer claim ( $-0.6 \pm 0.04$ ). These findings indicate that consumers respond to disease-specific warnings and risk-reduction claims in disease-specific ways, and suggest the value of evaluating risk perceptions in a disease-specific manner.

## ST 53

### Comparing direct and indirect assessments of perceptions of reduced risk for a modified-risk tobacco product

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Manufacturers seeking to make claims about modified-risk tobacco products (MRTPs) must assess consumers' perceptions of absolute and relative risks for these products. We cross-validate data from two methods: (a) indirect assessments based on the difference between absolute risk ratings for the MRTP and cigarettes; and, (b) direct assessments comparing the MRTP to cigarettes. We also examine indirect assessments of consumers who, on direct assessment, indicate they "don't know" the risk of the MRTP relative to cigarettes. U.S. adult current, former and never tobacco users ( $n = 18,234$ ) recruited from online research panels were shown advertisements stating that smokers who switch completely to Camel Snus (CS) could reduce their risk of lung cancer and respiratory disease. Respondents rated the absolute risks of CS and cigarettes, separately, on a 1-7 scale, and directly compared the risks of CS versus cigarettes for each disease. For lung cancer, the mean difference in absolute ratings for CS and cigarettes was  $-0.4 \pm 0.02$  for those who indicated no difference in risk, based on direct assessment;  $-2.1 \pm 0.02$  for those who indicated a lower risk for CS;  $-3.4 \pm 0.04$  for those who indicated CS presented no risk at all; and,  $-1.0 \pm 0.04$  for those who responded "don't know." Results were similar for respiratory disease. The tight correlation between direct and indirect modes of assessing perceived risk indicates reliability and validity for both methods. That respondents who indicated "don't know" in the direct assessment provided absolute ratings intermediate between 'same as smoking' and 'less risk' indicates their perceptions are very conservative; they do not believe CS is completely safe. The findings confirm the validity of these approaches for assessing perceived risks of MRTPs.

## ST 54

### **A cross-sectional survey to assess tobacco and nicotine product use behaviour in Japan**

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The rapid emergence of next generation products (NGPs), with their potential to reduce exposure to harmful substances, has substantially changed the tobacco and nicotine landscape in recent years. The impact of introducing NGPs on individuals and the population as a whole can change over time due to a variety of factors, including changes in tobacco use behaviour, consumer perceptions, and changes in the tobacco product marketplace. Population studies that generate data to demonstrate the public health benefits of introducing these NGPs are required, taking into account both users and non-users of tobacco products.

This cross-sectional survey aims to examine the current tobacco use behaviour in Japan with special focus on tobacco heating products (THPs). Product misuse, risk perception and health-related Quality of Life (QoL) will be evaluated under real-world conditions.

A face-to-face administered paper-based questionnaire is used for data collection. In order to create an environment which allows respondents to document real tobacco product use behaviour, the questionnaire will be self-administered. For the selection of 4,000 participants in our first wave study, a geographically stratified three-stage probability sampling will be applied. The sampling universe comprises all persons aged 20 years and older living in private households in three selected cities.

The survey comprises four main sections: sociodemographic information; lifetime tobacco use behaviour by product type; risk perception; and health-related QoL. Prevalence data generated from the survey will describe the use behaviour of never, current, and former users of tobacco products with an emphasis on THP users. In the absence of epidemiology, analytical modelling of survey data can be used to predict the population health impact of introducing a THP into the market place.

## ST 55

### **Determination of tobacco alkaloid enantiomers using reversed phase UPLC/MS/MS**

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N'-Nitrosornicotine (NNN), one of the carcinogenic tobacco-specific N'-nitrosamines (TSNAs), is on the Food and Drug Administration's list of harmful and potentially harmful constituents (HPHCs). Nornicotine, a compound of the demethylation of nicotine, is the

immediate precursor for NNN formation. Nicotine, nornicotine and NNN are optically active. The accumulation of the isomers of nicotine, nornicotine and NNN impacts their biological activity. Quantitative analysis of isomer alkaloids in tobacco is important for understanding the metabolomic mechanism and composition changes of the enantiomers of nicotine and nornicotine and potentially its biological activity. The objective of this study was to develop and validate a method to quantify the enantiomeric composition of the alkaloids in tobacco.

In this study, the method of determination of tobacco alkaloid enantiomers (including nicotine, nornicotine, anabasine and anatabine) in tobacco samples using a reversed phase ultra-performance liquid chromatography-tandem mass spectrometer (UPLC/MS/MS) has been developed. Tobacco samples (0.2 g) in 10 ml 70 % methanol were shaken for one hour on a reciprocal shaker. Extracts were filtered through a 0.22  $\mu\text{m}$  PTFE filter to remove the tobacco powder and then diluted 100 times before injection into the LC/MS/MS. All analyses were done on Waters ACQUITY UPLC H-Class System equipped with Xevo TQD triple quadrupole mass spectrometry. CHIRALPAK AGP and LUX-Cellulose-2 columns were used.

The method validated in this study is simple, fast and sensitive for the quantification of alkaloid enantiomers in tobacco leaf and has been applied to investigate the ratios of tobacco alkaloid enantiomers in different tobacco lines and tobacco products.

## ST 56

### **Photoionization time-of-flight mass spectrometry as a tool for the identification of selected isomers in the gas phase of mainstream cigarette smoke**

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To identify selected isomers in the gas phase of mainstream cigarette smoke, an on-line measurement system with a photoionization unit was built. With this instrument, photoionization efficiency (PIE) curves for constituents in the gas phase of mainstream cigarette smoke were established. The before mentioned system is set up as follows: single-port smoking machine, three-port injector and synchrotron radiation vacuum ultraviolet single photon ionization time-of-flight mass spectrometry. The PIE curves of a calibration gas containing 1,3-butadiene, 1,2-butadiene and 1-butyne as well as the gas phase constituents in mainstream cigarette smoke derived under the ISO (International Organization for Standardization) smoking regime were separately determined between 8.0 and 10.6 eV. The isomers of the earlier mentioned three compounds in the calibration gas and the gas phase constituents in mainstream cigarette smoke were characterized by experimental PIE curves and multiple linear regression (MLR) method. Our results showed that PIE curves combined with the MLR method were not only capable to provide a substantial and accurate characterization of the isomers in the gas phase of mainstream

cigarette smoke, but also to determine the mole percentages of each isomer at the same mass-to-charge ratio. These results can be used as the basis for further qualitative and quantitative analysis of gas-phase constituents in mainstream smoke by applying on-line and real-time analysis with soft ionization mass spectrometry, especially synchrotron radiation photoionization mass spectrometry.

## ST 59

### **An inter-laboratory comparison study focused on the determination of total NNAL in human urine**

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In an ongoing effort to fulfil the 2<sup>nd</sup> objective of the CORESTA Biomarker Sub-Group, periodic inter-laboratory comparison studies have been performed to evaluate the consistency of the quantitation for various harmful or potentially harmful exposure markers. To further this objective an evaluation of the measures of total NNAL was conducted. 4-Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) is the stable carbonyl reduced butanol metabolite of 4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNK has been identified as carcinogenic to humans by the International Agency for Research on Cancer. The measurement of total NNAL has been established as an acceptable biomarker to assess NNK exposure.

The inter-laboratory testing data provided by four separate laboratories was evaluated for consistency of quantitation. The initial results confirmed an analytically significant bias between the laboratories when quantitation was performed with standard calibrators prepared separately by each laboratory. Quality control samples prepared by supplementation with known amounts of the aglycone metabolite only showed a similar level of bias (35 %) to the multiple lots of urine collected from consented smokers that contain both the aglycone and glucuronide metabolites of NNAL (41 %). Comparability between the laboratories however was demonstrated when a uniform set of calibrators, which had been provided to each laboratory with the test samples, were used for quantitation.

The inter-laboratory comparison study effectively demonstrated the importance of such exercises to ensure the comparability of results provided by laboratories performing tobacco related research and to highlight the importance of the characterization and sourcing of quality reference materials to ensure comparability of results.

## ST 60

### **Assessing changes in biomarkers of effect in smokers who switch to a closed system electronic cigarette**

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Previous studies have demonstrated that when smokers switch to an electronic cigarette, they are exposed to significantly lower levels of carcinogens and toxicants. As a result, there is a reported significant reduction in biomarkers of exposure to levels similar to those observed with complete smoking cessation. Biomarkers of effect enable the assessment of the biological response to reduced exposure to cigarette smoke carcinogens and toxicants when consumers smoking conventional products switch to e-cigarettes. The aim of this study was to evaluate changes in biomarkers of effect when healthy smokers switch from conventional cigarettes to a typical closed system electronic cigarette. The study consisted of two parts; the first, a 5-day study under clinical confinement conditions, compared baseline blood samples from smokers who switched to e-cigarettes against those who quit conventional cigarettes unassisted after 5 days; the second study compared baseline blood samples from smokers who switched to e-cigarettes against samples taken two years later in a 2-year real-life ambulatory study. Specific and targeted analysis of potential biomarkers of effect in the samples resulting from the 5-day and 2-year clinical studies were performed, along with a non-biased untargeted metabolomics approach in the samples resulting from the 2-year clinical study. Most biomarker of effect data were similar across the study groups but a small separation between baseline and 2-year samples in the untargeted analysis was observed, specifically in amino acid and arachidonic acid metabolites. Given the significant reductions in exposure to carcinogens and toxicants observed when smokers switch to e-cigarettes, longer study periods may show more substantial effects; however, more research is required to demonstrate this. The findings presented here provide useful insights into study design for evaluating the harm reduction potential of e-cigarettes.

## ST 61

### **Novel biomarkers to characterize exposure to aldehydes from e-vapor products based on stable-isotope constituents**

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E-vapor products (EVP) are an emerging category of non-combustible tobacco products that hold the promise of harm reduction for users of combustible products. Degradation products such as aldehydes have received much attention lately, however human



exposure cannot be characterized due to absence of validated biomarkers of exposure to aldehydes. We evaluated the emission of aldehydes in EVP under typical usage conditions generated from e-liquid containing isotope-labelled nicotine (Nic), glycerol (G) and propylene glycol (PG) and the smoke of a conventional non-filter cigarette spiked with the same labelled compounds, serving as positive control. Urine samples for biomarker analysis were derived from a clinical study comprising experienced vapers (n=20) and cigarette smokers (n=5). This approach allows for the systematic evaluation of exposure to the e-liquid constituents and potential degradation products.

Several (bio)analytical methods were developed and modified for the quantification of labeled and unlabeled aldehydes in e-liquid aerosol as well as the labeled and unlabeled urinary biomarkers of those compounds (metabolites deriving from glutathione adducts, e.g. mercapturic acids).

The mainstream smoke of spiked cigarettes showed significant levels of labeled acrolein, crotonaldehyde, formaldehyde and acetaldehyde. The corresponding labeled metabolites for acrolein, crotonaldehyde and formaldehyde in urine were only quantifiable in the positive control group (cigarette smokers), none of them were quantifiable in the vapers.

In conclusion, our data proved the applicability of the stable-isotope labelling concept to unequivocally assess exposure to potential pyrolysis products derived from PG and G from EVPs and their metabolites in human urine.

## ST 62

### **Immune functions are perturbed to a greater extent with smoking rather than moist snuff use**

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Cigarette smoking is a major risk factor for lung cancer, chronic obstructive pulmonary disease, and cardiovascular disease (CVD). Existing epidemiological data suggest moist snuff consumption is generally associated with reduced health risks relative to smoking, although certain CVD mortality risks may be elevated compared to non-tobacco consumption. While smoking is known to induce oxidative stress and chronic inflammation, whether smokeless tobacco products alter immune responses is unknown. Our ongoing studies indicate that while smokers exhibit many perturbations in the expression of genes involved in immune regulation, there are minimal changes in gene expression between non-tobacco consumers (NTC) and moist snuff consumers (MSC). Our objectives were to 1) evaluate several markers related to immune regulation in smokers (SMK), MSC and NTC, and 2) perform functional analyses to better understand the effects of chronic tobacco use. Sixty-one markers associated with immune regulation were measured in peripheral blood mononuclear cells from SMK (n = 40), MSC (n = 40),

and NTC (n = 40). Among these, relative to NTC, seven markers were significantly suppressed in SMK, whereas in MSC, only perforin<sup>+</sup> in lymphocytes was significantly suppressed. In a multinomial logistic regression model consisting of race, heart rate, and systolic blood pressure, markers including granzyme B<sup>+</sup>, perforin<sup>+</sup> in lymphocytes, granzyme B<sup>+</sup>, and KLRB1<sup>+</sup> in CD8<sup>+</sup> cells remained as statistically significant predictors for classifying the three cohorts. Functional analysis revealed that cell-surface receptor signaling pathways and cell-cell signaling processes were downregulated in SMK relative to MSC, whereas chemotaxis, LPS-mediated signaling pathways, and the mitogen-activated protein kinase (MAPK) cascade were upregulated in SMK compared to MSC. Furthermore, a protein-protein interaction network of the 61 markers was constructed to visualize the immunosuppressive effects in SMK relative to MSC. In summary, this is the first study to demonstrate that moist snuff consumption, relative to smoking, is associated with minimal perturbations in inflammatory networks and immune function.

## ST 63

### **Metabolomics analysis identified reduced levels of oxidative stress and improved vitamin metabolism in smokers switching to an electronic nicotine delivery system (ENDS)**

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Switching to non-combustible tobacco products presents an opportunity for cigarette smokers to potentially reduce the harm associated with smoking. Electronic nicotine delivery systems (ENDS) are one type of non-combustible tobacco product that hold potential for harm reduction, since the vapor produced from ENDS contains far fewer toxicants than cigarette smoke. To investigate the biochemical effects of switching cigarette smokers to an ENDS, we assessed global metabolomic profiles of smokers in a seven-day clinical confinement study. In the first two days of this clinical study, the subjects used their usual brand of cigarettes and then switched to exclusive ENDS *ad libitum* use for five days. Urine and plasma samples were collected at baseline and five days after switching. The samples were analyzed using a mass spectrometry-based metabolomics platform. Random forest analyses of urine and plasma metabolomics data revealed excellent predictive accuracy (>97 %) of the 30-metabolite signatures that can differentiate the smokers before or after switching. In these signatures, most biomarkers are nicotine-derived metabolites or xenobiotics that were significantly reduced in urine and plasma after switching, suggesting a decreased xenobiotic load on consumers. Our results also show significantly decreased levels of plasma glutathione metabolites after switching, indicating reduced oxidative stress. In addition, increased urinary and plasma levels of vitamins and anti-oxidants were observed, suggesting enhanced bioavailability due to discontinuation of cigarette smoking and less interference from ENDS use. Together, our results suggest a less toxic chemical environment, reduced oxidative stress and potential beneficial changes in vitamin metabolism within a few days in combustible cigarette smokers after switching to an ENDS.

## ST 64

### **What fundamental tobacco science teaches us about heated tobacco products – assessment on THP1.0**

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Heated tobacco products are designed to reduce toxicant emissions in aerosol as compared to cigarette smoke. Different heating mechanisms have been used in this type of product, either using solid fuels (e.g. charcoal) or electrical energy (e.g. rechargeable batteries) to heat a tobacco consumable. In this work, fundamental tobacco thermophysics and thermochemistry principles developed for cigarettes are outlined to illustrate their relevance to understand the aerosol formation in heated tobacco products. Furthermore, these principles were applied to assess the extent of tobacco thermal conversion in a commercial heated tobacco product THP1.0 (commercially known as glo™) in a 5-step process. This covers thermogravimetric analysis on the tobacco reconstituted material (step 1), accurate measurement of the maximum and the range of temperatures the tobacco is exposed to (step 2), the levels of combustion markers (CO, CO<sub>2</sub>, NO and NO<sub>x</sub>) in the aerosol emission (step 3), the levels of thermal breakdown marker compounds (step 4), and finally the appearance of the consumable sticks post-use (step 5). The results showed that the aerosol in THP1.0 is mainly formed by distillation and evaporation, and not by tobacco combustion. This is critical to ensure that its aerosol contained significantly fewer toxicants, and any toxicants present in the aerosol are at much reduced concentration compared to those in cigarette smoke.

## ST 67

### **Study on combustibility and particle size distribution in smoke aerosols of cigarettes with different circumferences**

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In order to investigate the effects of cigarette circumference on its combustibility and particle size distribution in smoke aerosol, the solid phase temperature of combustion cone and the gas phase temperature in filter tip of the same brand cigarettes with three circumferences ( $\varnothing$  24.37 mm, normal;  $\varnothing$  20.06 mm, medium;  $\varnothing$  17.02 mm, slim) were tested by infrared thermal imager temperature measurement technology and thermocouple temperature measurement method, respectively. Meanwhile, a smoking cycle simulator and a fast particulate spectrometer DMS500 were employed to determine the particle size distribution in smoke aerosol under ISO smoking regime. The results showed that: (1) The solid phase temperature of the combustion cone of medium and

slim cigarettes was higher than that of normal cigarettes by 100 °C on average. (2) The gas phase temperature at the front of the filter of medium and slim cigarettes was higher than that of normal cigarette by 5 °C; however, the gas phase temperature in filters of the three sizes of cigarettes tended to be consistent due to the cooling effect of filters. (3) With the decrease of cigarette circumference, the average particle number concentration of aerosols for all puffs increased significantly, while the count median diameter (CMD) decreased, which indicated that the number of fine particles increased. (4) With the decrease of cigarette circumference, the average particle surface area of aerosols for all puffs increased significantly, and the area median diameter (AMD) reduced, which suggested that the total area of fine particle increased remarkably.

## ST 68

### **E-cigarette aerosol dynamics in a physical model of the adult human oral/pharyngeal/tracheal cavity**

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The objective of this work is to generate experimental data to validate a computational deposition model for e-cigarette aerosol. An adult human oral/pharyngeal/tracheal hollow prototype model was generated using a 3D printer from the CT scan of a 28 year old healthy male that had an internal volume of 120 cm<sup>3</sup>. The internal wall of the model was covered with a layer of cotton cloth that can be saturated with water to replicate the high humidity conditions in a human oral/pharyngeal/tracheal cavity. The physical model was placed in an oven set to normal human body temperature (37 °C), and measurements were taken under both wet and dry wall conditions. Deposition efficiency and hygroscopic growth from a MarkTen<sup>®</sup> e-vapor product using a prototype formulation were determined by measuring cumulative aerosol mass from five puffs (gravimetric). Selected chemical constituents (propylene glycol [PG], glycerin, and nicotine) from a single puff were measured by GC/MS analysis at the entrance and exit of the physical model. Humidity at the exit of the physical model was maintained at >95 % under wet wall conditions. A constant air flow rate of 1.1 L/min was maintained during all the measurements. Under dry wall conditions mean aerosol mass decreased 19.7 % due to the regional deposition to the wall. Under wet wall conditions, the aerosol mass increased by 161 % due to moisture uptake by the aerosol. The deposition of nicotine and PG is higher than the deposition of glycerin under both dry and wet conditions. These experimental data will be used to validate computational models developed by Altria Client Services LLC.

# CORESTA CONGRESS 2018

## SMOKE SCIENCE and PRODUCT TECHNOLOGY

### POSTERS

#### STPOST 01

##### **Cross-sectional surveys on the use of tobacco products in the general population and in users of IQOS in Germany, Italy, and London (2018-2020): introducing the study protocols**

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**Background:** Philip Morris International (PMI) launched cross-sectional surveys in Italy, Germany, and London in March 2018 following the commercialization of a heat-not-burn tobacco product, IQOS. This publication introduces the study protocols.

**Methods:** The objectives of these studies are to estimate the prevalence of tobacco use, describe past and current patterns of use, and explore their associations with self-reported health, motivation to use, risk perceptions and perceived aesthetic changes. The overall design of the surveys in the three markets is similar. The studies are conducted in two annual samples over three consecutive years: a representative sample of the general population, and an IQOS users sample registered in PMI IQOS user database. About 6,000 adults per year and market will be selected through multi-stage stratified sampling from the general population and will complete face-to-face computer-assisted personal interviews (CAPI). In addition, about 1,200 IQOS users per year and market will be randomly selected from the database and will complete the survey online using computer-assisted self-interview (CASI). The smoking questionnaire<sup>[1]</sup> will be used to assess the tobacco use patterns of the participants.

**Conclusion:** These surveys allow the assessment of the prevalence of tobacco use and will provide insights into use patterns and associated factors.

[1] Weitkunat R, Coggins CRE, Sponsiello-Wang Z, Kallischnigg G, Dempsey R. Assessment of cigarette smoking in epidemiologic studies. *Beitr Tabakforsch Int.* 2013;25:638-48

## STPOST 02

### **Quantification of nitrogen oxides in aerosol of heat-not-burn IQOS product and combination of aerosol collection with the aerosol generation for TSNA analysis**

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Nitrogen oxides (NO<sub>x</sub>), nitrogen monoxide (NO), and tobacco-specific nitrosamines (TSNA) are compounds that are analysed in the mainstream aerosol of heat-not-burn tobacco products, such as IQOS. The purpose of this study was to develop and validate a method able to collect in combination NO/NO<sub>x</sub> and TSNA from aerosol and to quantify them precisely and accurately. TSNA results were compared with the reference method, in which aerosol collection is done separately. The mainstream aerosol was collected, using a linear smoking machine under the Health Canada Intensive regimen, in bags for NO/NO<sub>x</sub> measurement after passing through a glass fibre Cambridge filter pad for TSNA analysis by liquid chromatography coupled with tandem mass spectrometry. The NO<sub>x</sub> in the bags were quantified by EcoPhysics CLD811 detector, and the results were converted to [µg/item] using an equation derived from the ideal gas law. By combining the aerosol generation, TSNA results showed relative mean differences of -0.52 to 1.26 % compared with the reference method, which is within the variability of the analytical method. For both NO and NO<sub>x</sub>, the obtained lower limits of quantification were below the lower working range limit (1 ppm), and instrumental repeatability was between 0.18 and 0.42 %. For NO, the r and IP limits were at 0.84 and 1.19 µg/item, respectively, at nominal content (12.6 µg/item), and for NO<sub>x</sub>, the r limit and the IP limit were at 0.89 and 1.02 µg/item, respectively, at nominal content (12.9 µg/item). Based on these results, the validation of the NO/NO<sub>x</sub> measurement has been successful. The method was shown to be precise and accurate over the ranges of concentration from 1 to 40 ppm (~0.87 to 35 µg/item) for NO/NO<sub>x</sub> in mainstream aerosol. In conclusion, it was demonstrated that the combination of aerosol generation does not impact the TSNA results.

## STPOST 03

### **Determination of nicotine, eugenol, menthol and aerosol formers in tobacco products and materials**

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The purpose of this study was to validate a unique method that simultaneously determines the concentration of nicotine, eugenol, menthol, and glycerin in Reduced-Risk Products tobacco products<sup>[1]</sup>, and menthol and triacetin in materials.

Analytes are extracted from tobacco products (cast leaf, cast leaf containing cloves, tobacco plug, and tobacco powder) and materials (filter, mentholated filter, and mentholated inner liner) using methanol containing 1,3-butanediol as internal standard and analysed using gas chromatography equipped with a flame ionisation detector (GC-FID). Results were reported as percent dry-weight-basis (calculation performed considering water content measured by Karl Fisher titrator) for tobacco products and percent by weight, milligram per filter, or milligram per cm for materials.

Linearity was demonstrated for all compounds ( $R^2 > 0.995$ ). The recoveries ranged between 90-107 % for concentrations in mg/mL and 80-110 % for concentrations in µg/mL for all targeted compounds in the tested matrices. In tobacco products, coefficient of variation (CV) of repeatability ranges between 0.52-2.06 % for nicotine, 0.91-2.25 % for glycerin, 0.72-3.62 % for eugenol, and 0.63-0.94 % for menthol. CV of intermediate precision ranges between 0.68-9.18 % for nicotine, 1.80-4.14 % for glycerin, 1.81-6.84 % for eugenol, and 2.07-9.59 % for menthol. In materials, CVs of repeatability of 8.98-17.85 % for menthol and 2.04-3.34 % for triacetin were obtained. CV for intermediate precision ranges between 8.98-18.35 % for menthol and 3.91-6.45 % for triacetin. Higher CVs for materials were obtained but mainly attributed to product variability.

The method was validated according to ICH guidelines, and validation results showed to be selective, precise, and accurate. A linear relationship was demonstrated over the tested concentration range.

[1] Reduced-Risk Products (RRPs) is the term used to refer to products that present, are likely to present, or have the potential to present less risk of harm to smokers who switch to these products versus continued smoking.

## STPOST 05

### Examining intra-individual and inter-individual variability of plasma nicotine PK parameters in e-vapor use by adult cigarette smokers in three studies

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Pharmacokinetic studies are often used to assess the rate and amount of nicotine delivery into the bloodstream during product use. Knowledge about intra-individual and inter-individual variability of pharmacokinetic (PK) parameters is important in the design of PK study for e-vapor products (EVPs). We analyzed two plasma nicotine PK parameters, maximum concentration ( $C_{max}$ ) and area under the curve (AUC) from three randomized controlled crossover PK studies ( $n = 27, 36, 38$ , respectively) in which various commercial and prototype EVPs were used by adult cigarette smokers under two different use conditions. The first condition was a controlled use of 10 inhalations of 4 second duration with 30 second intervals and the second condition was an *ad libitum* use condition (use for 10 minutes without restrictions on inhalation times and duration). A linear mixed model that included study product, sequence and period as fixed effect and subject as random effect was used to estimate the intra- and inter-individual variability. The average variability (CV %) across the three studies was larger under the *ad libitum* condition (intra-

individual mean = 32.3 % and inter-individual mean = 52.9 % for  $C_{max}$ ; intra-individual mean = 26.3 % and inter-individual mean = 54.3 % for AUC) than under the controlled condition (intra-individual mean = 31.6 % and inter-individual mean = 39.3 % for  $C_{max}$ ; intra-individual mean = 22.8 % and inter-individual mean = 36.2 % for AUC). The average intra-individual variability across the three studies for  $C_{max}$  and AUC was smaller than the average inter-individual variability under both conditions. In each study, the intra-individual variability for both parameters was smaller than the inter-individual variability. For the *ad libitum* condition, the inter-individual CV in  $C_{max}$  was 52 %, 52 % and 53 %, respectively in three studies and the inter-individual CV was all around 54 % for AUC in three studies, indicating consistency across studies. These variability estimates can inform design of future EVP PK studies.

## STPOST 06

### **Research on the application of microwave non-destructive testing technology for detection of capsule filter rod or capsule cigarette**

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In the filter rod capsule and cigarette capsule production process, the capsule defects, which include breakage, loss and position offset, occur easily because of the technology of capsule filling, filter rod cutting, and cigarette winding. Because the capsule is buried in the filter rod or cigarette, it is difficult to visually judge its normality without breakage. However, the dielectric constant of the capsule is different from that of the filter rod or cigarette. The difference of their signal will appear in the area whether there is a capsule or not. According to the principle of dielectric constant detection in microwave resonator, the signal of filter rod in the length direction was collected by a microwave resonator sensor. The leakage, location, and breakage of the capsule in the filter rod were detected according to the difference of the signal intensity. A detector was designed based on the microwave resonance cavity perturbation technique. The detector was mainly composed of a filter rod feeding device, a filter rod conveying device, a microwave detection unit and a sorting device. The designed detector and the established algorithm featured high accuracy, which was 0.1 mm for detecting the position of the capsule, and good recognition effect for defects or absence of the capsule. The detector provides a rapid and accurate quality detection method for capsuled-filter rods.



## STPOST 07

### Computer tomography-based analysis of the inner structure of tobacco products

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Modern cigarettes and tobacco heated products (THPs) often contain additional components, which are not visible externally. In addition, the inner structure is of great importance regarding the function, especially for THPs.

To drive the scientific research for innovative products that fulfill internal, actual and future regulatory standards, a reliable, reproducible and quantifiable method for measurements of the inner structure is required. The micro computed tomography ( $\mu$ CT) offers in this context a non-destructive possibility to examine the structure by providing high-resolution data without impacting the integrity of the object observed. Qualitative and quantitative analysis can be carried out. For example, the determination of the positions of capsules, carbon particles and threads or the determination of segment lengths with an accuracy of  $<0,1$  mm are possible.

In this study, the  $\mu$ CT technique is applied for the structural analysis of conventional and reconstituted tobacco (recon) rods. First, frontal planes of processed CT image data are derived and analyzed using the Fourier analysis. Subsequently, the resulting Fourier plots are evaluated applying a Gaussian fit. Finally, the main orientation of the structure, the corresponding dispersion and the relative proportion of the orientation relative to the overall structure for multiple frontal planes are determined.

The results show that similar main directions of approx.  $90^\circ$  (longitudinal rod direction) can be observed for tobacco and recon rods, but with different dispersions ( $17^\circ$  vs.  $25^\circ$ ). For tobacco rods the main direction depends on the frontal section considered. Certain cutting sections have a distinct direction, other sections do not show a dominant orientation.

The  $\mu$ CT-based analysis is a non-destructive and accurate scientific research tool for the assessment of quality relevant parameters of tobacco products. The proposed approach helps to develop innovative products and to comply with internal and regulatory standards.

## STPOST 08

### Effects of treatment time of ultraviolet irradiation and ozone on the internal quality of the redrying tobacco

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To investigate the effect of ultraviolet (UV) radiation and ozone on the internal quality of redrying tobacco, Qujing redrying tobacco (B2F) was selected as experimental material and irradiated by UV irradiation (producing ozone) and treated with ozone for different periods. The conventional chemical composition and aroma composition were then analyzed, and sensory evaluation was examined.

The results showed that the contents of total sugar and water-soluble sugar were raised by ozone treatment. The total sugar content was the highest after 0.5 h treatment, while water-soluble sugar content was the highest after 1.5 h treatment. The nicotine in the treated sample was significantly lower than that in the control, and nicotine content was the lowest after 2.0 h treatment. The ratio of sugar to nicotine was highest after 0.5 h treatment. The total aroma amount was the highest during the processing time of 1.0 h. The sensory evaluation showed that quality and quantity of aroma were best after 0.5 h treatment, and the aftertaste was weaker and strength was moderate.

By UV irradiation (producing ozone) treatment, the contents of total sugar and water-soluble sugar were higher than the control and the highest after 0.5 h treatment, except 2.0 h treatment time. After 0.5 h treatment, the nicotine in the treated sample was the lowest and the ratio of sugar to nicotine was the highest. The total aroma amount was the highest in the processing time of 1.0 h.

In summary, the quality of tobacco leaf was the best with the 0.5 h ozone treatment and the 1.0 h UV irradiation (producing ozone) treatment, with the latter being better than the former.

## STPOST 09

### **End-point detection of electronic nicotine delivery systems (ENDS) and tobacco heated products**

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Electronic nicotine delivery systems (ENDS) and heated tobacco products (HTPs) generate aerosol without the heat used for termination in machine smoking. Therefore, termination systems using the aerosol's optical density have been developed. Since both ENDS and HTPs can be vaped on the same machine, a flexible end-point detection system is required to accommodate both types of product, either to terminate vaping or to prompt product exchange in continuous vaping systems.

Our objective was to develop a system to provide both real-time and recorded characteristics of aerosol production of ENDS and HTPs with an in-built calibration capability to reliably define the termination setting. This can be configured with a puff engine as a stand-alone bench-top unit or integrated into a multi-channel vaping machine.

Aerosol density is sensed via optical fibres set at the outlet of the product. The source intensity is normalised by channel before each run. A graphical interface presents real-time information and sets the termination level. The peak opacity after each puff is recorded and signals are set at user-defined 'be prepared' and 'end-of-life' levels. The system can also characterise aerosol production where there is electronic control of product lifetime.

HTP aerosol is less dense than that of ENDS and tends not to follow a gentle decline over life. Puff-to-puff noise can be significant for ENDS, such that a simple trigger level underestimates product life. Intensity calibration significantly reduces channel-to-channel variation. We have previously demonstrated that cumulative opacity can be linked to aerosol yield and this is available to the user.

Detection of the optical density of the aerosol can define end-of-life and is also capable of providing yield information that is useful for R&D and QA. In-built calibration enhances the flexibility of the system, particularly where a wide range of products and product types are vaped.

## STPOST 10

### **Principal component analysis of volatile components in refill liquids for electronic cigarettes with different tastes**

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In this study the characterization of refill liquids for different electronic cigarettes with different tastes was compared. GC/MS and principal component analysis were employed to analyze the volatile components in refill liquids for five kinds of taste, which included Jasmine, Furongwang, Zhonghua, Li Qun and Yuxi. The results of GC/MS showed that the main component of the refill liquids included propylene glycol, glycerol and nicotine. Principal component analysis results showed that flavor components affecting the flavor type of the refill liquid can be grouped into four factors, namely jasmine flavor, fruit flavor, caramel flavor and rose flavor. The jasmine flavor factor has a larger impact on the Jasmine taste of refill liquids, and the fruity flavor factor has a larger impact on the Furongwang taste of refill liquids. Benzyl acetate and benzyl alcohol were the main aroma components affecting the Jasmine taste of refill liquids, phenol and ethyl maltol affected the Furongwang taste, indole affected the Zhonghua taste, 2,3,5,6-tetramethyl pyrazine, dihydro damascenone and benzene ethanol affected the Liqun taste, and phthalic acid-di(2-propylpentyl) ester and benzene ethanol affected the Yuxi taste. This study provided a theoretical support for the blending of refill liquids for electronic cigarettes.

## STPOST 11

### **Characterization of flavor transfer during smoking of cigarettes with flavor capsules and aromatized tobacco cigarettes using SPME-GCMS**

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Flavored tobacco has been used in cigarettes for many decades, but nowadays consumption of flavor capsule cigarettes is increasing in South American markets. Flavor capsules give the smoker the option of choosing to smoke the cigarette with or without flavor or to choose between two or more different flavors.

The aim of the present work is to analyze if there is a difference in flavor delivery between flavor capsules and aromatized tobacco to cigarette smoke.

Ten brands of cigarettes with flavor capsules and seven brands of aromatized tobacco cigarettes were used to test how flavor components transfer to smoke after smoking. The

flavors were analyzed on the tobacco or in the liquid within the capsule directly with a SPME fiber to identify their components.

Two cigarettes of each type were smoked with an intensive regime, using Cambridge filters and methanol traps at -20 °C after the cigarette holder to capture flavor components.

The Cambridge filter and the methanol were put in different flasks and the flavor components were captured with a solid phase microextraction (SPME). The SPME fiber was analyzed by GC-MS and spectrums were analyzed manually with Palisade and National Institute of Standards and Technology (NIST) libraries and with the Automated Mass Spectral Deconvolution and Identification System (AMDIS) using the Varian library. Flavor components were retained principally in the Cambridge filter.

Flavor capsules analyzed contained spearmint, peppermint, a mixture of both, red fruits with menthol, only menthol, and other fruit flavors. Aromatized tobacco contained chocolate, coffee, tea, menthol, applemint and red fruit flavors.

Between one and thirty compounds were identified in the different flavors, and an average of 40 % of them were identified as transferred to the smoke in the flavor capsule cigarette and also in the flavored tobacco cigarette.

No differences in flavor components delivery between flavor capsules and aromatized tobacco were found, both systems being equivalent in transferring flavor from the cigarette to the smoke.

## STPOST 12

### **Characterization of the Vitrocell® high throughput exposure modules using different tobacco product types**

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The development of whole smoke/aerosol exposure systems provides a means to conduct *in vitro* assessment of freshly generated whole smoke and aerosol from combustible and tobacco heating products (THP) as well as electronic nicotine delivery systems (ENDS). One challenge with such systems is ensuring sufficient throughput for *in vitro* toxicological studies in a timely manner. Vitrocell® has developed a high throughput whole smoke/aerosol exposure module designed to deliver concurrently up to seven different doses of smoke/aerosol and a clean air control to 48 wells of bacterial or mammalian cell cultures (six wells per dose). Characterization of this system was conducted with a series of experiments designed to assess smoke/aerosol delivery and biological responses from a Kentucky Reference 3R4F combustible cigarette or a commercially available THP. Dilution airflows consisting of 0.5-10 L/min for 3R4F and 0 (undiluted)-4 L/min for the THP were evaluated. Smoke/aerosol deposition was quantified using fluorescence measurements (Ex 355/Em 485) of captured particulate matter and chemical analysis (e.g. glycerol, nicotine) of either DMSO (3R4F) or PBS (THP)

traps within the module. Further characterization of the high throughput module was performed with the neutral red uptake (NRU) assay to determine the cytotoxic response to 3R4F whole smoke. Current results demonstrate a dose-dependent deposition of smoke/aerosol constituents and a characteristic dose-dependent decrease in cell viability as indicated by the NRU assay.

## STPOST 13

### **Genotoxicity evaluation of tobacco and nicotine delivery products: Part 1. Mouse lymphoma assay**

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*In vitro* studies have been widely used to support the toxicological evaluation of chemicals and complex mixtures including cigarette smoke. In this study a variety of test matrices from different tobacco and nicotine delivery products was assessed against a Kentucky reference (3R4F) cigarette.

The mouse lymphoma assay (MLA) is underpinned by OECD guideline 490 and ICH S2(R1) guidance and is a recognised *in vitro* genotoxicity test battery assay. The aim of this study was to assess the suitability of the MLA with a variety of tobacco and nicotine product test matrices. Testing was conducted in general accordance to OECD Guideline 490 and ICH S2 (R1) test guidance. The same samples were also assessed using the *in vitro* micronucleus assay; results are reported separately (Part 2).

Total particulate matter (TPM) from a 3R4F cigarette was compared against pad-collected aerosol matter generated from a commercial electronic nicotine delivery system (ENDS), a commercial e-liquid, and TPM from a commercial tobacco-heating product (THP) using the *in vitro* MLA. Exposures were conducted for 3 h ±S9 metabolic activation and 24 h -S9 conditions at concentrations up to 500 µg/mL.

Under all treatment conditions, 3R4F produced a clear positive response with regard to induction of mutation. In contrast, no marked induction of mutation was observed for the e-liquid, ENDS and THP. Based on the results of this study, the mouse lymphoma assay can be used effectively for the assessment of these test matrices.

## STPOST 14

### Genotoxicity evaluation of tobacco and nicotine delivery products: Part 2. *In vitro* micronucleus assay

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*In vitro* studies have been widely used to support toxicological evaluations of chemicals and complex mixtures including cigarette smoke. In this study a variety of test matrices from tobacco and nicotine delivery products were assessed against a 3R4F reference cigarette using the *in vitro* micronucleus assay (IVMN). Assays were conducted in general accordance to OECD Guideline 487 and ICH S2 (R1) guidance. Samples were also assessed using the mouse lymphoma assay (Part 1).

3R4F total particulate matter (TPM) was first assessed with CHO, V79 (both rodent) and TK6 lymphoblastoid (human) cell lines with 3 h exposures  $\pm$ S9 metabolic activation and extended -S9 treatments with/without a 1.5-2 cell cycle length recovery period at doses up to 500  $\mu$ g/mL. CHO, V79 and TK6 cells gave varied positive responses, with V79 being most responsive. The extended recovery treatment increased assay sensitivity for CHO and V79 cells; this was less clear in human TK6 cells. V79 cells were taken forward for further assessments.

3R4F TPM was compared against pad-collected aerosol matter generated from a commercial electronic nicotine delivery system (ENDS), a commercial e-liquid, and TPM from a commercial tobacco-heating product (THP) using the same treatment schedules described above.

Across all treatment regimens with V79 cells, clear negative responses were observed for the e-liquid, ENDS and THP samples, while 3R4F elicited a clear positive response. The most potent 3R4F responses were observed following extended treatment -S9 with recovery, suggesting this to be a more appropriate treatment schedule for the assessment of tobacco and nicotine product test matrices. Based on the results of this study the IVMN assay can be used effectively for the assessment of these test matrices.

## STPOST 15

### Temperature logging of next generation heat-not-burn aerosol

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The widespread use of heat-not-burn products (HNB) is in its infancy and consumer acceptance will involve comparison with existing conventional cigarettes. User perception may be influenced by aerosol delivery temperature and how this is modified by different puffing parameters. The objective of this study was to establish how aerosol

temperature varies as a consequence of changing puffing parameters for three commercially available HNB products.

Any change in aerosol temperature was explored using a vaping machine, a Cambridge filter holder modified with a K type thermocouple in the aerosol stream, logging temperature at 4 Hz. Three products, IQOS, glo™ and a Ploomtech, were tested. Experimental protocols were based upon changing the key puff parameters: duration; interval; volume and shape.

As puff count increased, Ploomtech generally increased aerosol temperature whilst IQOS and glo™ reached maximum aerosol temperature after two puffs (24 °C - 27 °C above ambient) with subsequent temperature decline. Increasing puff duration for constant volume/interval had little impact on the aerosol temperature profile of glo™ and IQOS. For Ploomtech and IQOS, changing volume yielded little change in aerosol temperature. For glo™, the lowest puff volume (35 ml) had lower aerosol temperatures. Changing puff interval for consistent puff condition (55 ml puff; 3 s duration) changed the aerosol generated from the glo™ in a not easily understood manner and had minimal impact on the temperature of the IQOS aerosol. The Ploomtech aerosols became hotter as the puff interval decreased (60 s = 1 °C rise, 15 s = 5° rise).

Aerosol temperature for IQOS and glo™ seems independent of the “intensity” of the puffing parameters chosen. Evidence is presented that after the initial two puffs a shorter puff interval yields marginally higher aerosol temperatures. The Ploomtech aerosol temperature increases for more “intense” regimes, shortening puff intervals having the most significant impact.

## **STPOST 17**

### **Evaluation of reduction in exposure to selected cigarette smoke constituents after switching to novel tobacco vapor product (NTV) use during a five-day confinement study in Japan**

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We recently developed a novel tobacco vapor product (NTV) that comprises a battery, a cartridge with a heater and nicotine-free liquid, and a tobacco capsule filled with tobacco blend. Vapor containing nicotine and flavor is produced by aerosol from the cartridge that passes through the capsule during inhalation. The results of the chemical analysis of vapor showed that most of the measured selected cigarette smoke constituents were below quantifiable levels, suggesting that switching to NTV reduces the exposure to these constituents. The present study aimed to investigate the level of exposure to selected harmful and potentially harmful constituents (HPHCs) of cigarette smoke in adult smokers who switched to NTV compared with those who continued to smoke their own brands of commercial cigarettes (CC). We also assessed the level of exposure in adult smokers who abstained from smoking (SA) as a benchmark of exposure reduction. Sixty healthy Japanese smokers were randomized to the NTV, CC, and SA groups for five days under confined conditions, and 15 biomarkers of exposure (BoEs) to 14 HPHCs and pyrene were



measured at baseline, day three, and day five (UMIN000025777). The levels of all BoEs were significantly reduced in the NTV group compared with the CC group. Of significance, the magnitude of the reduction in exposure to HPHCs observed in the NTV group (49 %-94 %) was close to that observed in the SA group (39 %-95 %). This result indicates that the level of exposure to smoke constituents can be reduced in smokers by switching to NTV from cigarette smoking compared with smokers who continued smoking CC, and the level of exposure can be reduced to the level observed when smokers abstained from smoking.

## **STPOST 18**

### **Evaluation of subjective effects and product use patterns of novel tobacco vapor product (NTV) use during a five-day confinement study in Japan**

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Some authorities have highlighted information relating to the abuse liability of tobacco products as key in reaching regulatory decisions. Although abuse liability should be assessed with multiple factors, one of the essential elements is the subjective effects of product use. In this study, we conducted a preliminary assessment to characterize some subjective effects during short-term use of a recently developed novel tobacco vapor product (NTV). The measurements were taken as part of a five-day, single center, randomized, open-label study with healthy adult Japanese smokers to assess exposure to selected smoke constituents after switching from conventional cigarette to NTV use (UMIN000025777). Each of the 20 participants who switched to NTV use (NTV group) or who continued to smoke their own brand of conventional cigarettes (CC group) were asked to complete measurements of subjective effects including product liking, satisfaction, smoking urges and withdrawal over the five-day period. Patterns of consumption and topography were also measured. Overall scores for liking, satisfaction, reduction in smoking urges and reduction in withdrawal in the NTV group did not exceed those observed in the CC group. Nevertheless, during the study period the scores for liking, consumption and number of puffs gradually increased in the NTV group but remained stable in the CC group. In conclusion, the subjective effect assessments included in this short-term study would suggest that the abuse liability of NTV would not exceed that of conventional cigarettes. However, the increase in the liking score, consumption and number of puffs during NTV use may reflect a course of adaptation to NTV use among participants who have not previously been familiar with tobacco products other than conventional cigarettes. Therefore, it remains to be assessed how such outcomes could change over prolonged observation.

## STPOST 19

### **Simultaneous analysis of five tobacco-specific N-nitrosamines in blood plasma of non-smokers and smokers using fully automated online solid phase extraction-liquid chromatography-tandem mass spectrometry**

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Tobacco-specific nitrosamines (TSNAs) in cigarette smoke are known as potential carcinogens. 4-(methylnitrosamino)-1-(3-pyridine)-1-butanol (NNAL) and its glycoside derivatives are established as biomarkers of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) exposure in smokers and non-tobacco users. Many studies have been focused on the determination of TSNA biomarkers of exposure in urine, whilst venous blood, due to its complex matrix effects, was not frequently considered for analysis. The purpose of this study was to develop an efficient and robust (precise as well as accurate) method for the simultaneous analysis of NNAL, NNK, N-nitrosoanabasin (NNA), N-nitrosoanatabine (NAT), and N-nitrosoanabasine (NAB) in human plasma by using online solid phase extraction-liquid chromatography-tandem mass spectrometry (online SPE-LC/MS/MS). Target compounds were concentrated and purified from blood plasma using a two-dimensional online SPE procedure. The required performance in chromatographic separation of the target analytes was achieved by using an Atlantis T3 column. A two-dimensional SPE clean-up process for the plasma samples was used by applying a combination of a PRS cation exchange and resin GP reverse phase column. The combination of these SPE column types resulted in a significant reduction of matrix background in the sample, therefore the clean-up significantly improved the sensitivity and accuracy of the method. The limits of detection (LODs) of this method for the five TSNAs ranged from 0.04 to 0.09 pg/mL, which are much lower than those reported in literature. The developed method was validated by determining the free and total TSNA levels in the plasma of 52 non-smokers and 61 smokers. Our results showed that this method combines high sensitivity with high repeatability. Furthermore, the automated online SPE procedure offers high throughput at high accuracy as well as precision level.

## STPOST 20

### **Factors influencing burning cone fallout of super slim cigarettes**

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The effects of four factors (individual weight, combustion improver content in cigarette paper, moisture content in cut tobacco and sliver content in cut tobacco) on the burning cone fallout of super slim cigarettes were investigated by an in-house developed

instrument. The results showed that the burning cone fallout ratio (BCFR) of super slim cigarettes decreased sharply with the increase of the individual weight of the cigarette, reduced obviously with a slight increase of moisture content in cut tobacco, lowered with the decrease of combustion improver content in the cigarette paper, and reduced slightly with the decrease of sliver content in cut tobacco. The data of a single factor analysis were normalized and the results suggested that a good linear relationship exists between BCFR and individual cigarette weight, the combustion improver content and the sliver content, while there was an inferior linear relationship between BCFR and the moisture content. Based on the single factor analysis, the influence of multiple factors on the BCFR of super slim cigarettes was simultaneously investigated by partial least squares (PLS) regression analysis using Minitab 17 statistics software. The independent variables and dependent variable presented significant correlations ( $p < 0.05$ ). BCFR was positively correlated with combustion improver content and sliver content and negatively correlated with the other two factors. Individual cigarette weight was the most significant influencing factor for BCFR, followed by the combustion improver content, the moisture content and the sliver content, which agreed with the results of single factor analysis. On the basis of these results, a specific cigarette was manufactured and the BCFR decreased from 58 % to 32 % by increasing its individual weight properly.

## STPOST 21

### **Cigarette combustion parameters and their effects on the deliveries of selected mainstream smoke constituents**

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In order to investigate potential dependencies between cigarette combustion parameters, such as temperature, pressure drop, etc, and the deliveries of selected constituents in mainstream smoke in real time, a set of on-line analytical systems were developed. The systems can simultaneously determine the gas flow distribution, pressure drop and combustion temperature in a cigarette during smoking. Cigarette samples having different air permeability were used to conduct the studies with the real time on-line analytical systems. The correlations between the gas flow distribution, pressure drop and combustion temperature were studied. Furthermore, the effects of the combustion parameters on the puff-by-puff deliveries of selected constituents, such as tar, nicotine, water and TPM in mainstream smoke were examined.

The results showed that: (1) There were very significant correlations among the gas flow through the burning cone, the gas flow through the tipping paper, the pressure drop and the combustion intensity; (2) Combustion parameters, especially the gas flow through the burning cone, were very significantly correlated to the deliveries of selected constituents in mainstream smoke; (3) The combustion intensity, that is defined as the product of the average combustion temperature and the number of corresponding data points, were very significantly correlated to the gas flow through the burning cone, the deliveries of selected constituents in mainstream smoke and the pressure drop below

800 °C. This correlation became less pronounced when the temperature in the burning cone was above 800 °C; (4) Significant correlations between the ventilation rate of filter and combustion parameters were found.

## STPOST 22

### **Simultaneous determination of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and cotinine in urine of smokers using two-dimensional liquid chromatography/tandem mass spectrometry**

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4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and cotinine are two efficient biomarkers of tobacco-specific carcinogen 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and nicotine. In this contribution, we developed an effective system of two-dimensional liquid chromatography coupled with tandem mass spectrometry for high-throughput simultaneous determination of NNAL and cotinine in smoker's urine. The strong cation-exchange (SCX) and the reversed-phase chromatography (RPLC) were adopted in the first and second dimensional studies. As mentioned below, the two-dimensional system is discussed. The orthogonality of this two-dimensional system was evaluated through the separation of NNAL and cotinine. In 2D-SCX/nRPLC, NNAL and cotinine can be fractionated in the same dimension (SCX: 5 µm, 35 mm × 2.0 µm i.d.) by step elution, followed by the molecular separation in the second dimension (RP: 5 µm, 150 mm × 4.6 µm i.d.) with binary gradient LC. In this work, the limits of detection of the method were 0.24 pg/mL and 0.054 ng/mL for NNAL and cotinine respectively. The recoveries of the spiked samples were 99.8 %-105.1 % and 111.9 %-114.2 % for NNAL and cotinine in urine, respectively. The precisions of the method were 0.43 %-3.07 % and 0.73 %-2.11 %. The method is useful for monitoring NNAL and cotinine in smoking and satisfactory to evaluate tobacco exposure.

## STPOST 23

### **Determination of 15 PAHs in mainstream cigarette smoke using concurrent solvent recondensation (CSR) injection combined with GC-MS/MS**

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Concurrent solvent recondensation (CSR) injection technique is known as one of the approaches to fulfill large volume injection for GC. With the advantages of large volume injection, complex steps such as concentration, enrichment and solid-phase extraction in the traditional methods can be simplified. The purpose of this study was to determine 15 polycyclic aromatic hydrocarbons (PAHs) in mainstream cigarette smoke using CSR injection technique combined with GC-MS/MS instrument. In this newly established

method, Cambridge filter pad was extracted by cyclohexane via ultrasonic after collecting cigarette mainstream smoke. Then the solvent was purified by magnesium trisilicate and filtered with 0.45 µm membrane. After filtration, the solution was injected into GC directly by CSR without enrichment. The introduction of CSR and GC-MS/MS could significantly promote the sensitivity and accuracy in the trace detection. In this work, PAHs displayed good linearity between 1 µg/L and 1000 µg/L while the limits of quantitation (LOQ) were below 0.8 ng per cigarette. Moreover, the recoveries were all between 85 % and 115 %. This work had the advantages of a high degree of automation, simple steps of pre-treatment, high sensitivity and accuracy. The established method proved to be adapted for the determination of PAHs in mainstream smoke of commercial cigarettes.

## STPOST 24

### **Influences of chronic alcohol consumption and critical enzyme regulation on the *in vivo* metabolism of NNK in mice**

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The progressive clarification of influential factors on the *in vivo* metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), one of the potent carcinogens in tobacco, could provide valuable information for precisely evaluating the health risk of tobacco products. The present study was designed to explore the influences of chronic alcohol consumption and critical enzyme regulation on NNK bioactivation in diverse animal models. The sensitive and rapid detection of NNK and its seven metabolites in mouse blood and tissues was achieved by UHPLC-HRMS method. Mouse models with alcohol-induced liver injury were then established and used to determine the variations of NNK metabolism due to organ damage caused by alcohol abuse. To study the role of mouse CYP2A5 in NNK metabolism, specific enzyme regulators (pyrazole and 5-methoxypsoralan) and Cyp2a5-null mouse were applied and compared. Furthermore, the effects of mouse DNA methyltransferase (DNMT) on the generation of DNA adduct (O6-mG) were investigated by using decitabine as a specific inhibitor. The results indicated that  $\alpha$ -methylene hydroxylation was the major pathway of NNK metabolism in mice, and alcohol-induced liver injury significantly promoted the  $\alpha$ -hydroxylation of NNK and the formation of O6-mG, which suggested that chronic alcohol consumption might increase the risk of carcinogenicity associated with NNK. Significant roles of CYP2A5 in NNK  $\alpha$ -hydroxylation (especially  $\alpha$ -methylene hydroxylation) was demonstrated through CYP2A5-regulated and Cyp2a5-null mouse models. It was also observed that DNMT activity was highly correlated to the generation of O6-mG through  $\alpha$ -methylene hydroxylation. These finds revealed the critical factors influencing the *in vivo* bioactivation of NNK and rendered important references for the precise evaluation of NNK exposure risk via individual lifestyle and genetic profile.

## STPOST 25

### **A simplified analytical method for determining metals in e-liquid**

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An analytical method for simultaneous determination of eight metals in mainstream cigarette smoke using inductively coupled plasma mass spectrometry (ICP-MS) was reported at the Tobacco Science Research Conference (TSRC) in 2009. The method does not require digestion during sample preparation. The method was modified for determination of 15 metals in e-liquid and reported at the 2017 CORESTA Smoke-Techno Joint Study Groups Meeting. In general, the sensitivity of the method to metals with a high ionization potential, such as arsenic and selenium, was influenced by the presence of organic components such as propylene glycol and glycerol, the so-called “matrix effect”. Under this modified method, the matrix effect was minimized by using calibration standards with methanol and an additional internal standard for the two metals, thereby improving the quantitation of the 15 metals. The complexity of these additional procedures and the need to increase the number of metals quantitated in an e-liquid sample, however, remained challenges to be solved. In this study, we used two approaches to simplify the procedure and quantitate more metals. The first approach was to optimize ICP-MS parameters instead of adding methanol to the calibration standards. The second approach was to optimize internal standards to increase the number of metals quantitated. We validated the two approaches with a verification test. Spiked e-liquid samples and calibration standards were diluted with a nitric acid solution and analyzed by an ICP-MS instrument equipped with a collision/reaction cell. The calculated recovery rates were sufficient to quantitate more than 20 metals in an e-liquid sample simultaneously. These two approaches contributed to simplifying the procedure and increasing the number of metals quantitated.

## STPOST 26

### **Determination of carbonyl compounds in gas vapour phase in phosphate buffered saline by LC-MS/MS for use with traditional tobacco and electronic nicotine delivery system (ENDS) products**

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The purpose of this study was to develop a liquid chromatography tandem mass spectrometry (LC-MS/MS) method to quantify selected carbonyl compounds (formaldehyde, acetaldehyde, crotonaldehyde, acrolein, acetone, butyraldehyde,

2-butanone (MEK) propionaldehyde, 2,3-butanedione (DA) and 2,3-pentanedione (AP)) in gas vapour phase (GVP) collected in phosphate buffered saline (PBS) for use with traditional tobacco and ENDS products. In this method the aerosol is trapped in impingers containing ice cold PBS. An aliquot of the solution is then diluted with 2,4-dinitrophenylhydrazine (DNPH) and a mixed solution of deuterated internal standards prepared in pyridine is added. Derivatisation is completed within an hour of GVP generation to be comparable to the GVP entering *in vitro* assay systems (Health CanadaT-502); analysis of samples is performed on the day of generation. The limits of detection (LOD) and limits of quantification (LOQ) for crotonaldehyde, acrolein, propionaldehyde, MEK and butyraldehyde are 0.03 µg/mL and 0.1 µg/mL respectively. The LOD and LOQ for formaldehyde, acetaldehyde, DA and AP are 0.03 µg/mL and 0.2 µg/mL respectively. The LOD and LOQ for acetone are 0.03 µg/mL and 7.0 µg/mL respectively (the LOQ for acetone was raised 7.0 µg/mL during validation due to unacceptably high variation at the lower levels as a result of the suspected presence of acetone in the background environment). The inter-run precision values (n=15) for all components is ≤21 %; the inter run accuracy values (n=15) for all components is ±24 %. The precision and accuracy values are affected by known instability of certain components, so in order to compensate, each analysis includes Quality Control samples that can be used to correct for any losses during collection and analysis. The method has been shown to be accurate, precise and linear with good correlation coefficients ( $R^2 > 0.999$  for formaldehyde, acetaldehyde, crotonaldehyde, acrolein, acetone, butyraldehyde, MEK and propionaldehyde; and  $R^2 > 0.992$  for DA and AP).

## STPOST 28

### Non-targeted analysis for differential screening of tobacco samples

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Non-targeted analysis of samples has broad applications across the food industry and in the evaluation of natural products. For example, this approach has been used by the food industry to evaluate purity and quality of fruit juices, olive oil and tea. Our lab explored applying a non-targeted analysis using LC/MS-TOF to differentiate between types of tobacco and tobacco leaf blends. This work can provide valuable information to improve our understanding of differences in types of tobacco, curing processes, tobacco origin and the impact of leaf blending. In addition, it may help us identify compounds that contribute to a tobacco's key characteristics. We used a Waters Acquity UPLC® with Waters Premier Quadrupole Time of Flight (QTOF) mass spectrometer with positive electrospray ionization in full scan mode. Chromatographic separation was conducted using a reverse phase gradient with a BEH C18, 2.1 × 100 mm 1.7 µm column from 5 % Acetonitrile/95 % Ammonium Formate to 95 % Acetonitrile/5 % Ammonium Formate over 16 minutes. The Waters MarkerLynx XS software was able to find hundreds of marker compounds in a variety of tobacco samples. Additional data processing in the software provided principal component analysis for the markers, and grouped the

tobacco samples in scores plots. Loadings plots provided insights as to which markers are responsible for the unique groupings. MarkerLynx XS software allowed for differentiation of tobacco types and leaf blends. Future work to identify key marker compounds using accurate mass analysis could provide insights into the differences in chemical composition of these tobacco samples.

## STPOST 29

### **Toxicological assessment of e-liquid formulations using *in vitro* genotoxicity and cytotoxicity assays**

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In the electronic nicotine delivery systems (ENDS) Premarket Tobacco Application (PMTA; 2016) Draft Guidance, the U.S. Food and Drug Administration (FDA) recommends a full assessment of the toxicological profile associated with new tobacco products, using *in vitro* toxicology (e.g. genotoxicity and cytotoxicity) studies. As part of a toxicological hazard assessment, we tested flavor varieties of e-liquids used in MarkTen® e-vapor products (a total of 14 formulations) and two carrier formulations (propylene glycol, glycerin, with 0 % or 5 % nicotine) in a standard battery of *in vitro* cytotoxicity (neutral red uptake [NRU]) and genotoxicity (Ames and micronucleus [MN]) assays according to OECD guidelines, using the maximum doses suggested for mixtures. The e-liquid formulations were characterized for key ingredients (propylene glycol, glycerin, and nicotine). All the formulations were non-cytotoxic per NRU assay (viability >80 %). None of the e-liquids were mutagenic in the Ames assay, however some reduction in background lawn was observed with the carrier formulation at the high (5 %) nicotine content. In the MN assay, 3/14 MarkTen® flavor formulations induced a weak but statistically significant increase in micronuclei formation, resulting in positive or equivocal findings according to OECD 487. All three flavor formulations were further evaluated in an *in vivo* combined genotoxicity (MN and Comet; OECD 474/489) assay and found to be negative for genotoxic endpoints. Therefore, consistent with the International Conference on Harmonization (ICH) S2(R1) genotoxicity testing guideline (2012), the tested e-liquids were regarded as negative for genotoxicity under the conditions of the assays.



## STPOST 30

### Effects of exposure to e-cigarette aerosols compared with cigarette smoke on 3D human buccal and small airway cultures: a systems toxicology assessment

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Considerable attention has been given toward the reduced risk potential of e-cigarettes (e-cigs). Most *in vitro* studies have focused on testing e-liquid formulations directly on submerged 2D cultures. Here, we examined the effects of exposure to the whole e-cig aerosols compared with the mainstream cigarette smoke (CS) using human 3D organotypic cultures. Buccal and small airway cultures were exposed at the air-liquid interface over 28 min to 112-puffs of undiluted aerosols generated from an e-vapor product, containing different e-liquids [with aerosol formers alone (CARRIER), with 4 % nicotine (BASE), with 4 % nicotine and flavors (TESTMIX)] or to diluted CS in a Vitrocell® exposure system. Nine independent exposures were conducted for a robust assessment. Concentrations of the deposited nicotine and carbonyls in the exposure chamber were measured as markers of exposure. Biological endpoints include histology, cytotoxicity, pro-inflammatory mediators, and gene microarray. Alterations in morphology and cytotoxicity were not observed in buccal culture exposed to undiluted e-cig aerosols, despite a 2-fold higher nicotine deposition compared to the diluted CS exposures. A similar lack of cytotoxicity and morphology changes was observed in the small airway following e-cig aerosol exposures (with a 10-fold higher nicotine deposition compared to CS exposures). CS exposures resulted in an increase in pro-inflammatory mediators in the media in both buccal and small airway cultures. In addition, compared with e-cig aerosols, CS exposures showed greater biological impacts in the global gene expression, including impacts on cell fate, proliferation, stress, and inflammatory response networks. Following 24 h post exposure, e-cig aerosol-induced changes in small airway cultures reverted to the levels of the air-control cultures. Among different e-vapor aerosols, there were no significant differences in any of the biological end points tested.

## STPOST 31

### **The relationship between pharmacokinetic parameters and the amount of e-liquid used by cigarette smokers under two e-vapor product use conditions**

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Plasma nicotine pharmacokinetic (PK) parameters have been used for assessing the rate and amount of nicotine uptake from use of e-vapor products (EVPs). The purpose of this study is to examine the relationship between each of the two PK parameters, the maximum concentration ( $C_{max}$ ) and area under the curve (AUC) of plasma nicotine, and the amount of e-liquid used (AEU) as measured by cartridge weight change of each product under two different conditions (10 puffs with a 4-second puff duration and 30-second puff interval versus *ad libitum* use of approximately 20 puffs for 10 minutes). The data were from two clinical studies ( $n = 70$  adult smokers in each study) in which nicotine PK parameters and other outcomes were evaluated for 6 MarkTen® EVPs (three EVPs in each study) containing nicotine from 2.5 to 4.0 % by weight in the e-liquid. An ordinary least squared regression model was used to examine the relationship.  $C_{max}$ , AUC and AEU were significantly greater under the *ad libitum* than the controlled condition for all products. On average, AEU ranged from 35 to 40 mg under the controlled condition, and from 50 to 70 mg under the *ad libitum* condition. Significant (positive) linear relationships were observed between  $C_{max}$  and AEU and between AUC and AEU for all products under both study conditions. Under the controlled condition, AEU explained approximately 20 % of the variance in the  $C_{max}$  and 36 % in AUC, while under the *ad libitum* condition, AEU explained approximately 69 % and 77 % of the variance in  $C_{max}$  and AUC, respectively. These findings showed a linear relationship between PK parameters and AEU in the study products, with stronger relationships observed in the *ad libitum* condition compared to the controlled condition. The relationship could be the base for prediction of PK parameters from AEU when additional factors are considered.

## STPOST 32

### Characterization and *in vitro* testing of whole smoke condensates from combustible cigarettes

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Health Canada recommends *in vitro* testing of separately collected gas and particulate phases of cigarette smoke for cytotoxicity and genotoxicity assessment. While these test materials have shown cytotoxicity and genotoxicity in literature, there has been limited data on their chemical characterization, although the gas phase samples are recommended to be tested within an hour of collection. Herein we describe an alternative whole smoke collection procedure which combines gas and particulate phases as “condensates” in ethanol.

Mainstream 3R4F cigarette smoke was generated using Health Canada Intense smoking regimen and collected on a Cambridge filter pad (CFP) connected in series to an ethanol-filled impinger in ice-bath. The CFP was immediately extracted with the impinger content to produce the whole smoke condensates in ethanol. Selected smoke constituents (nicotine, 1,3-butadiene, acrylonitrile, isoprene, benzene, toluene, acetaldehyde, formaldehyde, acrolein and crotonaldehyde) in condensates were compared to the whole smoke. The condensates were tested using standard *in vitro* assays: Neutral red uptake (NRU) for cytotoxicity, Salmonella mutagenicity (Ames), and micronuclei (MN) in TK6 cells for genotoxicity.

Recovery of nicotine in condensates was 99 % in comparison to whole smoke. Remaining constituents were also detected in the condensates, however, their relative proportions varied. The 3R4F condensate was cytotoxic in the NRU assay (1/EC<sub>50</sub>: 20.83 mL/mg TPM), mutagenic in the Ames assay (TA1537 and TA98), and genotoxic in the MN assay. The proposed collection method captured both gas and particulate phase of a whole smoke and the resulting condensates could be simultaneously used for chemical as well as *in vitro* biological characterization.

## STPOST 34

### Characterization of an air-liquid-interface (ALI) *in vitro* exposure system (VITROCELL® VC1/7 and Ames 48) using a prototype e-vapor product

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Direct delivery of aerosol or vapor to the apical surface of cells (ALI) allows more relevant exposure for *in vitro* toxicological evaluation of inhalable chemicals. In this study, we quantitatively characterized the aerosol delivery in a commercially available ALI *in vitro*

exposure system (VITROCELL® VC1/7 puffing machine and Vitrocell® Ames 48 [Ames 48]) using a prototype e-vapor product (MarkTen® e-cigarette with a prototype e-liquid containing propylene glycol, glycerin, nicotine, and water). The e-vapor product, with a fully-charged battery, was puffed using a 55 ml puff over 5 seconds, with a 30 second inter puff period, by a VC1/7 puffing machine. As specified by the manufacturer, e-vapor aerosol was pulled into the VC1 puffing machine and then pushed into the exposure system over 8 seconds. Aerosol mass was collected and measured gravimetrically following the first 20 puffs at the exit of each puffing unit (seven VC1s) and the inlet and outlet of the AMES 48. The average aerosol mass delivery (calculated as measured mass/total product weight loss × 100 %) was 68.6 %, 49.1 %, and 46.6 %, respectively, with about 0.48 %-0.66 % of aerosol mass delivered to the exposure inserts. Results suggested about 50 % aerosol loss in the aerosol transportation path (VC1 and tubing) prior to entry into the exposure system. To minimize the aerosol loss and consequently increase the aerosol delivery to the inserts, we revised the aerosol delivery method by shortening the aerosol transportation path. With the revised puffing method, the estimated aerosol delivery at the inlet of the *in vitro* exposure system was about 93.5 %-95.3 %, with increased aerosol delivery to 1.0 %-1.2 % in the exposure inserts.

## STPOST 35

### **Simultaneous determination analysis of nicotine, propylene glycol, glycerol and menthol in vapor of tobacco vapor products**

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Recently, in addition to e-cigarettes, tobacco vapor products, such as directly heated products and indirectly heated products, have been gaining popularity in some markets. CORESTA has developed a Recommended Method for simultaneous determination of nicotine, propylene glycol and glycerol analysis in vapor of e-cigarettes (CRM 84), but not for tobacco vapor products.

The purpose of this study was to develop an analytical method for simultaneous determination of nicotine, propylene glycol, glycerol and menthol in vapor of tobacco vapor products.

First, we tried to check the applicability of CRM 84. The vapor of directly heated products on the market was collected and analyzed according to CRM 84 using the DB-ALC1 column. As a result, it was found that triacetin contained in the filter of the sticks for directly heated products overlapped the peak of nicotine. Therefore, to improve the method, the DB-WAX column was adopted, which is known to be able to separate nicotine and triacetin. It was confirmed that all target peaks were separated by the analysis of a standard mixture of nicotine, propylene glycol, glycerol, menthol and triacetin. Furthermore, by using the column with a smaller diameter, all target components in the vapor of tobacco vapor products were separated with shorter analysis time. It was confirmed that this method was applicable to e-cigarettes and tobacco vapor products (directly heated products and indirectly heated products).

In conclusion, a new analytical method was developed for simultaneous determination of nicotine, propylene glycol, glycerol and menthol in vapor of e-cigarettes and tobacco vapor products in a short analysis time.

## STPOST 36

### Analysis of the TPD2-related flavor compounds in cigarettes by GC-MS/MS and LC-MS/MS

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The new European Union Tobacco Products Directive (TPD2) came into force in May 2016. The European Commission has established a priority list of 15 additives referred to in Article 6 of TPD2. The purpose of this study was to develop quantitative analysis methods of three flavor compounds (geraniol, maltol and guaiacol) among the additives in the priority list because there is no international standard method for their quantification for cigarettes. In order to obtain highly selective analytical results based on their chemical properties, the gas chromatography tandem mass spectrometry (GC-MS/MS) method was used for guaiacol and geraniol, and the liquid chromatography tandem mass spectrometry (LC-MS/MS) method was used for maltol. For quantitative analysis, methanol extracts and water-acetonitrile (9:1, v/v) extracts from cigarette samples were used for GC-MS/MS and LC-MS/MS, respectively. The results showed that guaiacol and geraniol had good correlation coefficients ( $R^2 > 0.999$ ) and good average recoveries (addition concentration of 0.5  $\mu\text{g/g}$ ) of 99.8 % and 101 % with relative standard deviations (RSD) of 3.8 % and 0.8 %, respectively. Commercial and reference cigarettes (CM8 and 3R4F) contained 1.32  $\mu\text{g/g}$  to 1.45  $\mu\text{g/g}$  and 0.06  $\mu\text{g/g}$  to 0.17  $\mu\text{g/g}$  of guaiacol and geraniol, respectively. The results also showed that maltol had a good correlation coefficient ( $R^2 > 0.999$ ) and good average recoveries (addition concentration of 0.5  $\mu\text{g/g}$ ) of 101 % with RSD of 5.3 %. Commercial and reference cigarettes contained 1.22  $\mu\text{g/g}$  to 3.62  $\mu\text{g/g}$  of maltol. These methods showed high selectivity, good linearity, and high reproducibility for analysis of geraniol, maltol, and guaiacol.

## STPOST 37

### Simultaneous determination of sugar alcohols in cigarettes by LC-MS/MS

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Sugar alcohols are used generally and widely as sweeteners and humectants in the food industry. On the other hand, it is known that some sugar alcohols are present in tobacco leaf and tobacco products such as cigarettes. However, there is no method for simultaneous determination of nine sugar alcohols (erythritol, xylitol, pinitol, sorbitol, mannitol, myo-inositol, maltitol, lactitol, palatinit) in cut filler. The purpose of this study was to develop a new method for simultaneous determination of these nine sugar alcohols in cut filler of cigarettes. The sugar alcohols were extracted with ultrapure water, and the water extract was then ultrafiltered and the ultrafiltrate diluted by a factor of 100 with a water-acetonitrile mixture (2:8, v/v). Subsequently they were analysed by the liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) in order to obtain highly selective analytical results. The results showed that the nine sugar alcohols had good correlation coefficients ( $R^2 > 0.999$ ), limits of detection were approximately 20  $\mu\text{g/g}$  and good average recovery rates (addition concentration of 100  $\mu\text{g/g}$ ) ranged from 98 % to 115 % with relative standard deviation from 2.4 % to 13.3 %. Consequently, this method had high selectivity and good linearity and reproducibility for simultaneous determination of the nine sugar alcohols in cut filler. Furthermore, sugar alcohols in cut filler of reference cigarettes (3R4F, CM8) and some commercial cigarettes were analysed by this method. The results showed that certain sugar alcohols existed in the reference cigarettes and the commercial cigarettes tested.

## STPOST 39

### The use of high content screening (HCS) in human primary lung cells to assess e-liquids of differing flavors and nicotine concentrations

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Due to the current scientific debate and evolving regulatory landscape, new assays are needed to quickly determine the subtle biological response of e-liquids for both stewardship and product development purposes.

For stewardship, all ingredients in the e-liquid were first assessed for carcinogenic, mutagenic and reproductive (CMR) properties and respiratory sensitizing potential, using the scientific literature and/or *in silico* tools. If no major alerts were detected, the e-liquids were assessed using a range of *in vitro* assays including high content screening (HCS). This technique utilises a range of biologically relevant cellular end points (e.g. GSH depletion, Apoptosis, NfKB etc.), in human primary lung cells (NHBEs).

This poster will discuss the data generated for two experimental base liquids (PG/VG with different nicotine strengths 1.2 and 2.4 %), four commercial e-liquids (all 2.4 % nicotine strength), and TPM generated from 3R4F cigarette. As expected, the 3R4F TPM was active and induced most of the HCS end points at concentrations, for the majority, two orders of magnitude lower (minimum effective concentration, MEC range 0.001-0.008 %) than the e-liquids (MEC range 0.05-2.98 %). Base liquids were generally inactive in most of the HCS end points measured, with effects at lower MECs seen with higher nicotine strengths. There were differing effects of flavors on specific HCS endpoints.

Due to the osmotic nature of e-liquids, cells were exposed to a maximum concentration of 3 % for 24 hours. A concentration of 3 % is in excess of levels of exposure attainable through normal e-cigarette usage. Work is currently underway to determine local cellular concentrations of e-liquid aerosol exposure in humans. The HCS assay enables prioritisation of flavors and the data is used as part of a wider overall assessment framework. We are currently developing methodology to investigate HCS endpoints using aerosol, to cover the possible impacts of any thermal degradation products.

## STPOST 40

### **Analytical methods for the determination of aldehydes in e-vapor aerosol and cigarette smoke and their urinary biomarkers of exposure**

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Potential exposure to aldehydes, such as acrolein, formaldehyde or acetaldehyde, formed due to thermal degradation of the e-liquid constituents propylene glycol (PG) and glycerol (G) during the consumption of e-vapor products (EVPs), have received much attention in the scientific and public health community in recent years.

An e-liquid, containing stable isotope-labelled nicotine, PG and G, and conventional non-filter cigarettes spiked with the same labelled compounds, serving as a positive control, were used to assess the formation of aldehydes in EVP aerosol and mainstream smoke. In addition, urine obtained from a clinical study with 20 vapers and 5 smokers using the labelled products was used to investigate the aldehyde related mercapturic acid metabolites and glutathione adducts.

Mass selective detection allows the unequivocal discrimination between labelled and unlabelled compounds. Hence, analysis of the labelled aldehydes by means of LC-MS/MS revealed the amounts exclusively formed from PG and G during vaping/smoking and their metabolites in human urine. Aerosol analysis comprised the simultaneous detection of 13 aldehyde derivatives after trapping with 2,4-dinitrophenylhydrazine (DNPH) solution. Urinary bioanalysis included the mercapturic acids metabolites of acrolein and crotonaldehyde using previously validated assays, while metabolites of formaldehyde (thiazolidine-4-carboxylic acid (TCA)) and acetaldehyde (methyl-thiazolidine-4-carboxylic acid (MTCA)) were investigated by means of a newly developed and validated LC-MS/MS method.

The methods are suitable to differentiate between labelled and unlabelled aldehydes and therefore allowed for the analysis of aldehydes specifically formed due to the degradation of PG/G under vaping and smoking conditions as well as the excretion of the respective metabolites in urine.

## **STPOST 41**

### **LC-MS/MS multi-method for the determination of mono- and dicarbonyls in e-liquid and e-cigarette vapor**

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It is generally accepted that e-liquids and e-cigarette vapor may contain mono- and dicarbonyls. This class of compounds derive either from propylene glycol, glycerol or flavor substances in e-liquids or from thermal degradation during the vaping process. Since some of the mentioned carbonyls are toxic, there is great interest to investigate their levels in emissions, especially from reduced risk products.

In this work we present the development and validation of an analytical method which is capable to quantitatively determine twelve monocarbonyls (formaldehyde, acetaldehyde, crotonaldehyde, propionaldehyde, butyraldehyde, acetone, acrolein, 2-butanone, benzaldehyde, cinnamaldehyde, acetoin, acetol) as well as four dicarbonyls (glyoxal, methylglyoxal, diacetyl, 2,3-pentanedione) in e-liquid and e-cigarette vapor. The large number of analytes in combination with their presumably low concentrations provides the need for a highly selective and sensitive analytical method such as high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry (HPLC-MS/MS) operating in negative mode. Prior to injection, e-liquid and e-cigarette vapor samples were diluted with 2,4-dinitrophenylhydrazine for derivatization. Within 23 min total chromatographic run time, 16 carbonyls together with their corresponding isomers could be unambiguously identified. Four isotope-labelled internal standards were monitored to control the reproducibility of the sample preparation and analytical method.

The described method was comprehensively validated showing reasonable results regarding recovery (e.g. 83-126 % in e-cigarette vapor) or reproducibility (e.g. inter-day RSD for e-liquid <20 %, except glyoxal). In one of the selected e-liquid samples glyoxal, methylglyoxal and acetol could be detected at concentrations of 8.4, 25 and 9.6 µg/g e-liquid, respectively. Vaping of the same e-liquid according to CRM 81 using a closed tank-system as device provided the following results: glyoxal, methylglyoxal and acetol at concentrations of 0.4, 2.6 and 1.5 µg per 25 puffs each, respectively.

In summary, a straightforward, robust, sensitive, selective and reliable method for the determination of 16 carbonyls in e-liquid and vapor of e-cigarettes has been developed and validated.



## STPOST 42

### **Observation of wicking behavior of an electronic nicotine delivery system (ENDS) device using weight-time measurements**

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The rate of liquid uptake (wicking) of an e-vapor liquid is an important factor for the performance of an electronic nicotine delivery system (ENDS) device. A common method for quantifying the wicking rate uses Lucas-Washburn theory. Here the time required for a liquid to travel a certain height in the material due to capillary action is determined. An alternative to this traditional height-time approach to measuring the wicking rate uses the mass uptake of the liquid by the material over time. Weight-time measurements consider the rate of liquid traveling throughout the entire wick whereas height-time measurements only consider a single observable edge. A modified Lucas-Washburn equation serves as a model equation for these weight-time measurements. This modified equation gives a capillary constant, an effective composite rate constant for the entire wicking process that includes both liquid parameters and wicking dimensions. The mass uptake of a variety of e-liquids by a wick made of fiberglass strands offers a test case to apply this method. A tensiometer serving as a hanging balance monitored the mass uptake of an e-liquid into an ENDS device wick over the time interval required for saturation. The calculated capillary constants from these experiments correlate with the liquid parameters of the modified Lucas-Washburn equation: viscosity, surface tension, and liquid density. The results show that weight-time measurements offer a direct and reproducible method to understand the wicking process observed in ENDS devices.

## STPOST 43

### ***In vitro* micronucleus (MN) assay using TK6 cells: review of historical positive and negative control data**

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TK6 human lymphoblastoid cells are widely used in the *in vitro* MN assay as a part of regulatory genotoxicity testing for pharmaceuticals, but have seen relatively limited use in testing of other test materials, including tobacco-related products. As an established cell line, they do not exhibit the donor to donor variability observed in primary cells such as human lymphocytes. Unlike other routinely used p53-deficient cells (CHO, V79 and L5178Y), TK6 cells are p53-proficient, capable of DNA repair and normal cell cycle regulation, and generally have lower spontaneous MN frequencies and a lower rate of

false or misleading positive results. We have used TK6 cells extensively to test a variety of e-liquids and e-vapor condensates, and total particulate matter (TPM) from combusted cigarettes. Here we present historical positive and negative control data, compiled from these OECD TG487- and GLP-compliant studies. The average negative control values (%MN) for the three treatments ranged from 0.93-1.03 with comparable ranges between different vehicles (negative controls). The known positive controls (two clastogens and one aneugen), run concurrently, gave consistent and robust responses in %MN induction and ranged from 6.36-10.53. The consistency of results is critical since OECD guidelines place emphasis on the use of historical controls in evaluating individual assay acceptability and the biological relevance of results, and demonstrate the utility of this test system for evaluating tobacco- and ENDS-related products.

## **STPOST 44**

### **Non-targeted analysis using gas chromatography mass spectrometry to evaluate stability of e-vapor products**

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In the newly regulated landscape of tobacco products there is an increasing need to fully characterize the chemical composition of aerosol in new products. Although aerosol from e-vapor products are considerably less complex than aerosols from heat-not-burn (HNB) or mainstream smoke from cigarettes; there are still challenges that arise from the chemical composition of the matrix, variety of flavors used, and the potential for chemical interactions to occur during storage. There is additional complexity associated with efficiently collecting both volatile and semi-volatile compounds delivered in the aerosol from e-vapor products. We have developed an automated workflow for data analysis that includes mass spectral deconvolution, peak detection, library searching and reporting.

Aerosol samples were collected using a 55 mm Cambridge filter pad (CFP) with a trailing impinger containing 10 mL of ethanol chilled to -70 °C, to ensure both volatile and semi-volatile compounds are captured. Samples were analyzed on an Agilent GC/MS system (7890B with 5977A) using a Restek Stabilwax® GC column (30 meter × 0.25 mm ID × 0.25 µm film) with an infused 5-meter integra guard column. Our workflow includes both Agilent MassHunter Unknowns Analysis and Automated Mass Spectral Deconvolution and Identification System (AMDIS) software for identification of extraneous peaks. Identification of compounds not present in mass spectral libraries includes secondary analysis using high resolution mass spectrometry on a GC-Orbitrap™ with EI and CI ionization modes, allowing for the identification of molecular formulas within 5 ppm of mass accuracy. Compound identification was confirmed through the use of reference standards.

## STPOST 45

### Ames assay cytotoxic assessment using bacterial lawn integrity with 35 mm plate spread technique

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**Background:** The Ames assay is commonly used to evaluate the mutagenic potential of tobacco products for regulatory purposes. A significant increase in the number of His+ revertant colonies observed in agar containing Salmonella bacteria and a test chemical indicates mutagenicity. To assess cytotoxicity in this assay, thinning of the background bacterial lawn is microscopically determined. With the development of new *in vitro* exposure systems that permit continuous cellular exposure to smoke or aerosol, the Ames assay method required modification by spreading the bacterial solution on top of agar in a 35 mm plate. However, studies detailing the use of bacterial lawn thinning as an indicator of cytotoxicity are needed.

**Objective:** To determine if microscopic analysis can detect thinning of the bacterial background lawn on top of the agar with or without metabolic activation (S9). We hypothesize that thinning can be generated by limiting the concentration of histidine/biotin in the spread solution.

**Methods:** Ames assay was performed using a 35 mm agar plate spread technique with a bacteria solution (with or without S9) containing varying amounts of histidine-biotin. Salmonella strains (TA98, TA100, TA102, TA1535, TA1537 and TA97a) were used to determine the number of His+ revertants and lawn thinning after 48/72 hours of incubation.

**Results:** We observed the reproducible thinning of the background lawn by limiting the amount of histidine/biotin. Lawn thinning was clearly observed without S9. However, the presence of 10 % S9 compromised the lawn assessment but was improved by reducing the S9 to 5 %. Using 5 % S9, the positive and negative control values for revertant colonies were within historical control ranges (similar to 10 % S9) for all strains.

**Conclusions:** Thinning of the background bacterial lawn was clearly observed in all strains using the Ames assay with spread technique in the presence or absence of 5 % S9 and microscopic analysis.

## STPOST 46

### Validation of an *in vitro* test battery to evaluate smokeless tobacco products

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Evaluating potential cytotoxicity and genotoxicity of smokeless tobacco products poses unique challenges for *in vitro* testing. Each product is a complex mixture of vegetable matter, and may include a packaging component as part of the final product. Therefore, such products are evaluated in a manner similar to packaging products or medical devices – incubated with various fluids to extract leachable components which are subsequently applied to the test system. Also, individual extract constituents may be dilute and/or innocuous, requiring large dose volumes to detect any effect. Here we report validating sample preparation, and basic processing and handling parameters, for an *in vitro* test battery including the bacterial reverse mutation (Ames) assay, *in vitro* micronucleus in TK6 cells, and neutral red uptake (NRU) cytotoxicity assay. Two commercial smokeless tobacco products (one flavored, one unflavored) were shredded and sieved, and then sonicated and extracted in artificial saliva according to published procedures. Initial results in an Ames screen (using tester strains TA98 and TA100 ±S9, pre-incubation treatment, and dose volumes up to 200 µL/plate) revealed: moderate to severe cytotoxicity for the flavored product at a dose of 200 µL/plate; discoloration of the plates at the highest dose levels (without interference with automated scoring); and a lack of mutagenicity for both extracts under these conditions. Preliminary results using nicotine as a surrogate marker indicate that the extracts are stable for at least one month at -70 °C. Additional experiments in the full assays, and to qualify other flavor markers or tobacco constituents, are in progress.

## STPOST 47

### Effect of ethanol concentration on chemical composition of tobacco extracts

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Ethanol is widely used as a solvent in the chemical, pharmaceutical, and biological industries, for the extraction of various natural products, including those from tobacco. In this study, an ethanol-water system with four different concentrations of ethanol was used for extracting products from flue-cured tobacco by reflux. Changes of chemical composition including the volatile compounds in the extracted fractions were measured.

The extracts were also blended into a typical flue-cured cigarette tobacco blend for sensory evaluation. With the decrease of ethanol concentration, the following results were observed: 1) content of total sugar and reducing sugar in tobacco extracts gradually increased; 2) total alkaloids gradually decreased, and total nitrogen gradually increased; 3) range changes in total alkaloids and total nitrogen were smaller; and, 4) tobacco extracts of volatile alcohols, aldehydes, ketones, and esters were gradually reduced. With this system, the extraction can be effectively controlled according to the needs of cigarette formulations. It was found that tobacco extract with a high concentration of ethanol had a stronger effect on the top notes of cigarette tobacco blends and tobacco extract with a moderate and low-concentration of ethanol had a stronger effect on the body-base notes of cigarette tobacco blends. The developed technology can be used to improve the sensory quality of cigarettes by effectively modifying their flavour and taste. The requirements of the related cigarette tobacco blend for improving the formulation of the top notes, the body notes, and the base notes can be satisfied.

## STPOST 48

### **Six-month systems toxicology inhalation/cessation study in *ApoE*<sup>-/-</sup> mice to investigate cardiovascular and respiratory exposure effects of two reduced risk tobacco products compared with conventional cigarettes**

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Cigarette smoking causes adverse health effects that may occur shortly after smoking initiation and lead to the development of cardiovascular disease, respiratory disease (chronic obstructive pulmonary disease), and various cancers. To reduce the risk of smoking-related diseases, Philip Morris International is developing reduced risk products (RRP) to which adult smokers can switch instead of continuing to smoke cigarettes.

Engaging a systems toxicology approach combining physiological, histological, and -omics endpoints, the effects of a six-month exposure to cigarette smoke (CS) or to aerosols from two RRP, the carbon heated tobacco product (CHTP) and the tobacco heating system (THS), were investigated in *ApoE*<sup>-/-</sup> mice. In addition, the impact of cessation or switching to CHTP aerosol exposure after three months of CS exposure was evaluated.

Our results demonstrated that exposure to CS at a concentration of 28.0 µg nicotine/L causes adverse effects on the lungs, including increased lung volume, lung inflammation, aortic plaque formation, and a dysregulation of the heart transcriptome. In contrast, exposure to either THS or CHTP aerosol at matched nicotine concentrations did not induce lung inflammation or enhance plaque development. Cessation or switching to CHTP aerosol exposure reversed lung inflammatory responses and halted progression of aortic plaques. Transcriptomics analysis revealed that multiple biological pathways were

impacted significantly in heart tissue by CS exposure but not by exposure to CHTP or THS aerosols. Both cessation and switching to CHTP aerosol reduced these perturbations to levels similar to those in sham-exposed animals.

In conclusion, in this study, exposure to aerosol from either THS or CHTP had minimal adverse respiratory and cardiovascular effects. In addition, cessation or switching to CHTP aerosol exposure delayed the progression of CS-induced atherosclerotic and lung emphysematous changes.

## STPOST 49

### Vacuum photoionisation TOF-MS as technique to analyze complex gas mixtures on-line and in real time

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Within the last few years e-cigarettes and other new smoking/vaping products have become more and more commonly used. There are two general ionization techniques that are covered by vacuum Photo-Ionization Time-of-Flight Mass-Spectrometry (PI-TOFMS). On the one hand there is SPI (Single-Photon-Ionization) ionizing a wide range of organic molecules and on the other hand there is REMPI (Resonance-Enhanced-Multi-Photon-Ionization) focusing primarily on aromatic structures. The complementary use of SPI and REMPI can especially access new information.

SPI uses varying ionization light sources based on lamps or lasers. The respective wavelengths/ionization energy limit the range of ionizable organic compounds being roughly in a range of 7 to 11 eV (177 nm to 112 nm). Because most matrix gases such as oxygen, nitrogen, carbon dioxide and especially water vapour have higher ionization energies of 12 eV and more, they will be suppressed efficiently. Depending on the specifically used REMPI method, at least two photons are used for ionization. This ionization mechanism requires a stable excitable intermediate state that is primarily present and accessible in aromatic structures.

Soft photoionization can be applied in various research fields and applications dealing with complex gas mixtures that need to be observed in real time. A high temporal resolution especially enables the investigation of fast and dynamic processes. Faced with a wide range of cigarettes and new smoking products such as e-cigarettes and tobacco heating products (THP), photoionization enables a puff resolved investigation of released compounds starting with nicotine but also the HPHCs (harmful or potentially harmful compounds). In a further step, not only the temporal release but also the spatial occurrence in the cigarette, can be observed. Chemical heat maps can be produced to understand formation and degradation processes within the cigarette.

The PI-MS approach can be also transferred to other applications needing a temporal and spatial analytical resolution.

## STPOST 50

### A risk-based approach for the validation of bespoke smoking analyser software

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**Background:** The Smoking Analyser 7 (SA7), a device for measuring consumer's puffing topography and mouth-level exposure, has been used extensively by British American Tobacco (BAT) since 1999. Due to the ever-changing nature of technology and the regulatory environment, a more modernised, future-proof version of the existing software was designed, developed and validated in line with BAT's commitment to scientific data integrity. As the U.S. Food and Drug Administration (FDA) explicitly recommends employing "validated methods of analysis" for Modified-Risk Tobacco Product Application (MRTPA) submissions, a risk-based approach to data collection system validation is now more critical than ever before.

**Aim:** To develop and validate a modernised version of BAT's smoking analyser software following a Systems Development Lifecycle (SDLC) aligned with pharmaceutical industry standards (Good Automated Manufacturing Practice, GAMP5) and BAT Good Research Practice (GRP).

**Method:** GAMP5 is a recognised risk-based approach for Computer Systems Validation (CSV), widely used by FDA-regulated pharmaceutical companies as a key part of their Quality Management Systems (QMS). BAT R&D IT have developed a tailored QMS, built upon GAMP5, that describes the appropriate level of validation required for a computer system based on system criticality, complexity and novelty of the solution. Due to the bespoke nature of the SA7 software and relatively high operational risk, a GAMP Software Category-5 approach to validation was applied.

**Results:** The replacement SA7 software was successfully delivered and validated in accordance with the plan set out during initiation of the project. By following the GAMP5-based approach, end-users can be confident that the software meets their exact requirements, works as intended and ensures that the correct controls are in place to help maintain data integrity during and post-completion of the project.

## STPOST 51

### Simultaneous measurement of cilia beat frequency, goblet cell hyperplasia and TEER endpoints in EpiAirway™-FT treated tissues may reveal underlying toxicity mechanisms *in vitro*

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The EpiAirway™-FT (full thickness) tissues (Mattek Corporation, Ashland, USA) contain tight junctions, ciliated and other bronchial cell types making it an ideal model for testing aerosolised substances *in vitro*. Toxicity is not a single endpoint but a combination of

events that lead to tissue remodelling. By analysing cilia beat frequency (CBF), trans-epithelial electrical resistance (TEER), post-treatment tissue depth in conjunction with goblet cell hyperplasia (GCH) per tissue, we investigated this multi-endpoint approach for understanding different mechanisms of toxicity *in vitro*.

We treated EpiAirway™ tissues for 6 days (basolaterally) with several chemicals and recorded data for CBF, TEER, tissue depth and GCH.

IL-4, IL-17 and IL-13 resulted in up to 4-fold increases in GCH. Reduction in TEER was observed with Platelet Activation Factor (PAF) at 0.001 µM and above. Treatment with staurosporine appeared to reduce tissue depth and affect CBF (which was also affected by treatment with PAF at >0.001 µM).

We also exposed tissues to Adenosine triphosphate (ATP), Procaterol Hydrochloride (USAN), Hydrogen Peroxide (H<sub>2</sub>O<sub>2</sub>) or Benzalkonium Chloride (BC) or a pH range (4 to 10) and analysed after 0.5 and 2 hours. Untreated tissues had an average CBF of 17.62 Hz (n=40). ATP (0.01 mM) and USAN (0.01 mM) increased CBF (as expected) to 19.68 Hz and 20.42 Hz respectively and BC (3 mM) decreased CBF (as expected) to 2.5 Hz. H<sub>2</sub>O<sub>2</sub> (0.1 mM) unexpectedly increased CBF (19.55 Hz), even at 10 mM (19.37 Hz). Only pH4 adversely affected CBF yielding ciliostasis. When exposed to 3R4F cigarettes (Health Canada Intense regime / 88 minutes / Vitrocell® VC10® machine [VitroCell GmbH, Germany]), CBF was slightly elevated at 2 L/min compared to the air control and was undeterminable at higher concentrations.

These data show that toxicity is a complex process but by analysing multiple endpoints, elucidation of the underlying mechanism could be possible using this 3D tissue model.

## STPOST 52

### **Biological test procedure for fresh generated cigarette smoke and e-cigarette aerosols under air-liquid interface (ALI) exposure in 24 and 96 multi-well plates**

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Cigarette smoke and e-cigarette aerosol contain particulate matter/liquid droplets in the solid phase as well as volatile substances in the gas phase. Both fractions are of potential toxicological relevance and should be considered when assessing biological activity of cigarette smoke and e-cigarette aerosol. Experimentally, rapid ageing of the mixtures leads to problems regarding trapping and effective delivery of both fractions to the cells. Therefore, dedicated exposure systems have been developed allowing the exposure of biological *in vitro* systems to freshly generated smoke/aerosols.

Imperial Brands' Smoke Aerosol Exposure In Vitro System (SAEIVS) was designed to expose cells in multi-well plates (MWP) at the air-liquid interface (ALI). SAEIVS enables *in vitro* testing of aerosols generated from different product categories encompassing tobacco products and e-cigarette devices. Using this system, up to five tobacco products or e-cigarette devices can be puffed simultaneously and deliver the smoke/aerosol in



undiluted or diluted form to defined rows of wells. Two exposure chambers allow parallel exposure of the same smoke and aerosol in different dilution levels per plate. Furthermore, the separate chambers enable exposure to the same product in different MWPs.

The cytotoxicity of the products is tested by exposure of BEAS2B cells on Type I collagen matrix in 96 MWPs. The collagen layer guarantees stable exposure conditions over an extended time period sufficient for the testing of several hundreds of puffs. Genotoxicity testing is performed with hamster lung cells V79 grown on inserts and placed in 24 MWPs. Mutagenicity testing is conducted by bubbling a bacterial suspension directly with freshly generated smoke/aerosol. *Salmonella typhimurium* strain TA100 was used as it is highly sensitive to mutagenic substances in both fractions of smoke/aerosol.

The presented methods by Imperial Brands allow testing *in vitro* of aerosols generated from different product categories encompassing tobacco products and e-cigarette devices.

## STPOST 53

### Use of human derived cell lines to increase the biological relevance of cigarette condensate, non-tobacco materials and e-liquids testing

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The *in vitro* testing strategy of tobacco products is mainly based on the testing of extracts from cigarette smoke condensates. An accepted industry wide assay battery has been developed over recent years which delivers reliable results for the toxicological assessment of tobacco products. The testing battery comprises the neutral red uptake (NRU) assay, the *in vitro* Micronucleus (IVM), and the Ames test. This combination of tests evaluates relevant biological endpoints and is also used for the toxicological evaluation of non-tobacco materials (NTM) such as ingredients and, more recently, e-liquids. All methods are based on the corresponding OECD guidelines which leave some choices regarding the specific bacteria strains used in Ames test and cell lines utilized in NRU and IVM assays. To increase relevance for toxicity assessment, human cell lines were established whenever possible to measure cytotoxicity and genotoxicity of smoke extracts, non-tobacco materials and e-liquids. Human liver (HepG2) and bronchial cells (BEAS-2B) were used in the NRU assay where both demonstrated high sensitivity against cigarette condensate and delivered good results for e-liquids. The IVM assay was performed with the human lymphoblastoid cell line TK6 as extension to the V79 rodent cells. According to literature false positive effects are significantly reduced when using TK6 in comparison to widely used rodent cell lines. The assay battery was completed by mutagenicity testing using the Ames test. Here, representative results using different human cell lines with cigarette smoke condensate and e-liquids in the NRU and IVM tests are shown. Results of cigarette smoke condensate induced bacterial mutagenicity are shown for the two *Salmonella typhimurium* strains TA 98 and TA 100.

In summary this poster shows representative results that demonstrate the general applicability of IB's biological *in vitro* testing for the assessment of next generation products (NGPs).

## STPOST 54

### Patterns of use behaviors in a sample of Japanese heat-not-burn tobacco product (IQOS) users

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Innovative tobacco products are being developed with the potential to advance tobacco harm reduction efforts and reduce the risk of smoking-related diseases. Their potential beneficial public health impact depends upon smokers' acceptance to switch to these products instead of continuing to smoke cigarettes. Thus, post-market monitoring is important to evaluate actual use.

This online survey describes patterns of use behaviors in Japanese current users of a novel heat-not-burn tobacco product (commercialized under the brand names "IQOS" and "HeatSticks"). It was conducted in 2016-2017 with 2,000 participants (81.6 % males; average age 38.5 years; 56.8 % had completed higher education). 74.8 % [95 %CI 72.7-76.7 %] started using IQOS within the 12 months prior to the survey and 98 % [97.2-98.6 %] were previously using other tobacco products. Of those who were still smoking cigarettes, 52.1 % [49.1-55.1 %] quit smoking within the 12 months prior to the survey and had switched to IQOS.

63.4 % [61.2-65.6 %] reported exclusive IQOS use, 20.6 % [18.7-22.5 %] reported dual-use with cigarettes, and 7.3 % [6.1-8.6 %] reported poly-use with cigarettes. Average daily total consumption of tobacco products (*HeatSticks* and cigarettes) was 19.1 [18.6-19.6] in the whole sample and 24.8 [23.5-26.1] for dual IQOS and cigarettes users. Average daily consumption of *HeatSticks* was 16.8 [16.3-17.3] for exclusive users, 13.8 [12.9-14.6] for dual IQOS and cigarette users, and 12.8 [11.3-14.2] for poly-users. Average consumption of cigarettes per day was 11.0 [10.1-12.0] for dual cigarettes and IQOS users, and 12.8 [11.1-14.5] for poly-users.

In conclusion, these findings show that majority of IQOS consumers in this sample consisted of former smokers who switched to exclusive IQOS use. IQOS users who continued to smoke reported a higher total consumption of tobacco products, however, their average daily consumption of 11 cigarettes was lower compared to a national average daily consumption of 15.5 cigarettes in Japanese smokers (2015 Japanese National Health and Nutrition Survey).

## STPOST 55

### **A randomised, controlled study to evaluate the effects of switching from cigarette smoking to using tobacco heating products on health effect indicators in healthy subjects**

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Preclinical assessments and 5-day exposure clinical studies have shown that toxicant emissions are lower in tobacco heating products (THPs) compared to conventional cigarettes. However, it is unclear if these reductions are sustained and if they translate to reductions in smoking-related health risks.

The aim of this study is to test the hypothesis that reduction in exposure to toxicants will result in changes in biomarkers of biological exposure (BoBE) and biomarkers of exposure (BoE) when smokers switch to using THPs compared with smokers who continue to smoke, and that these changes are directionally similar to changes seen in smokers who cease smoking, over 12-months in an ambulatory setting.

This novel study, conducted in the U.K. (ISRCTN81075760), has been approved by a local Research Ethics Committee and run in accordance with ICH-GCP. Subjects will be of either gender (aged 23-55 years). Regular smokers will be randomly allocated to either continue to smoke their own brand cigarettes, or switch to smoking a THP for 360 days. A separate smoking cessation group will be made up of regular smokers who intend to quit and will be provided with assistance with quitting (NRT/varenicline/counselling). The last group will be participants who have never smoked. Subjects will attend a total of 13 non-residential clinic visits plus a Screening Visit and a Follow-up Visit over a period of 12 months. BoE, BoBE, physiological endpoints and questionnaire assessments will be assessed in this study. Safety evaluations will include adverse events, vital signs, clinical laboratory evaluations, physical examinations, electrocardiogram, and lung function tests.

The primary outcome measures will include changes in selected BoE, BoBE, biochemical, physiological and psychometric endpoints at baseline, 90 days and 360 days.

Data from this study will advance our scientific understanding of the changes in BoBE to cigarette smoke toxicants in smokers who switch to using a THP.

## STPOST 56

### **Application and use of e-cigarettes HPHC methods for the analysis of heated tobacco products**

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The interest in e-cigarette research has resulted in the development of methods for the quantitative analysis of chemical constituents present in their aerosols. The emissions of e-cigarettes contain not only nicotine, propylene glycol, glycerin, water and flavours, but also nicotine related impurities and thermal degradation products. We have developed and validated analytical methods for the analysis of e-cigarette aerosols including the compounds listed in the U.S. FDA Draft PMTA guidance document. These include primary constituents, aldehydes, VOCs, organic acids, metals, aromatic amines, nicotine related impurities and benzo[a]pyrene. An emerging tobacco product; heated tobacco products (HTPs) produce an aerosol by the heating of tobacco. Since tobacco is not combusted in these products, the majority of the resulting aerosol is comprised of water, humectants and volatile compounds. Given the similarities between e-cigarettes and HTPs, we hypothesized that e-cigarettes methods would also be suitable for analysis of HTPs emissions. In this study, we have applied our e-cigarette methods to the analysis of the iQOS HEETS HTPs. The aerosols were collected under intense smoking condition, using a device holder designed for e-cigarettes, and analyzed for HPHCs. We found that methods developed and validated for e-cigarettes could be successfully applied for the analysis of HTPs emissions. With the exception of nicotine and water, the emissions from HTPs were found to be more similar in emissions to e-cigarette aerosol than cigarette smoke. We found that e-cigarette methods were well suited for the analysis of HTPs due to the lower calibration ranges and selected compound list as compared to methods developed for conventional cigarettes. The iQOS HEETS HTPs were compared against the 3R4F reference cigarette and we found that VOCs, aromatic amines, benzo[a]pyrene and TSNA were reduced by 99 % and carbonyl compounds were reduced by 87 % when tested under intense smoking conditions.

## STPOST 57

### **Assessing the potential population health impact of market authorization of e-cigarettes in the U.S.**

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When evaluating a Premarket Tobacco Product Application (PMTA) or Modified Risk Tobacco Product Application (MRTPA), the Food and Drug Administration (FDA) employs a Population Health Standard, which requires evaluation of risk and benefit to the population as a whole; including tobacco users and non-users. In the absence of

epidemiological data, computational models are valuable tools for predicting the likely impact of introducing a new tobacco product on the U.S. population. Using best modeling practices, we have developed and validated an Agent Based Model that assesses the impact of introducing a new product into a hypothetical population by estimating changes in tobacco use prevalence and premature death prevented for a Modified Case Scenario (where both cigarettes and e-cigarettes have authorization to be marketed in the U.S.) against a Base Case scenario (where cigarettes are the predominately used tobacco product and e-cigarettes do not have authorization to be marketed). The model allows us to integrate information about relative risks between existing and new products, with learnings from published literature, national dataset analyses and consumer perception studies that provide insights about potential behavioral changes, which may occur when a new product is introduced into the market. Survival probabilities of current and former e-cigarette users were determined by combining statistical models with excess relative risks (ERR). Base Case transition probabilities were obtained from nationally representative databases. The Modified Case transitions probabilities were estimated from an analysis of Wave 1 and Wave 2 data from the Population Assessment of Tobacco and Health (PATH) study. Employing these transition probabilities and an ERR value of 0.05 for e-cigarette use compared to smoking, we demonstrate a net benefit to the population of ~600,000 premature deaths prevented, along with a reduction in cigarette smoking prevalence, over the simulation follow-up period. Results from sensitivity analysis will also be discussed.

## STPOST 58

### **Developing fit-for-purpose self-report instruments for assessing consumer responses to tobacco and nicotine products: the ABOUT Toolbox initiative**

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Assessing the full potential of reduced risk products (RRP), for individual users and the population as a whole, requires the assessment of consumer perception and behavior associated with tobacco and nicotine products (TNP) with different exposure and risk profiles. In this context, psychometrically sound self-report instruments are needed to allow accurate comparison between RRP and other tobacco and nicotine-containing products. Consistent with best practice guidelines, including the U.S. Food and Drug Administration's "Guidance for Industry Patient-Reported Outcome Measures: Use in

Medical Product Development to Support Labeling Claims”, fit-for-purpose, reliable, and valid instruments are now being applied to tobacco regulatory research. The present paper presents the ABOUT Toolbox (Assessment of Behavioral Outcomes related to Tobacco and nicotine products) initiative, resulting from an ongoing collaborative effort in close partnership with scientific experts from academic and commercial organizations with expertise in the fields of nicotine addiction, motivational aspects of consumer perception, and relevant areas on approaches to measurement (e.g. Patient Reported Outcomes, cross-cultural adaptation, psychometrics, regulatory submissions). This communication (1) describes the methodological steps followed for the development and validation of the measurement instruments included in the ABOUT Toolbox and (2) presents a summary of the high-priority, tobacco-related domains (e.g. perceived risks, dependence, product use history) that are currently covered in the ABOUT Toolbox. By making the ABOUT Toolbox available to the tobacco research and public health community, we envision a rapidly expanding knowledge base, with the goals of (1) supporting consumer perception research to allow comparisons across a wide spectrum of TNPs, (2) enabling public health and regulatory communities to make better-informed decisions for future regulation of TNPs, and (3) enhancing surveillance activities associated with smoking-related disease.

## STPOST 59

### **Quantification of nicotine related impurities in novel, oral tobacco-derived nicotine products**

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VERVE® Discs and Chews are oral, non-dissolvable, tobacco-derived nicotine products. All tobacco products sold in the U.S. are regulated by the FDA and this category of products will ultimately require a market authorization through the premarket tobacco application (PMTA) pathway. This regulatory pathway requires “Established shelf life of the product to include data establishing the stability of the product through the stated shelf life.” The U.S. and European Pharmacopeias recommend purity specifications for nicotine intended for pharmaceutical products; however, there are no official purity specifications recommended for the tobacco-derived nicotine added to novel tobacco products. We developed a sensitive, selective, and robust liquid chromatography-mass spectrometry (LC-MS) method utilizing an Agilent 1260 HPLC/6150 Mass Spectrometer with a Waters C18 column to quantify nicotine impurities. The method accuracy and precision was found to be acceptable at 88-103 % and 1.6-6.7 %, respectively. The limit of detection (LOD) was found to be 0.24 mg/g and 0.06 mg/g for VERVE® Discs and Chews, respectively, which was sufficient to quantify nicotine-related impurities listed in Pharmacopeia guidelines. An overview of the challenges and solutions that transpired during method validation for these unique matrices is provided. In addition, we provide nicotine impurity results in VERVE® Discs and Chews and monitored these impurities over time. This selective and sensitive method provides data suitable for quantitative risk assessments and for stability studies.

## STPOST 60

### Evaluation of novel, oral tobacco-derived nicotine products for HPHCs

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In May 2016, the U.S. Food and Drug Administration (FDA) issued a final rule to deem e-cigarettes, cigars and all other tobacco products to be subject to the Federal Food, Drug, and Cosmetic Act (the FD&C Act), as amended by the Family Smoking Prevention and Tobacco Control Act (Tobacco Control Act). Manufacturers of regulated tobacco products are required to report to FDA quantities of harmful and potentially harmful constituents (HPHCs) by November 8, 2019. FDA has not issued specific guidance for reporting HPHCs for novel oral tobacco products, such as those containing tobacco derived nicotine, as they have for certain other regulated tobacco products. In the absence of specific guidance from FDA, we measured HPHCs in VERVE® (oral, non-dissolvable, tobacco derived nicotine products) according to the requirements for smokeless tobacco, recognizing that these products do not meet the statutory definition of a smokeless tobacco product. The objective of this work was to modify and validate existing analytical methods to measure HPHCs in two product variants. An overview of the challenges and solutions that transpired during method development and validation for these unique matrices is provided. Also, the HPHC results are compared to other commercially available oral tobacco products and an oral nicotine replacement therapy (NRT) product. Results show the absence of detectable levels or significant reductions in HPHCs compared to traditional oral tobacco products and comparable HPHC results to the NRT.

## STPOST 61

### A 6-month inhalation study in *Apoe*<sup>-/-</sup> mice to investigate cardiovascular and respiratory exposure effects of e-vapor aerosols compared with cigarette smoke

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Chronic exposure to cigarette smoke is a risk factor for the development and progression of cardiovascular disease and chronic obstructive pulmonary disease. Considerable attention has been given towards the potential reduced harm of e-cigarettes (e-cigs). Here, *Apoe*<sup>-/-</sup> mice were used to evaluate lung inflammation, atherosclerosis

development and the underlying molecular changes upon 6-month exposure to mainstream (MS) cigarette smoke (CS) from a reference cigarette 3R4F or to e-cig aerosols generated using capillary aerosol generators from various e-liquids (“CARRIER” containing humectants [propylene glycol, glycerin] and water, “BASE” containing humectants, water and 4 % nicotine, and “TEST” containing humectants, water, 4 % nicotine, and flavors). *Apoe*<sup>-/-</sup> mice were exposed to CS and the e-cig aerosols (“BASE” and “TEST”) at a matched nicotine concentration for 3 hrs/day via whole-body inhalation. The CARRIER exposures were set to match the total particulate matter for “TEST”. Aerosol particle sizes were within the respirable range, from 0.71-0.90 µm for 3R4F MS and 0.74-1.28 µm for e-cig aerosols. Pulmonary inflammation and atherosclerotic plaque areas, as well as cholesterol concentrations in serum or lipoprotein fraction, were quantified at months 3 and 6. In contrast to CS, exposure to e-cig aerosols resulted in no increase in leukocyte counts, serum cholesterol concentration, aortic plaque formation, and lung matrix metalloproteinase activity compared to fresh air. Furthermore, no such changes were observed between the exposures to CARRIER, BASE, and TEST aerosols. In conclusion, e-cig aerosols did not induce disease mechanisms related to atherosclerosis and lung inflammation that were elicited by CS in the *Apoe*<sup>-/-</sup> model.

## STPOST 62

### **Nasal mucociliary clearance is a physiologically-relevant biomarker of tobacco effect/potential harm**

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Chronic cigarette smoking increases the risk of several diseases including chronic obstructive pulmonary disease (COPD). The pathophysiology of COPD can be attributed to multiple components: mucociliary dysfunction, airway inflammation and structural changes. Respiratory mucociliary clearance (MCC) is a primary defence mechanism and cigarette smoking has been reported to prolong MCC; whereas, the effect of electronic nicotine delivery systems (ENDS) on MCC has not been previously studied. Nasal Mucociliary Clearance (NMC) using a saccharin transit time (STT) method is a non-invasive, well established clinical procedure to assess MCC. We assessed NMC as a Biomarker of Effect (BioEff) in a single-center, three-cohort, ambulatory, clinical study. Thirty-five healthy adult subjects, consisting of 15 smokers (SMK), 5 ENDS users (ENDSU), and 15 non-tobacco consumers (NTC), were enrolled for evaluation of NMC STT. A saccharin particle (~0.5 mm) was placed 1 cm behind the anterior end of the subject’s inferior turbinate. The time of the saccharin placement and when a subject perceived a sweet taste was recorded, and STT was calculated. The average NMC STT for the SMK, ENDSU, and NTC cohorts were 12.1, 7.2, and 6.4 min, respectively. Our results demonstrated that NMC STT was significantly higher in SMK, but not in ENDSU, compared to NTC cohorts ( $p = 0.037$ ). NMC STT was not significantly different statistically when comparing SMK with ENDSU. This study demonstrated NMC as a potential and physiologically relevant biomarker of tobacco effect in SMK. Due to the small sample size of ENDSU cohort, additional study is needed to understand the effects of ENDS on nasal mucociliary clearance.



## STPOST 63

### Challenges and opportunities in cigar science

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An independent Cigar Working Group has been formed by interested cigar stakeholders (e.g. manufacturers and testing laboratories). The goals of the Working Group are to explore opportunities for engagement with the Food and Drug Administration (FDA) on issues relating to the manufacturing, analysis and testing of cigars. Recent deeming by the FDA places the burden on cigar manufacturers to obtain authorization for new products not on the market as of February 15, 2007, through one of three regulatory pathways. However, scientific research necessary to support regulatory submissions for cigars is limited and some of what exists, due to a lack of standardization in the field, is lacking in rigor. Though cigars and cigarettes are both combustible tobacco products, cigars, unlike cigarettes, have not been the subject of widespread scientific study. A Cigar Science sub-group was formed within the Cigar Working Group to explore the challenges and opportunities within the cigar science space. The group has identified the following as areas where research and development is warranted:

1. Quantity and breadth of scientific literature on cigars
2. Cigar use patterns and smoking topography
3. Cigar reference products
4. Standardized, validated analytical methods for chemical constituents
5. Lack of a common set of defined terms or terminology

The outcome of this collaboration will ensure that: a) future research on cigars is standardized across laboratories, b) cigar terminology will be consistent and c) future cigar research is robust and reproducible. These key factors will ensure that questions of public health are appropriately answered.

## STPOST 64

### You can't analyze for everything, or can you?

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Some regulators only want data on a product's emissions and/or certain constituents in the tobacco or e-liquids (e.g. U.S. FDA harmful and potentially harmful constituents [HPHC]). Other regulators want information on new constituents formed during processing (e.g. possible reaction of reducing sugars with amino acids and proteins in the tobacco) or storage/shipment (e.g. possible formation of acetals in an e-liquid containing aromatic aldehydes and propylene glycol). Regulators have also called for data to show

that products have been manufactured correctly. Numerous techniques and methods have been reported in the literature, conference proceedings, and legacy documents that can be used to provide data to the regulators. However, many of the techniques and methods require complex instrumentation and highly-trained laboratory personnel such as found at the major tobacco and e-vapor companies and commercial laboratories. Consequently, something simpler is needed. One approach is liquid chromatography (e.g. LC, HPLC), but not with the column technology used in the past (e.g. methods for casings on tobacco). The new technology involved the so-called Type C silica and permits the columns to be used in both the traditional reverse-phase (RP) and new aqueous-mobile-phase (ANP) modes. Thus, samples of e-liquids or tobaccos, diluted with or extracted with 50/50 (v/v) acetonitrile/water, can be chromatographed under normal RP conditions and alternate separations to resolve coeluting peaks performed under ANP conditions. This can be done without changing columns. Examples will be provided using a Cogent Phenyl Hydride column and a YMC Triart C18 column with gradients of water-acetonitrile for RP separations and acetonitrile-water for ANP separations. Examples will be provided for complex e-liquids, heavily processed commercial tobacco products (e.g. pipe tobaccos) and artificial salivas exposed to e-cigarette aerosols.