CORESTA CONGRESS 2016

ABSTRACTS

Presenter's name is underlined when it is known that the main author (listed first) did not present the paper

CORESTA CONGRESS 2016

PLENARY SESSION

INVITED SPEAKERS

Bridging the dangerous gap between industry science and public health

HUMAN D.

HEALTH Diplomats Sàrl, 8 Place de la Tour, CH-1270 Trélex, Switzerland

Both the United Nations (UN) and World Health Organization (WHO) have called for a whole-of-society, whole-of-government approach and multistakeholder action to prevent and control non-communicable disease. Tobacco is a major causative agent in the pathogenesis of these diseases. Recently, there has been a significant increase in industry investment in tobacco harm reduction science and product development. Although a switch from tobacco to safer forms of tobacco and nicotine could potentially transform public health, the short and long term risk and impact remain to be fully characterised. Industry science can meaningfully contribute in this process and be the catalyst for change towards significantly reduced risk tobacco and nicotine products. The following questions will be addressed during the presentation:

- Why has tobacco industry science consistently been ignored by public health?
- How can public and individual health benefit from industry science?
- What is the preferred future for industry-led tobacco harm reduction science?

What immediate steps need to be taken by industry R&D leaders?

Sixty years of tobacco agronomy: evolutions and challenges

VERRIER J.L.(1); PAPENFUS H.D.(2); PRAT. M.(3)

(1) Bergerac Seed & Breeding, La Tour, 24100 Bergerac, France
(2) Alliance One International Services Ltd., Building A, Riverside Way, Camberley, Surrey GU15 3YL, U.K.
(3) JT International Germany GmbH, Diedenhofener Strasse 20, D-54294 Trier, Germany

Tobacco leaf is a challenging production, labour-intensive, requiring special skills and competences at all steps. In the last century, this prompted tobacco producing countries to establishing institutes or university extension services, to perform applied research and provide assistance to growers. Exchange of information and results between these bodies became a necessity, which played an important stake in the establishment of CORESTA, in 1956. Since this, many evolutions occurred, which may tentatively be resumed as such:

- Firstly, technical innovations dramatically changed the production practices: topping and sucker control, bulk curing barns in flue-cured, float system seedling production, improved irrigation and soil cultivation practices, new cultivars and crop protection agents (CPA), etc.
- Second, the pathogens attacking tobacco evolved, became more widespread, acquired the capacity to bypass cultivar resistances, or became tolerant to new CPAs.
- Thirdly, a diversification of tobacco / nicotine delivery products occurred: not only cigars and cigarettes, but also shisha, snus, roll your own, etc… each of them require specific type(s) of tobacco leaf, therefore specific cultivars and production techniques.

Each of these changes added their own complexity / constraint layer to the other, thus increasing the global complexity of the leaf production activity, therefore the training and education need for growers.

While CORESTA played an important role in coordinating key collaborative efforts to addressing these issues, the tobacco industry also has changed. Applied tobacco research and extension bodies supporting leaf production tended to be reduced, or even disappeared in some countries. There is now a challenge faced by the leaf sector worldwide, with a dramatically increased need of training and information.

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PLENARY SESSION

PRIZE

Working together and sharing knowledge to produce robust scientific data on tobacco products

PURKIS S.W.

Retired – formerly with Imperial Tobacco Limited, Bristol, U.K.

There is much interest in understanding tobacco and smoke composition, measuring component yields and finding ways to produce reduced harm products. In an ideal world, preparatory work in method development and maintenance and the setting of reasonable measurement tolerances around methodology should be done in shared fora with participation from regulators, manufacturers, contract laboratories and suppliers. CORESTA, the TSRC and other such conferences or workshops are important examples of settings for knowledge sharing. Activities in ISO TC126 and its working groups as well as participation in local or global collaborative studies during method development or maintenance are other settings for experiencing the practical challenges and developing expertise.

Much work has been done by a wide range of scientists to share knowledge by presenting and publishing data on issues related to existing or upcoming regulations:

- Benchmark studies requested in individual countries that have demonstrated the strong relationship of many smoke analytes with tar and CO and identifying those analytes that might be more dependent on growing or curing conditions.
- Collaborative studies on method development and data reproducibility through CORESTA and ISO and other annual studies.
- Identifying issues associated with smoking regime intensity within ISO Working Group 10 and ensuring that learning is documented in ISO Technical Reports.
- Extracting available data from internal and external sources, for example, to show:
  - that good agricultural practices can lead to a reduction of agrochemical residues in raw tobaccos,
  - relationships between smoke analytes and blend precursors,
  - relationships between smoke analyte yields produced under different smoking regimes,
  - statistical methods to identify best approaches for dealing with smoke nicotine ratios and product differentiation.
- Increasing awareness of CORESTA successes and relevance by publication on its website.

The challenges are immense for both regulatory and industry scientists.

- An open forum should be made available to find smart ways to meet these objectives. Developed expertise and discussion should lead to more trust, less misunderstanding and to “scientific” reports that survive a high level of public scrutiny.
- A high individual laboratory workload with participation by a substantial number of participants in collaborative studies will be required to provide sufficient and robust data to produce robust measurement tolerances.
  - ISO and CORESTA could play an important role in helping to conduct such studies.
  - Smoke methods need to be robust under all mandated smoking regimes and tolerances will need to be set for all requested analytes.
  - Smoking regimes will also have to be agreed for other tobacco products and modified methods and different sets of tolerances may be required.

This is only part of the challenge because manufacturers and growers will need to reduce harmful constituents or their precursors and provide additional laboratory analysis time and resource for such research. They will then need to demonstrate to regulators what is and what is not practicable.

CORESTA CONGRESS 2016

PLENARY SESSION

INTERGROUP PAPERS

IG 01

Enhancing sustainability in tobacco production: energy efficient curing barn development for smallholder growers in Zimbabwe

MUNANGA W.(1); KUFAZVINEI C.(2); MUGABE F.T.(2); DIMBI S.(1)

(1) Tobacco Research Board, P.O. Box 1909, Harare, Zimbabwe
(2) Chinhoyi University of Technology, P. Bag 7724, Chinhoyi, Zimbabwe

Most of the over 70 000 tobacco growers in Zimbabwe are small scale tobacco growers who rely on wood-fuelled old conventional barns. However, these barns are inefficient, with Specific Fuel Consumption (SFC) ratio as high as 14 kg wood. This level of fuel consumption is higher than desirable and can impact the environment. To curb the potential environmental impact, a number of strategies such as the establishment of woodlots and the use of alternative energy sources have been implemented. The development of low cost energy efficient barns is an additional strategy that can aid in the sustainable growing of tobacco. An energy efficient barn (the rocket barn) originally developed in Malawi and modified for Zimbabwean conditions was adopted for use by small scale growers. However, this barn has a low capacity (0.5 ha) and is costly to build, hence the need for a larger capacity and low cost barn design. The main objective of this study was, therefore, to design and evaluate a higher capacity, low cost and energy efficient barn for tobacco curing. The Kutsaga Counter-Current 1 barn (KCC 1) with a curing capacity of 1.5 ha and based on a counter current principle was subsequently developed. The curing efficiency of the barn was evaluated against the energy efficient rocket barn and the standard conventional barn. The results showed that the barn utilizes 3.5 kg of wood to produce a kg of cured tobacco within 7 days compared to an average of 4.25 kg and 5.32 kg in the rocket and conventional barns, respectively. Given the high fuel use efficiency and a larger capacity, this study recommends the use of the KCC1 by small scale tobacco growers.

IG 02

High efficiency precision editing of the tobacco genome

DEWEY R.E.; MATSUBA Y.; SMITH W.A.

North Carolina State University, Dept. of Crop Science, Campus Box 8009, Raleigh, NC 27695, U.S.A.

Emerging genome editing technologies are revolutionizing the manner in which large, complex eukaryotic genomes may be modified. These technologies are based on the ability to design and introduce custom endonucleases that create double-stranded breaks at unique sites within complex genomes. Depending on the experimental design, the introduction of double-stranded breaks within the genome can be used to either inactivate the targeted gene, or introduce more subtle modifications such as changing a specific amino acid within the encoded protein, a process known as gene surgery. Targeted mutagenesis via designer nucleases is far superior to traditional chemical- or radiation-based approaches commonly used by plant breeders, as these

newer technologies do not leave undesirable secondary mutations scattered randomly throughout the genome, which requires extensive backcrossing to eliminate. The modification of plant genomes using genome editing technologies also holds great advantage over conventional transgenic approaches, as no foreign DNA remains in the end product, since the mutation-inducing nuclease construct can be easily segregated away in subsequent generations. Despite the absence of foreign DNA, the question of whether plants modified in this manner are “GM”, and how they should be treated by regulatory agencies is a matter of on-going debate, particularly within the European Union.

We are utilizing two classes of designer nucleases, CRISPR-Cas9 and custom-designed meganucleases, to modify the tobacco genome. To date, we have introduced targeted knockout mutations into 15 different tobacco genes using these systems. For some constructs, we have observed directed gene mutations in as many as 75% of the plants transformed. These results, together with the progress we are making toward developing the more technically difficult gene surgery techniques in tobacco, will be discussed.

IG 03

The fitness for purpose of existing tobacco product test methods - identifying the gaps

WRIGHT C.; TICHA J.; MARINER D.C.

British American Tobacco, R&D Centre, Regents Park Road, Millbrook, Southampton SO15 8TL, U.K.

Future developments in the regulation of cigarette products may include requirements for the accurate measurement and reporting of chemical constituents of cigarette smoke. The scope of constituent testing may span a shortlist of substances – for example the Framework Convention on Tobacco Control (FCTC) Articles 9 and 10 Working Group proposed priority list of substances (often described as the TobReg list) – to the complete suite of "Harmful and Potentially Harmful Constituents in Tobacco Products and Tobacco Smoke", but the technical challenge remains the same – to deliver representative product testing data with acceptable comparability between laboratories. Key to achieving this requirement is the development and application of rugged analytical methods in a harmonised manner.

Currently available methods for the determination of a significant proportion of smoke constituents do not provide the required analytical reproducibility to enable the direct comparability of testing data generated by different laboratories. This is problematic when attempting to establish thresholds for product acceptance or regulatory intervention. The presentation will use summarised results of mainstream cigarette smoke testing for products in different regional markets (nearly 2000 samples and 5 replicates) to illustrate the distribution of constituent levels in different markets and for those chemical constituents of regulatory and toxicological significance. The presentation will discuss the analytical performance requirements in terms of reproducibility and ruggedness necessary to distinguish between products in different quartiles of constituent abundance. The reproducibility of published methods will be compared with these targets.

IG 04

The fitness for purpose of existing tobacco product test methods - bridging the gaps

WRIGHT C.; TICHA J.; MARINER D.C.

British American Tobacco, R&D Centre, Regents Park Road, Millbrook, Southampton SO15 8TL, U.K.

Continuing the themes introduced in the previous contribution (IG 03), this contribution will compare existing analytical methods for the measurement of constituents of cigarette smoke with approaches adopted in other regulated sectors in which analytical methodologies are well established (including food and environment) to measure the same or similar classes of substance, including polycyclic aromatic hydrocarbons, carbonyl compounds and volatile organic compounds.

The presented work will review approaches to improve the reproducibility of analytical methods and how these can be applied for the determination of cigarette smoke constituents. The presented work will review existing good practice with regard to the ruggedness of analytical methods (i.e. the ability to tolerate minor changes in matrix, dynamic range and method conduct) and illustrate potentially viable alternative analytical methods that can achieve the required reproducibility, with focus on establishing the necessary selectivity of measurement and accuracy of quantification.

Because some proposals for product regulation depend upon constituent measurement relative to nicotine, its determination is a key analytical parameter that will be discussed in detail in the presentation, including the performance of methods currently used within the tobacco industry, potential impact upon the interpretation of results and approaches to improving the precision and accuracy of nicotine determination.

The presented work will summarise the observations made and suggest possible ways in which analytical reproducibility can be assessed, improved and harmonised.

CORESTA CONGRESS 2016

POSTER SESSION

INTERGROUP POSTERS

APSTPOST 01

The CORESTA Standard of Cooperation – document management

TRINKIES W.(1); COLARD S.(2); GUITTON P.M.(3)

(1) British American Tobacco Germany GmbH, Weiherstraße 26, D-95448 Bayreuth, Germany
(2) SEITA-Imperial Tobacco, 48 rue Danton, 45404 Fleury-les-Aubrais, France
(3) CORESTA, 11 rue du Quatre Septembre, 75002 Paris, France

The vision of CORESTA is "*to be recognised by our members and relevant external bodies as an authoritative source of publically available, credible science and best practices related to tobacco and its derived products*."

To pursue this vision, CORESTA develops analytical methods, produces technical, study and reference reports, and publishes guides and recommendations on best practices and proper usage of reference products. CORESTA documentation is available from its website at www.coresta.org and reflects the work done within the Association.

In order to improve overall efficiency, CORESTA determined that the steps leading to this work output needed to be formally structured and documented in order to ensure sustainable improvements. To further this goal, the CORESTA Standard Task Force was launched in 2012 to streamline the cooperation process and ensure that all steps were properly marked and reported to ease the follow-up of the on-going work and further archiving.

Based on the process description as presented at CORESTA 2015 events, this poster will introduce the type of harmonised documents, starting from a new work item proposal up to the documents produced and made publically available. It shall inform all experts who have to use these documents during their CORESTA activities in the future and enable them to apply this process properly.

Further perspectives on the future works to be undertaken by the CORESTA Standard Task Force and the corresponding impacts on the way of working will also be presented.

APSTPOST 02

The CORESTA Website

MANSION B.(1); LAURES F.(1); DE TERVARENT N.(2); GUITTON P.M.(2); MARIGNAC E.(2); COLARD S.(3); JACK A.M.(4); STEVENS R.(5); WAGNER K.A.(6)

(1) Mamasam, 48 avenue de la République, 94300 Vincennes, France
(2) CORESTA, 11 rue du Quatre Septembre, 75002 Paris, France
(3) SEITA-Imperial Tobacco, 48 rue Danton, 45404 Fleury-les-Aubrais, France
(4) University of Kentucky, KTRDC, 1401 University Drive, 202B KTRDC Bldg., Lexington, KY 40546, U.S.A.
(5) ITG Brands, 420 N. English Street, Greensboro, NC 27405, U.S.A.
(6) Altria Client Services LLC, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.

Over the past few years, CORESTA work procedures and communication policies have been evolving in line with the association's vision is to be "*an authoritative source of publically available credible science and best practices related to tobacco and its derived products.*"

As part of the evolutionary process, the CORESTA Website has been redesigned and modernised to provide more information on the work and documentation produced by the association's Working Groups, in addition to giving users' easy accessibility to contents and an efficient, friendly browsing experience.

The CORESTA Website Task Force, created in 2015 to develop the website, works with the selected developer to oversee the upload of content, liaise with the relevant CORESTA groups to determine user requirements, determine the features to be implemented, and monitor the step by step progress of the new system. The Task Force works in tandem with the CORESTA Standards Task Force whose work is to streamline the cooperation process, formalise procedures and formally document outputs.

The public section of the website (first phase of development) was launched end of April 2016 and provides users with information on the association, details about the Working Groups, easy access to published documents, abstracts, CORESTA presentations (with author permission), meeting details, and general information.

The new website has also been tailored to provide CORESTA Members with password access to a general private section with additional information and features (second development stage) and a private collaborative area for CORESTA Working Groups (third development stage) is planned.

This poster will give an overview of the current website features and future developments.

CORESTA CONGRESS 2016

WORKSHOPS

PRESENTATIONS

WORKSHOP EXTENSTION & TRAINING

APW 01

Extension and training

BRUCE R.

JT International S.A., 8 rue Kazem Radjavi, CH-1202 Geneva, Switzerland

Fast moving Regulatory and consumer-driven influences on manufactured tobacco products naturally translate into a need to exert correspondingly reactive or even pro-active influences over the production and supply of leaf tobacco. In order to exert such influences over leaf produced, effective extension provision is required and in turn, associated training is required for extension workers.

As leaf market structures in major leaf exporting countries have been progressively liberated to function on more free market terms over the past two decades, the volumes of leaf supplied have frequently matched or fallen short of demand levels but more importantly, the makeup of supply volume, in terms of leaf styles, qualities and integrity is frequently mismatched with manufacturer demand. Anticipating the probable increased future regulatory restrictions, not only on manufactured products, but also on aspects of leaf trade, will necessitate assurances on the provenance and traceability of leaf used in tobacco products sold for consumption. Without effective extension, the required response will not be fulfilled. The need has never been greater for comprehensive provision of GAP-based extension to ensure competitive, viable production of leaf that meets the quality and integrity needs of manufacturers.

Private funding has become increasingly more important in support of Research Programmes as public funding has dwindled and unless effective extension can be provided to link Research outcomes with commercial application, much of the Research done could potentially be regarded as an expensive academic pursuit. Extension should not be regarded as an expensive necessity but rather a competitive enabler.

APW 02

Strengthening science transmission capability: a case of Yunnan experience in disseminating the technology to farmers and field technicians in the tobacco production chain

ZHANG Limeng(1); YU Yanbi(2); LIN Zhonglong(2); HUANG Zhihua(1)

(1) Yunnan Tobacco Company, Yuxi Branch, Fenghuang Road No. 102, Yuxi City, 653100, P.R. China
(2) Yunnan Tobacco Company of CNTC, Tobacco Tower, 31 Tuodong Road, Kunming, Yunnan, 650011, P.R. China

As one of the most important tobacco growing regions in the world, Yunnan has established a worldwide reputation not only for its super-scale tobacco production but also for the excellent internal qualities of its tobacco. The tobacco yield from Yunnan in 2015 accounts for nearly 40% of production in China and 20% of the world production, respectively. Obviously, besides the unique natural environment, science innovation and the technical extension system have played an extremely important role in contributing to the high quality of tobacco in Yunnan. Over the past ten years, scientists have made great progress in key technologies such as variety breeding, soil improvement, accurate fertilisation, disease control, standardised production and scientific curing. How to properly transmit to the growers and field technicians these sciences and applied technologies? In Yunnan experience, the first step is to transfer the science and technology into technical standardisation. Based on this, Yunnan Tobacco Company has explored an effective model of science transmission, which is described as “top-to-base four levels” and “triple three” system. In the “four levels”, the top level is the Scientific Management Department of Yunnan Tobacco Company which is responsible for the planning of scientific innovation, extension and formulating policies. The second level is the Yunnan Academy of Tobacco Agriculture Science responsible for fundamental scientific research. The third level is the Tobacco Production Technology Center at municipal level which is the main force for the tobacco extension and training. The base level is the basic technical station in the branch company at county level, which is the performer for science transmission. This kind of “top to base” research system shortened the length of the science transmission chain, and increased the efficiency of scientific research and extension. Meanwhile, the “triple three” system is complementary to science transmission. In the “triple three” system, the first “3” represents promoting science transmission in three dimensions including transferring achievements by projects, ensuring the efficiency of transmission by training and education, and assessing the effect of transmission. The second “3” represents three characters of the transmission system, including pertinence, efficiency and persistence. The last “3” represents three kinds of transmission methods, combination of single and multiple technology transmission; combination of transmission in directional area and in different areas; combination of transmission by independent research and joint research. This science transmission system has proved to be effective over the years in tobacco production in Yunnan. As an example, the applied science transmission, biological control of *Myzus persicae* (Sulzer) on tobacco by parasitoids was introduced in this way.

APW 03

Educational efforts for U.S. tobacco agronomists and farmers

VANN M.C.

North Carolina State University, Dept. of Crop Science, Campus Box 7620, Raleigh, NC 27695, U.S.A.

United States grown tobacco has a longstanding reputation for high quality and, thus, strong global demand. This reputation is a result of many factors, not the least of which is educational programs for agronomists and growers alike. At present, tobacco is produced in at least 14 U.S. states, most of which have state level support for scientists and field agronomists in localized

tobacco producing regions. It is the goal of these individuals to provide timely, up-to-date, unbiased production information to producers. The information exchange among researchers, extension agents, field agronomists, and growers is vital to the sustainability of the U.S. industry. The information contained within this presentation will provide the background for and current status of these state funded initiatives and discuss the role of newer programs, such as GAP Connections.

APW 04

Translating research output into the grower's reality: tobacco training and extension for smallholder growers in Zimbabwe

DIMBI S.; KHUDU G.

Tobacco Research Board, P.O. Box 1909, Harare, Zimbabwe

The Tobacco Research Board (TRB) has the mandate to carry out all tobacco research in Zimbabwe. The main objective of the research programme is to enable tobacco growers to maximise value from responsible and sustainable tobacco production practices. Annually, therefore, some 150 research projects on variety development, plant health enhancement, best tobacco production practices for increased production and good agricultural practices are undertaken by the research team of 220 professional, technical and support staff. Once generated, the research information on specific technical aspects of production is appropriately packaged and disseminated to growers. This is done using several researcher-grower interaction platforms that include calendar-based training sessions, field days, field discussion groups and on-farm demonstration trials. For example, the Kutsaga Tobacco Improved Productivity Sites (TIPS) are one such platform that TRB has used to reach out to a host of growers annually. These sites are group-based learning hubs set up in all the tobacco growing areas of Zimbabwe, where TRB scientists extensively interact with growers and tobacco extensionists and impart skills in sustainable and profitable tobacco production. In these forums research results are converted to information that the grower can use, and participants are provided with focused, local, relevant and practical tobacco growing skills for their specific area. These sites, through which most of the small scale training and advisory is done, have enabled researchers to personally interact with some 400 extensionists and well over 20 000 growers within the season and translate research findings into the grower's reality. This paper discusses the TIPS and other extension tools with a personal touch, which TRB has continued to provide to the Zimbabwean grower for decades.

WORKSHOP NICOTINE

APSTW 01

What is the rationale behind TobReg's "Global nicotine reduction strategy"?

HENNINGFIELD J.E.

Pinney Associates, 4800 Montgomery Lane, Suite 400, Bethesda, MD 20214, U.S.A.
The Johns Hopkins University School of Medicine, Division of Behavioral Biology, Department of Psychiatry and Behavioral Sciences, Baltimore, MD 21224, U.S.A.

In 1994 Benowitz and Henningfield proposed a regulatory strategy to reduce the nicotine content of cigarettes to levels that would greatly reduce the risk that smoking could lead to addiction or that such cigarettes could readily sustain addiction. The Food and Drug Administration (FDA) determined that such a strategy merited consideration and further research. A 1998 report

commissioned by the American Medical Association (AMA) to Henningfield et al. summarized conditions necessary to implement such a strategy. The AMA endorsed the concept. One condition was ready access to alternative forms of nicotine. This strategy was discussed at several World Health Organisation (WHO) TobReg meetings with little consensus (e.g. WHO SACTob, 2003). Increasing requests for evaluation by parties to the WHO Framework Convention on Tobacco Control (FCTC) converged with implementation of FDA regulation (2009) and the emergence of electronic nicotine delivery systems (ENDS) as a nicotine delivery alternative that might make nicotine reduction viable. With the FDA putting millions of dollars into research, data began to emerge to provide the basis for evaluation by TobReg and the topic was addressed at the 2013 TobReg meeting with initial recommendations released in a report to the FCTC Conference of Parties in 2014, and published as a WHO TobReg Advisory Note in 2015. The report calls for additional research and discusses several conditions to enable the implementation of such a strategy with an explicit caution that "the strategy is not recommended in the absence of developed capacity for market surveillance and product testing" and by implication other conditions discussed in the report. Additional rationale and research since TobReg's evaluation will be discussed in this presentation. Conditions considered vital by TobReg to minimize unintended consequences will also be discussed.

APSTW 02

What does smoking behaviour tell us about the importance of nicotine to consumers?

MARINER D.C.

British American Tobacco, R&D Centre, Regents Park Road, Millbrook, Southampton SO15 8TL, U.K.

Although it is widely accepted that nicotine is not the cause of smoking-related diseases, nicotine plays an important role in smoking and for many smokers may be the determining factor in their use of cigarettes. This presentation will highlight the key aspects of nicotine's role in smoking behaviour and briefly identify other, non-nicotine factors which influence the acceptability of cigarettes.

APSTW 03

Tobacco reduced nicotine content and leaf production

MARTELLINI B.

Ultoco Services S.A., 82 rue de Lausanne, CH-1202 Geneva, Switzerland

The World Health Organization (WHO) Study Group on Tobacco Product Regulation (TobReg), which is mandated to provide the WHO Director General with scientifically sound and evidence-based recommendations about tobacco product regulation, published an advisory note to address the reduction of dependence potential of manufactured cigarettes by reducing their nicotine content to levels that cannot cause or sustain addiction. TobReg stated that no specific amount of nicotine has yet been identified as the absolute threshold for addiction, and yet TobReg recommends reducing nicotine levels to at least 0.4 mg/g of cigarette tobacco filler.

The purpose of this presentation is to examine different approaches to address the reduction of nicotine content in leaf tobacco and the expected impact these could have on leaf production.

Varying degrees of nicotine reduction in the leaf can be achieved through cultural practices, modification of the plant's genome, and through the removal of nicotine from the leaf after harvest. The modified cultural practices approach has an impact on the yield and the value of the

leaf and consequently on the income of the farmers. The genetics approach may take several years to achieve a plant which meets the nicotine target, is adapted to the growing conditions of different countries, and may also have legal implications. The nicotine removal approach will likely result in tobacco material which cannot be directly used in cigarettes, as well as negatively impact tobacco's natural flavour. Further discussion should take place so that proposed regulations can be realistically achieved and practical to implement, and not negatively impact tobacco farmers' income.

APSTW 04

Prospects for genetics-based reduction of nicotine content in tobacco

LEWIS R.S.

North Carolina State University, Dept. of Crop Science, Campus Box 7620, Raleigh, NC 27695, U.S.A.

Consideration is being given to a mandated lowering of nicotine levels in tobacco products. Nicotine is a plant natural product, where the ultimate level of accumulation is dependent upon plant genetics, environmental conditions, and production practices. Multiple approaches exist to utilize plant genetic variation to reduce nicotine content in tobacco leaves, and thus tobacco products. This includes the use of naturally-existing variation in genes encoding for enzymes involved in the nicotine biosynthetic pathway, or in genes encoding for transcription factors that regulate expression of the biosynthetic genes. Methods also exist to create novel variability in such genes through random induced mutation or via use of gene editing technologies. Expression of genes involved in nicotine biosynthesis can also be upregulated or downregulated through the use of transgenic methodologies. To date, a major challenge in developing low-nicotine tobacco cultivars has been the observation that such materials frequently exhibit reduced cured leaf quality. With some approaches to reduce nicotine content, increased levels of other metabolites is to be expected. Some of these metabolites might be considered undesirable. Methods that might ultimately be adopted will likely be dependent upon genetic complexity, potential trade-offs with cured leaf quality, intellectual property considerations, and industry acceptance of certain plant breeding techniques.

WORKSHOP TOXICANT CEILINGS

STW 01

Toxicant ceilings: the regulatory landscape

MARINER D.C.

British American Tobacco, R&D Centre, Regents Park Road, Millbrook, Southampton SO15 8TL, U.K.

The WHO Study Group on Tobacco Product Regulation has proposed ceilings for the nicotine-corrected levels of selected toxicants in cigarette emissions, and a process by which these could be implemented. Regulators in a number of jurisdictions either require currently, or have proposed, the reporting of toxicant emissions, and a number have the regulatory scope to introduce product standards which could include ceilings. As an introduction to this workshop, this presentation will summarise the various proposals.

STW 02

Implications of product testing: method variability and suitability and ratio of smoke emissions to nicotine

INTORP M.

Reemtsma Cigarettenfabriken GmbH (an Imperial Brands PLC Company), Albert-Einstein-Ring 7, D-22761 Hamburg, Germany

Regulatory authorities are discussing the measurement of certain smoke constituents, and this may eventually lead to discussion of ceilings. For this purpose internationally standardised methods are required to enable the generation of reliable data. CORESTA has strongly supported the development of robust methods to deliver information on method variability based on sound science. This information will enable regulatory authorities to take method variability into account when considering setting ceilings for smoke constituents.

To date, CORESTA has published six recommended methods encompassing 25 compounds. Four of these methods have already been submitted to ISO as potential ISO standards. This process includes a series of collaborative studies involving a number of laboratories to assess the reproducibility and repeatability (R and r) of each method.

All studies conducted were statistically designed to investigate effects of parameters that could contribute to within-lab and between-lab variability. As a result, the method variability is recorded in the published recommended methods. Overall reproducibility data shows a significantly higher variability compared to the analytical methods for tar, nicotine and carbon monoxide currently applied by authorities for measurement and ceilings in the EU. This fact should be considered in case ceilings for further smoke analytes are proposed.

Additionally, WHO TobReg has proposed nine priority compounds for regulation and recommends reporting levels for smoke compounds in terms of per milligram of nicotine. In TobReg's view the sale or import of cigarette brands that have yields above these levels should be prohibited. An unintended consequence of the introduction of independent multiple analyte ceilings based on a ratio to nicotine would be to prohibit the sale of many products with low smoke constituent yields within a given market with the result that the average smoke constituent yields in the market would rise.

STW 03

Toxicant ceilings - a leaf production perspective

WINEGARDNER M.R.

Universal Leaf Tobacco Co., Inc., P.O. Box 25099, Richmond, VA, 23260, U.S.A.

Regulatory proposals to limit tobacco product constituent levels pose questions and challenges for leaf production. Each constituent has its own biological or chemical synthesis and physiological pathway determined by the tobacco variety and commonly influenced by leaf production variables such as geological factors, crop growing conditions and cultural practices. Further, constituent levels often differ across the anatomy of the plant and the age of the leaf material. Research targeting the impact of these variables is critical in determining potential mechanisms to moderate constituent levels in leaf. The challenge is increasingly complex when attempting to reduce multiple, naturally occurring constituents simultaneously. It is important to also consider the relationship between levels in leaf and those in final consumer products, especially if analysing constituent levels in ratio to nicotine yield as suggested in some regulatory proposals. With knowledge of pathways of constituent synthesis and/or accumulation, various strategies can be employed to influence toxicant levels during leaf production, each of which would require research and development supported by analytical testing and feasibility assessments. Strategies could include cultivar selection, new cultivar development, cultural

practice modification or other supply chain changes. The selection and implementation of toxicant limits requires a science-based, stepwise approach, supported by validated methods, robust sets of testing data and changes in leaf production supported by farmer dialogue and field extension services. Potential impacts include changes in farm production yields and costs, which need to be analysed and addressed to minimize negative impacts to farmers and suppliers. Given the number of targeted constituents in various chemical families, there may arise situations where modifications to leaf production may require balancing opposing impacts to two or more toxicant levels. This fact necessitates responsible limit setting addressing risk, feasibility and uncertainty associated with an agricultural crop, in a biological system which is often impacted by an ever changing environment.

STW 04

Opportunities for toxicant reduction

BETSON T.

British American Tobacco, Globe House, 4 Temple Place, London WC2R 2PG, U.K.

Regulators are arguing the need to collect information on specific toxicants in smoke with the aim to possibly introduce ceilings in the future. In order to comply with potential ceilings, leaf technology, blending and product design can be used to reduce particular smoke toxicants. A review of currently available approaches will be presented. This will focus on the priority nine smoke toxicants proposed for mandated ceilings by the WHO TobReg study group. The presentation will cover leaf precursor reduction through leaf agronomic practices, grade selection, blending and filter technology.

WORKSHOP REFERENCE PRODUCTS

STW 05

Design, manufacture, and availability of reference tobacco products

JI H.; CANETE S.; CHAMBERS O.

University of Kentucky, KTRDC, Center for Tobacco Reference Products, 1401 University Drive, Lexington, KY 40546, U.S.A.

As the science of tobacco analysis continues to advance, there is a need for appropriate tobacco reference materials. There is natural variability in the measurement of tobacco product constituents coming from the raw inputs, but measurement variability also comes from the design, the manufacture and the evolving science of tobacco analytics. The call for certified tobacco reference materials and their potential application in product research and regulation suggests a need to understand this variability. While these products can provide a reference point to help researchers obtain more accurate data, differences in tobacco reference products suggest that certain uses are more appropriate depending on specific design characteristics. It is clear that there is a need for a variety of reference tobacco products, covering a range of design parameters and constituent delivery. The availability of tobacco reference products will be reviewed and differences in design characteristics will be discussed to provide some background information for a discussion of reference tobacco product characterization/certification, variability and appropriate use.

STW 06

Reference product characterization

GILLMAN I.G.

Enthalpy Analytical Inc., 800-1 Capitola Dr., Suite 1, Durham, NC 27713, U.S.A.

This presentation will focus on how reference and/or monitor tobacco products are characterized and then used by analytical testing facilities to assure method performance during the analysis of tobacco products. Reference tobacco products are commonly used by testing facilities to verify ongoing method performance, detect analytical method drift and to qualify or validate new analytical techniques or methods. To be fit for this use, reference tobacco products must be well characterized including information on product uniformity and stability for all compounds of interest.

How reference tobacco products are characterized, by testing laboratories, along with their use in qualifying and validating new analytical methods will be presented. The advantages and disadvantages of common reference and/or monitor tobacco products will be also presented including the 3R4F, 1R5F, CM8, 1R6F, IP-2, NIST SRM 1082 cigarettes and the CORESTA CRP series of smokeless tobacco products.

STW 07

Suitability of reference products and observations from the Cigarette Variability Task Force (CVAR) Phase 1 Study

MORTON M.J.; FLORA J.W.

Altria Client Services LLC, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.

Reference products are an invaluable laboratory tool. Several of the uses of reference products will be discussed including analytical method QA/QC, laboratory performance comparison, and as an adjustment tool for gauging product results when tested in different laboratories. Observations will be shared from the Cigarette Variability Task Force (CVAR) Phase 1 Study that could aid in the choice of reference products.

STW 08

Proficiency testing, a tool for laboratory comparison and regulatory compliance

SYKES M.; ANDERSON D.

Fera Science Ltd (Fera), National Agri-Food Innovation Campus, Sand Hutton, York YO41 1LZ, U.K.

Taking part in a proficiency test (PT) gives a laboratory an independent assessment of their performance. These results can be used for many purposes, as an interlaboratory comparison, for long-term trend analysis, continuous assessment for quality systems/regulatory purposes, for customer confidence and for regulatory compliance. Details of the proficiency test samples used will be outlined to provide a basis for discussion of tobacco proficiency test matrix variability, challenge and frequency of schedule. These results will lead to whether PT tobacco samples should be characterised / certified further so that they may be used as reference materials post proficiency test.

CORESTA CONGRESS 2016

AGRONOMY & LEAF INTEGRITY and PHYTOPATHOLOGY & GENETICS

ORAL PRESENTATIONS

AP 01

Managing tobacco nematodes using isothiocyanate products

JOHNSON C.S.

Virginia Tech, Southern Piedmont Agriculture Research and Extension Center, 2375 Darvills Rd, Blackstone, VA 23824, U.S.A.

Globodera tabacum solanacearum (TCN; tobacco cyst nematode) remains an important parasite of flue-cured tobacco in Virginia; effective nematicides remain necessary when susceptible cultivars are planted and crop rotations are short. Contact nematicides such as Nemacur (fenamiphos) and Temik (aldicarb) are no longer available, while soil fumigants containing methyl bromide or chloropicrin have been withdrawn or require cumbersome additional practices and documentation. Metam sodium products generate methyl isothiocyanate (MITC) after soil application, and were shown to reduce TCN populations in soil and in roots and to increase flue-cured tobacco growth and yield in Virginia research conducted in 1998-1999, 2001-2006, and 2009-2010. Allyl isothiocyanate (AITC), chemically related to MITC, can be generated via degradation of Brassica crop residues, and experiments conducted in 2011-2013 found variable TCN management arising from early pre-transplant incorporation of varying rates of mustard seed meal. Dominus, the first US EPA-registered “biofumigant”, contains 96% AITC. In a 2014 trial, 187 L Dominus/ha significantly reduced the number of TCN/g of feeder root compared to an untreated control, while smaller reductions linked with 93 and 140 L/ha were not statistically significant. Numeric reductions in nematodes/g root where AITC had been applied were not significant in 2015. Phytotoxicity observed with the higher Dominus rates in 2014 did not occur in 2015. Rate-dependent increases occurred in tobacco vigor up to topping where Telone II (1,3-D) or AITC had been applied versus the untreated control, and yield was significantly higher where Telone II or 187 L/ha Dominus were applied compared to Velum Total, Vydate, 94 L/ha Dominus, and the untreated control.

AP 02

Characterisation of root-knot nematode (*Meloidogyne javanica*) resistance mechanism in tobacco

MAKUNDE P.T.; MAHERE T.S.; DIMBI S.; GARWE D.

Tobacco Research Board, P.O. Box 1909, Harare, Zimbabwe

Resistance to root-knot nematode (*Meloidogyne* spp) has been identified, incorporated, and deployed in Kutsaga commercial cultivars of tobacco, *Nicotiana tabacum*. These cultivars have varying resistance levels to root-knot nematode. The classification of the varieties was based on root gall indices obtained at final reaping, after the varieties have been grown in fields with high nematode populations. However, little is known about the mechanism of root-knot nematodes

resistance in local tobacco varieties. The objective of this study, therefore, was to determine the nature of root-knot-nematode resistance in the Kutsaga root-knot resistant varieties. Trials were conducted in the greenhouse to investigate the role of tobacco root diffusates in the hatching and survival of infective juveniles, the rate of penetration and development of root-knot nematodes in test varieties. Six tobacco varieties (K RK26, K RK29, K RK66, T71, T72 and K RK64) with moderate to high resistance to root-knot nematode were used in the study. A known root-knot nematode susceptible cultivar K M10 was included as a control. Results showed that root diffusate had no inhibitory or delaying effect on hatching and survival of infective juveniles and the tested tobacco varieties deployed different mechanisms of resistance. These included the suppression of root penetration by infective juveniles and the inhibition of development and reproduction of the nematodes after penetration. These results are discussed in detail in this paper.

AP 03

Meloidogyne species isolated from tobacco root knots in Japan

MIYAMOTO Y.; MIKI J.; KOGA K.

Japan Tobacco Inc., Leaf Tobacco Research Center, 1900, Idei, Oyama, Tochigi 323-0808, Japan

Root-knot nematodes (RKNs) cause severe and contagious plant diseases worldwide. Their host plants vary, with nematodes adversely affecting not only tobacco but also many other plants. The RKN genus (*Meloidogyne*) includes many plant-parasitic species/races that attack tobacco, such as *M. incognita* with races 1, 2, 3, and 4, *M. arenaria* with races 1, and 2, *M. javanica*, and *M. hapla*. It is important to ascertain what RKN species/races are damaging in tobacco fields because RKN-resistant sources differ from one another. Nevertheless, no report of the relevant study has elucidated the RKN species/races occurring in tobacco fields in Japan. This study was conducted to investigate RKN species/races occurring in tobacco fields in Japan.

We developed a simple method of RKN species classification from single root-knots using PCR-RFLP. We collected root samples from various Japanese tobacco fields in 2015. The various RKN races were identified using North Carolina standard hosts. We confirmed the existence of four RKN species in the investigated tobacco in Japan. Root-knots infected by *M. arenaria* were confirmed in a field growing a *M. incognita* resistant variety. The race diversity was not confirmed in the tobacco fields investigated for this study.

AP 04

Kutsaga releases a Katambora Rhodes grass variety (G HR 1) for nematode management under tighter rotations

SHAVA J.G.; MAKUNDE P.T.; MAGAMA F.; SHAMUDZARIRA M.

Tobacco Research Board, P.O. Box 1909, Harare, Zimbabwe

Although the use of resistant varieties is effective, Katambora Rhodes grass (*Chloris gayana*) has been used for a long time in rotations not only to suppress nematodes but also to improve the soil structure in tobacco farming systems. This grass was introduced into Zimbabwe in the early twentieth century from the Cape Province of South Africa. Over the years, however, Katambora Rhodes grass has lost its nematode suppression capability probably because of gene dilution since it is naturally out-crossing. Through recurrent breeding, a new Katambora grass variety (G HR1) with superior root-knot nematode suppression capability was developed at Kutsaga. The objective of this study was to evaluate the nematode suppression potential of G HR1 in tobacco rotations. Field trials were conducted at Kutsaga Research Station for three seasons. A split plot

design with grass variety as the main factor and tobacco variety as the subplot factor was used. The land was first planted to the nematode susceptible cultivar K M10 for a season, to boost the nematode populations before being planted to G HR1 and the Old Katambora Rhodes grass for periods of 12, 24 and 36 months. Thereafter, plots were planted to tobacco (varieties K M10 and K RK26). A no grass control was included in the trial. Root galling and yield assessments were then conducted on the tobacco crops. Additionally, soil samples were collected at the end of each season to determine the root-knot infective juvenile populations after one, two and three seasons of grass. Results showed that G HR1 is superior to the Old Katambora Rhodes grass in its nematode suppression capability and is now recommended for use in shorter rotations of 12-18 months compared to 36 months recommended in the traditional grass fallow.

AP 05

Working to improve black shank control in flue-cured tobacco

BERTRAND P.F.(1); MOORE J.M.(1); SHEW D.(2); LEWIS R.S.(3); VON WALDNER M.(1)

(1) University of Georgia, 4604 Research Way, Tifton, GA 31793, U.S.A.
(2) North Carolina State University, 2510 Thomas Hall, Raleigh, NC 27695, U.S.A.
(3) North Carolina State University, 4310 Williams Hall, Raleigh, NC 27695, U.S.A.

Black shank is a concern for farmers growing tobacco on land infested with *Phytophthora nicotianae*. The first superior *Php* gene variety (NC-71) was introduced in 1995. The *Php* gene confers complete resistance to the wild type of *P. nicotianae* (race 0), whereas traditional FL-301 resistance confers only partial resistance. By 2004, black shank was increasing where the *Php* gene was used as the sole means of control. This was due to a new pathogen race, designated race 1, selected in apparently mixed race populations as race 0 was eliminated by the *Php* gene. Black shank losses increased sharply in 2013, confirming that *Php* resistance would no longer control black shank in Georgia. A series of trials was begun in 2013 to improve black shank control. A 2013 trial of varieties and breeding lines conducted in a black shank site showed disease incidence of >85% in popular varieties (K-326, NC-71, and NC-196). Eleven breeding lines containing *Wz* genetics derived from *Nicotiana rustica* developed <10% disease. Very heavy rain during and just after transplanting in 2014, resulted in the highest black shank losses in 20+ years. In several cases where losses were high, mefenoxam had been used. Sensitivity tests performed on 30 isolates from such fields found all to be sensitive to mefenoxam. Mefenoxam is known to be prone to leaching with heavy rain. Trials conducted in 2014 found the varieties used for black shank control (NC-196 and GF-318) had only moderate to low levels of FL-301 resistance, providing 36.3% and 21.6% control respectively compared to K-326, the standard of low FL-301 resistance. These same trials identified three varieties that showed relatively good control compared to K-326 (NC-925, 81.4%; CC-143, 70.0%; GL-395, 63.5%). Black shank losses continued to be high in 2015. Variety trials conducted in 2015 confirmed the 2014 results. Flupicolide, a new chemical control option, reportedly less prone to leaching than mefenoxam, was tested in 2015. Control with a layby application of flupicolide (140 grams/ha) versus mefenoxam (560 grams/ha) was equal (p=0.05). Varieties with superior FL-301 resistance coupled with less leaching prone chemical options should improve black shank control. Resistance based on *Wz* genes offers a tool for further variety improvement.

AP 06

Inactivation of *NtCPS2* allele linked with the major QTL for black shank resistance derived from Beinhart 1000

SHINJO A.; KOGA K.; HYODO H.; NOGUCHI S.

Japan Tobacco Inc., Leaf Tobacco Research Center, 1900 Idei, Oyama, Tochigi 323-0808, Japan

The cigar tobacco variety Beinhart 1000 is well known to have high level of partial resistance to black shank caused by *Phytophthora nicotianae*. The researchers at the North Carolina State University reported that it's quantitative trait loci (QTLs) associated with the resistance and the major/novel QTL on linkage group 15 among them is closely linked with *NtCPS2* gene which encodes an enzyme for the biosynthesis of *cis*-abienol that is undesirable for the flavour of flue-cured and Burley tobacco. The linkage between the major QTL and *NtCPS2* allele must be broken to utilise in breeding.

The objective of this research was to produce a useful resistance source by inactivating the *NtCPS2* allele linked with the major QTL. For the efficient screening of mutated non-functional *NtCPS2* allele, the F1 seeds from a cross of Beinhart 1000 and K326 were treated with ethyl methanesulphonate. The *NtCPS2* locus of F1 plant consists of functional and non-functional *NtCPS2* allele derived from Beinhart 1000 and K326 respectively.

The *cis*-abienol production in leaves of F1 plants was analysed by thin-layer chromatography. As the result, 27 F1 plants which did not accumulate *cis*-abienol were selected from approximately 4,000 F1 plants. Two independent F1 plants harbouring a mutation-introduced *NtCPS2* allele were identified among them by DNA sequence analysis. Each sequence of *NtCPS2* allele in the selected two plants had a nonsense mutation and a missense mutation.

AP 07

Breeding for blue-mould resistance in Burley and flue-cured tobacco: an overview of results

VERRIER J.L.; MALPICA A.; L'HUMEAU J.

Bergerac Seed & Breeding, La Tour, 24100 Bergerac, France

Conventional, non-GMO blue-mould (*Peronospora tabacina*) resistance has been transferred to *Nicotiana tabacum* L. from interspecific hybridisation works tracing back to years 1965-70, involving in particular the species *Nicotiana debneyi*. Partial resistance behaviours, expressed mainly at the adult plant growth stage, could be recovered in *N. tabacum*. Breeders have included these sources in their programs in attempts to obtain highly performant tobacco varieties with partial resistance levels. This implies solving two problems: firstly the partial resistance should be fully transferred, secondly the "linkage drag" often present in interspecific resistance sources should be removed or repressed so that the performance of the obtained variety is acceptable. A significant part of the Burley and flue-cured breeding efforts of the former Institut du Tabac Bergerac (ITB), then Bergerac Seed & Breeding (BSB), is devoted to this. The breeding material is evaluated for blue-mould resistance in infested field nurseries, naturally contaminated, with late transplantation to secure optimal conditions for expression of the resistance. In non-infested, treated field tests transplanted at usual dates, the same material is assessed for yield and quality. Databases gathering all results over several years were built and exploited to assess the importance of the linkage drag. Results show no relation between blue-mould resistance levels and days to flowering or days to leaf maturity, in both tobacco types. In Burley, the only trait which seems related to blue-mould resistance is the alkaloid content, with resistant varieties being higher. In flue-cured, higher resistance levels are mainly linked with

lower yields and quality (leaf body and colour). Knowing that the breeding effort towards blue-mould resistance is older in the Burley part of the programme, this suggests that the linkage drag has been overcome in Burley, except for the alkaloid content, whereas in flue-cured it is not fully overcome yet.

AP 08

Pythium myriotylum, the dominant Pythium species associated with root rot of tobacco seedlings produced using the float tray system in Zimbabwe

MUFUNDA F.; MUZHINJI N.; KASHANGURA C.

Tobacco Research Board, P.O. Box 1909, Harare, Zimbabwe

Pythium root rot caused by the oomycete Pythium species is the most serious disease affecting tobacco seedlings produced under the hydroponic float tray system in Zimbabwe. Currently, knowledge of the occurrence and genetic diversity of the Pythium species involved in Pythium root rot in tobacco float seedling production is limited. In this study we established the identity and genetic diversity of the Pythium species affecting tobacco seedlings in Zimbabwe. A total of 120 isolates were collected from tobacco float seedling production sites at Kutsaga Research Station and Banket, the two most commercial tobacco seedling sites in Zimbabwe in the 2015-2016 tobacco growing season. Sequence analysis of the internal transcribed spacer regions (ITS1 and ITS4) of ribosomal DNA (rDNA) confirmed all the isolates to be *Pythium myriotylum*. Phylogenetic analyses of the internal transcribed spacer region of the *P. myriotylum* isolates showed positive selection and sequence diversity giving four distinct clades according to the geographical origin. This is the first comprehensive study to determine and characterise Pythium species associated with root rot in tobacco float seedling production in Zimbabwe. Knowledge about the occurrence, distribution, and genetic diversity of Pythium species will help in the implementation of integrated control strategies of Pythium in Zimbabwe.

AP 09

Effect of organic nitrogen source and application rate on the yield and quality of flue-cured tobacco

VANN M.C.; FISHER L.R.; WHITLEY D.S.

North Carolina State University, Dept. of Crop Science, Campus Box 7620, Raleigh, NC 27695, U.S.A.

Organic carbon-based sources of nitrogen, such as livestock waste or by-products, have not been recommended for the production of flue-cured tobacco. At present, there is significant demand for organically produced leaf, and in these systems the use of synthetic nitrogen is prohibited. The effect of organic nitrogen from animal by-product to the yield and quality of organically produced flue-cured tobacco are not presently known. Research was conducted in 2012 to evaluate the effect of two organic nitrogen sources: Nature Safe (13-0-0) and Nutrimax (12-1-0) applied at three different rates: 17 kg N below the recommended rate, the recommended rate, 17 kg N above the recommended rate. In 2013, 34 kg N above recommended rate was added for evaluation. A single treatment consisting of synthetic 28% Urea-Ammonium-Nitrate (UAN) applied at the recommended rate was evaluated as a control. All nitrogen was applied in one-half rate split applications 10 days after transplanting and at layby. After transplanting, tissue samples were collected at layby, at topping, and after curing to quantify nitrogen utilization. Leaf yield, quality, and chemistry were recorded as well. Due to weather variation, results were relatively inconsistent; however, the synthetic UAN treatment commonly had the highest total nitrogen content later in the season, though this factor was not always an indicator of significantly

higher yield. Organic nitrogen application rate tended to have a greater impact on leaf chemistry than did organic nitrogen source, as total nitrogen content in leaf tissue was found to have increased along with applied organic nitrogen in three of the four environments. Nature Safe application did result in higher leaf yield in one environment when compared to Nutrimax. Results from this study indicate that either organic nitrogen source is likely to be acceptable for tobacco production and that application rates above recommendation are not necessary for optimum yield to be realized.

AP 10

Effect of organic nitrogen source and application timing on the yield and quality of flue-cured tobacco

MASON J.H.; VANN M.C.; FISHER L.R.; WHITLEY D.S.

North Carolina State University, Dept. of Crop Science, Campus Box 7620, Raleigh, NC 27695, U.S.A.

Mineralization rates of organic nitrogen are extremely dependent upon factors such as soil pH, temperature, and moisture. Flue-cured tobacco is extremely sensitive to nitrogen availability; therefore, many questions have been presented regarding appropriate application programs for organic tobacco systems. It has been theorized that organic nitrogen application at layby could result in late-season mineralization and uptake, thus creating management and curing issues. To address these concerns, research was conducted in North Carolina 2012 and 2013 to evaluate the impact of two organic nitrogen sources: Nature Safe (13-0-0) and Nutrimax (12-1-0) when applied at three different timings: 100% broadcast prior to bedding, 50% broadcast prior to bedding/50% sidedress at layby, or 50% sidedress after transplanting/50% sidedress at layby. Nitrogen application rate was the same for all treatments and was targeted at a rate appropriate for each environment. In addition, a single treatment of synthetic 28% Urea-Ammonium-Nitrate was included as a control and was applied 50% sidedress after transplanting and 50% sidedress at layby to reflect recommended practices employed by conventional producers. Tissue samples were collected at layby, topping, and after curing to evaluate nitrogen accumulation during the season. After curing, leaf yield, quality, and chemistry were determined as well. In general, yield was not impacted by nitrogen source or application timing; however, increases in leaf quality, nitrogen uptake, and total alkaloids were observed where nitrogen application occurred after transplanting. These results likely indicate that nitrogen use efficiency was improved when the nutrient was placed in closer proximity to the rooting zone of the plant.

AP 11

Linking liming and soil nutrients availability: bridging the gap on knowledge of the effect of liquid liming agents as soil pH correction tools for improved yields and quality of flue-cured tobacco in Malawi

MTONGA Y.P.(1); MUMBA J.B.(1); GOMONDA R.W.J.(1); KHUMBANYIWA A.G.(1); LOCKINGTON I.(2)

(1) Agricultural Research and Extension Trust (ARET), Private Bag 9, Lilongwe, Malawi
(2) Greenbelt Fertilisers Ltd, P.O. Box 30582, Kanengo, Lilongwe, Malawi

Malawi soils are highly weathered and acidic resulting in fixation of Phosphorus (P), Potassium (K) and Nitrogen (N) into insoluble forms with iron (Fe) and aluminium (Al) which are inaccessible by plants. Soil pH correction through liming to neutralise acidity to amenable levels for plant growth is an important cultural practice for improved productivity. But currently, the standard cumbersome method of ploughing and immediately manually applying of dry-calcitic-lime at two

tons/ha as a blanket recommendation nine months before transplanting poses implementation challenges amongst resource challenged small-holder farmers who channel all their energies to current crop rather than the future one. With unavailability of tractors in such small-holdings, liming is almost non-existent. The adoption rate of liming is 2% this affects productivity per unit area. As a mitigation measure targeting small-holder farmers, two liquid-liming agents, MAG-LIME-FLO and CAL-LIME-FLO, with two controls: the standard dry-calcitic practice and plot with no lime, were investigated for their efficiency in correcting pH, improving nutrient availability, on increasing yields and quality in a trial laid out in randomised complete block with six replications. Data collection was on pH at transplanting, 6, 10, 14 weeks after transplanting and assessed for yield and quality. The results showed that liquid-limes started correcting pH as early as six weeks after transplanting with MAG-LIME-FLO and CAL-LIME-FLO increasing soil pH by 15% at six weeks and by 25% five months later. Liquid-limes increased P availability by 70%, K by 56%, Ca by 79% and Mg by 77%. Liquid-limes had 94% yield advantage over non-limed plots and significantly outperformed the dry-calcitic-lime by 50%. Handling costs showed that liquid-limes had reduced application and transportation costs of 70% over dry-calcitic-lime. These findings are of significant benefit to Malawi small-holder farmers who are yet to embrace pH correction initiatives through liming as an integral part of good agricultural practices.

AP 12

Developments in tobacco fertiliser evaluations and research in Zimbabwe over the past 15 years: an overview

RUKUNI D.

Tobacco Research Board, P.O. Box 1909, Harare, Zimbabwe

In Zimbabwe, tobacco is grown on some 100 000 hectares of predominantly sandy textured soils of low fertility. Thus achieving good yields and quality leaf heavily depend on appropriate and adequate fertilisation. The Tobacco Research Board (TRB) has over the years supported tobacco growers by evaluating all granular and foliar fertilisers, soil and pH amendments and growth stimulants targeted for use on tobacco to ensure that they are effective and provide the right quantities of the requisite minerals and nutrition to enable the attainment of high yields and quality. Additionally, the TRB has occasionally collaborated with agrochemical companies to develop fertilisers to suit specific production systems. Over the last 15 years, some 35 granular and foliar fertilisers, quick-acting soil and pH amendments, and plant growth stimulants were tested, while two fertiliser formulations for use in the float tray system were developed. Fertiliser evaluations showed that while all granular basal fertilisers tested were effective, foliar fertilisers were generally found to have no positive effect, and thus were not recommended for use on tobacco. Additionally, most so-called quick-acting soil and pH amendments, and growth stimulants were found to add no extra benefits in well fertilised crops. In contrast, the Kutsaga-developed float fertilisers were very effective in the production of high quality tobacco transplants and have since been commercialized and are widely used by tobacco and vegetable growers. This paper gives an overview of the agronomic products evaluated by the TRB in the past 15 years for the enhancement of leaf yield and quality. Additionally, current products on the market and possible research gaps will be discussed.

AP 13

Dark tobacco response to boron

BAILEY W.A.; RODGERS J.C.

University of Kentucky, Research & Education Center, 1205 Hopkinsville Street, Princeton, KY 42445, U.S.A.

Boron deficiency has been documented on several farms in the dark tobacco production area of Kentucky and Tennessee U.S.A., most commonly in the Purchase Area of Kentucky (Murray/Mayfield KY area). Typical symptoms of boron deficiency include leaf breakage approximately 2 inches from the stalk and possibly stunting/yellowing/malformation of the terminal bud. In some cases, high soil pH has been a contributing factor as boron becomes less available as soil pH approaches 7 or higher. Field experiments were conducted in 2014 and 2015 at a private farm near Mayfield KY where boron deficiency had been documented on the tobacco crop in 2012 and the soybean crop that followed in 2013. Treatments included transplant water-simulated (TPW) applications of boron made immediately after transplanting, or foliar applications made at 3 to 4 weeks after transplanting. Boron rates used were 0.25, 0.5, or 1.0 lb B per acre. Boron source was boric acid (Borosol® liquid, 10% B). In 2014, extensive transplant injury was seen from 0.5 and 1.0 lb B/A applied as TPW applications. Injury resulted in 34 to 66% injury from boron at 0.5 to 1.0 lb B/A, respectively, and as much as 29% stand loss at the 1 lb B/A rate. However, foliar boron applications were beneficial, with tobacco showing increasing positive visual growth responses to increasing rates of boron. 2014 yields were reflective of negative responses seen to TPW applications and positive responses seen to foliar applications. In 2015, injury from TPW applications was not obvious as it was in 2014, presumably due to much wetter conditions during and following transplanting. Growth and yield responses in 2015 were also less than those seen in 2014. This experiment is being repeated in 2016 and these results will also be discussed.

AP 14

Enhancement of tobacco leaf quality with foliar sprays of potassium sulphate

MARCHAND M.(1); LUYTEN N.(1); MELGAR R.(2); COMETTO A.L.(3)

(1) Tessenderlo Group, Troonstraat 130, 1050 Brussels, Belgium
(2) Estación Experimental Pergamino, Instituto Nacional de Tecnología Agropecuaria, Av. Rivadavia 1439 C1033AAE, Buenos Aires, Argentina
(3) Independent Consultant

Tobacco is heavily fertilised with potassium and other nutrients to satisfy its needs in many parts of the world. The tobacco cluster region of Salta and Jujuy in Northwestern Argentina usually applies between 200 to 300 kg/ha of K_2O as sulfate or nitrate throughout the growth cycle. The direct supply and assimilation of potassium (K) to the leaves during its exponential growth stages may improve K content in the leaves, which guarantees a good maturation at drying and combustibility. K may also influence dry matter accumulation directly, resulting in higher leaf yields.

The objective of this work was to confirm previous investigations in several countries concerning the effect of foliar sprays with soluble sulphate of potash (SOP) on the commercial yield and quality of flue-cured Virginia tobacco.

Trials were conducted on Virginia tobacco in collaboration with the Instituto Nacional de Tecnología Agropecuaria (INTA) and Finca Experimental La Posta over a three year period in Perico, Jujuy, Argentina. The control was treated with the normal fertilisation programme consisting of 800 kg/ha of a 13-11-27 NPK and 100 kg/ha of granular SOP. The latter was reduced to 75 kg/ha for parcels that received foliar sprays. Three foliar sprays of SOP were

applied (6, 12 or 18 kg/ha/spray in the first year and 3, 6 or 9 kg/ha in consecutive years) as a complementary treatment to base fertilisation.

On several occasions, the parcels treated with foliar sprays of SOP consistently gave more high quality leaves. Results suggest that foliar sprays of SOP as complimentary treatment to the base fertilisation can increase yield of high quality leaves, which offers an economic advantage to the farmer, thus confirming previous results in different cropping conditions. On average, higher yields of first class leaves with foliar sprays of SOP amounted to an additional profit of about US$280 per hectare.

AP 15

Fertigation of flue-cured tobacco with a combination of water soluble and straight fertilisers, for enhancing nitrogen and potassium use efficiency

MAHESH M.N.V.A.; RAMAKRISHNA B.; MANI M.; SRINIVAS P.

ITC Limited - Agri Business Division-ILTD, Research Department, Rajahmundry, Andhra Pradesh 533 107, India

Nutrient application through drip irrigation (fertigation) with water soluble fertilisers, is an established practice to achieve nutrient use efficiency in flue-cured tobacco production. However, there has been a steady price escalation and erratic availability of water soluble fertilisers, in the recent past, which prompts to identify an effective combination of nutrient source, time, dose and schedule of application, to harness myriad advantages of fertigation. The study aims at establishing a combination of water soluble and straight fertilisers, method of application, scheduling application time and dosing of nutrients. Nutrient use efficiency, productivity and quality of flue-cured tobacco, were studied to establish the influence of various nutrient combinations, method, dose and time of application.

Experimental trials were conducted in 2014 and 2015, in irrigated flue-cured tobacco growing tracts of Alfisols in Northern Light Soils (West Godavari District, India). A combination of two water soluble fertilisers - potassium sulphate & potassium nitrate and four straight fertilisers - Single super phosphate, ammonium sulphate, potassium sulphate and urea in different combinations and rates were used for the study. Fertigation treatment combinations were compared with drip irrigation with manual fertiliser application. Nitrogen (N) and potassium (K) at 75% rate, through a combination of urea, potassium nitrate and potassium sulphate resulted in 3353 kg/ha which is similar to the productivity levels achieved with 100% nitrogen and potassium with same combination of fertilisers (3406 kg/ha), indicating a saving of 25% nitrogen and potassium. The practice of fertigation with same combination, also resulted in a significantly higher productivity compared to manual application of fertilisers, with drip irrigation (3143 kg/ha). A grade index of 82 with the above fertiliser combination indicates no significant shift in quality. Similar uptake levels of nitrogen and potassium were recorded at 75% application rate in comparison with nitrogen and potassium uptake of 95 kg and 83 kg respectively, with 100% nutrient dose and no significant changes in chemical characteristics like total alkaloids as nicotine and total sugars were observed.

Application of nitrogen and potassium through fertigation, delivers a dual advantage of nutrient optimisation coupled with enhanced productivity levels, leading to a sustainable tobacco production.

AP 16

Role of polyamines in leaf ripening of low alkaloid tobacco varieties

NÖLKE G.(1); VOLKE D.(1); CHUDOBOVA I.(1); HOUDELET M.(1); LUSSO M.(2); FREDERICK J.(2); WAREK U.(2); STRICKLAND J.(2); XU D.(2); FISCHER R.(1); SCHINKEL H.(1); SCHILLBERG S.(1)

(1) Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Forckenbeckstrasse 6, 52074 Aachen, Germany
(2) Altria Client Services LLC, Research, Development and Regulatory Affairs, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.

Traditional breeding and molecular approaches have been used to develop tobacco varieties with reduced nicotine and secondary alkaloid levels. However, low-alkaloid (LA) tobacco varieties often show impaired leaf ripening and senescence leading to poor leaf quality upon curing. An analysis of the nicotine biosynthesis pathway suggests possible interactions with polyamine and ethylene pathways, potentially having an influence on leaf ripening and senescence. Therefore, we investigated the role of polyamines in tobacco leaf ripening by analyzing free, soluble conjugated and insoluble conjugated polyamine fractions (putrescine, spermidine and spermine) in leaves and roots of LA and normal alkaloid (NA) tobacco varieties grown in the field and a greenhouse. Data revealed that levels of free and conjugated forms of putrescine and spermidine were higher in LA plants compared to NA controls. Fractions of the conjugated forms increased with leaf ripening and senescence in roots and leaves of LA plants. Furthermore, a positive correlation between polyamines, chlorophyll content and phenotype development in LA plants was observed. Treatments of LA plants with polyamine biosynthesis inhibitors reduced free putrescine content in roots and leaves similar to NA plant levels, thus leading to partial reversion of the LA phenotype; however, conjugated polyamines and chlorophyll levels were not affected. Our data indicate that the unbalanced crosstalk among nicotine, polyamine and ethylene biosynthesis is involved in delayed senescence but may not be the only factor responsible for impaired leaf ripening.

AP 17

Using RNP CRISPR to create ultra-low nicotine tobacco

SHEN Y.(1); QI D.(2); YANG J.(1); KUDITHIPUDI C.(1); XU D.(1); WAREK U.(1); STRICKLAND J.(1)

(1) Altria Client Services LLC, Research, Development and Regulatory Affairs, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.
(2) Eurofins Lancaster Laboratories, c/o Altria Client Services LLC, Research, Development and Regulatory Affairs, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.

Nicotine is the most abundant alkaloid in cultivated tobacco (*Nicotiana tabacum*), typically constituting more than 90% of total alkaloids. Our research showed that nicotine levels in PMT-RNAi and PR50-RNAi lines were reduced more than 95% and 80-90%, respectively, and both lines had significantly better leaf quality after curing than *nic1nic2* mutant controls. In order to generate low nicotine lines, CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 technology was combined with synthetic-guide RNAs (sgRNA) and a ribonucleoprotein (RNP) delivery technology (sgRNA complex) prior to validation in tobacco protoplasts against two PR50 homologues. The sgRNA complex was validated in tobacco protoplasts by PEG transfection. A treated protoplast fraction was collected for fragment analysis to validate gene editing location and the remaining fraction was subjected to tissue culture and plant regeneration. Fragment analysis and fragment sequencing were performed on microcalli and plants for insertion and deletion mutation confirmation. Since sgRNA complex technology in combination with protoplast transfection does not involve any foreign DNA and thus the resulting plants should be considered non-GMO.

AP 18

Reduction of nicotine content in tobacco leaves by modification of transporter gene expression

FREDERICK J.; XU D.; DAVIS G.A.; LION K.; WAREK U.; STRICKLAND J.

Altria Client Services LLC, Research, Development and Regulatory Affairs, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.

Current tobacco lines with reduced nicotine content derived from *nic1nic2* mutants have inferior leaf quality and undesirable smoking characteristics possibly due to the metabolic consequences of blocked nicotine biosynthesis. Allowing root production of nicotine but inhibiting transport to leaves might alleviate some of these consequences. The objective of this study was to identify genes involved in transportation of nicotine from roots to leaves. Two species of *Nicotiana* (*N. alata* and *N. otophora*) produce alkaloids in roots but detectable levels are not found in leaves. RNA-seq was used to compare gene expression in *N. tabacum* with the two non-transporting species. Annotated transport related genes that were highly expressed in roots of *N. tabacum*, but were not detected in *N. alata* or *N. otophora* were targeted. Twenty-three targeted genes were evaluated in *N. tabacum* var. Narrow Leaf Madole using RNAi. Down-regulation of several genes resulted in various levels of decreased leaf alkaloids compared to control. This study suggests that at least three genes are involved in alkaloid transport from roots to leaves and may allow development of tobacco lines with reduced alkaloid levels. Reducing the levels of leaf alkaloids but not changing the biosynthesis pathways involved might also reduce unintended impacts on leaf quality.

AP 19

Discovery of the Nic1 locus in *Nicotiana tabacum* L.

ADAMS A.; PRAMOD S.; LUSSO M.; XU D.

Altria Client Services LLC, Research, Development and Regulatory Affairs, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.

In *Nicotiana tabacum* L., four major alkaloids, including nicotine, are synthesized. It has been known for some time that two independent loci, Nic1 and Nic2, are key biosynthetic regulators and that the effect of the Nic1 locus is about two-fold greater than the effect of the Nic2 locus on nicotine levels. Although, seven ERF genes within the Nic2 locus were previously characterized, little is known about the Nic1 locus. In this study, we focused on identifying the Nic1 locus in Burley tobacco. The TN90 genome was sequenced. To identify the Nic1 locus, the near isogenic lines (NILs), Burley 21 (BU21, *Nic1Nic2*), High Intermediate (HI, *Nic1nic2*), Low Intermediate (LI, *nic1Nic2*) and Low Alkaloid BU21 (LA, *nic1nic2*), were also sequenced. By comparing sequencing data of the 4 NILs to TN90, we identified a putative Nic1 region. To improve elucidation, sequencing was also performed on bacterial artificial chromosomes created from tobacco genomic DNA. We have now identified a large chromosomal region that spans both borders of the Nic1 locus. Primers for genes within Nic1 locus were tested and validated along with known Nic2 ERF189 primers on two F2 populations (~510 individuals each) segregating for nic1 and nic2 loci. Genotypic segregation ratios of 9:3:3:1 as well as alkaloid levels consistent with identified genotypes were observed. Identification of the Nic1 genomic location facilitates not only characterization of its functional genes but also development of SNP markers to enable high throughput screening and identification of low alkaloid traits in breeding populations.

AP 20

Ultra-low nicotine tobacco lines with improved leaf quality

KUDITHIPUDI C.(1); MORRIS J.W.(1); LUSSO M.(1); SHEN Y.(1); QI D.(2); DAVIS G.A.(1); LION K.(1); XU D.(1); WAREK U.(1); STRICKLAND J.(1)

(1) Altria Client Services LLC, Research, Development and Regulatory Affairs, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.
(2) Eurofins Lancaster Laboratories, c/o Altria Client Services LLC, Research, Development and Regulatory Affairs, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.

Nicotine is the most abundant alkaloid in cultivated tobacco (*Nicotiana tabacum*), typically constituting more than 90% of total alkaloids. The remaining fraction consists of three minor alkaloids: nornicotine, anabasine and anatabine. Genetic control of nicotine biosynthesis in tobacco and subsequent development of low alkaloid traits have been derived historically from *nic1nic2* mutant lines. This unique genetic resource has been used to successfully produce tobacco lines having approximately 95% nicotine reduction compared to a *Nic1Nic2* control. However, the quality of cured leaf from these mutant lines, as measured by the USDA Grade Index, is commercially undesirable. The objective of this study was to develop new ultra-low nicotine tobacco lines having nicotine levels comparable to *nic1nic2* mutants while maintaining a USDA Grade Index of cured leaf comparable to a *Nic1Nic2* control. PR50, a key nicotine biosynthetic pathway regulator, and Putrescine N-methyltransferase (PMT), the enzyme involved in the first committed step of nicotine biosynthesis, were used. Transgenic tobacco plants containing separate RNAi constructs of PR50 and PMT were generated and grown in the field. After harvest and curing, alkaloid levels and cured leaf Grade Indexes were measured. Cured leaf nicotine levels in PMT-RNAi lines and PR50-RNAi lines were reduced approximately 95% and 80-90%, respectively, compared to *Nic1Nic2* controls. In addition, cured leaf samples of PMT-RNAi and PR50-RNAi lines had quality Grade Indexes similar to *Nic1Nic2* controls and significantly higher than samples from *nic1nic2* mutant lines.

AP 21

Evaluation of three transgenic and three conventional varieties for low alkaloid production with three nitrogen variables

CHEEK J.A.; VANN M.C.; LEWIS R.S.; FISHER L.R.

North Carolina State University, Dept. of Crop Science, Campus Box 7620, Raleigh, NC 27695, U.S.A.

Nicotine is one of the most studied and scrutinized plant secondary metabolites. In the United States, its concentration in tobacco products now falls under the jurisdiction of the Food and Drug Administration and it has been speculated that increased regulation might occur. Between 2014 and 2015, research was conducted in six North Carolina growing environments to evaluate three transgenic tobacco lines altered for decreased alkaloid (nicotine) production. Transgenic varieties were derived from K-326, with each having a different location at which the transgenic event took place. These three transgenic varieties: DH22A, DH32, and DH16A, along with three conventional varieties: K-326, NC-95, LAFC-53 were randomized over three different nitrogen rates, for a total of 18 treatments. Nitrogen application rates were 70%, 85%, and 100% of the recommended rate for each environment. Nitrogen was applied at a base rate of 30 lbs N/acre after transplanting, with the difference being applied through 28% liquid Urea-Ammonium-Nitrate at layby. After topping, leaf length, leaf width, SPAD readings, leaf discs, stalk height, and leaf counts were collected. Leaf yield, grade, and value were quantified after curing and composite samples from all four stalk positions were analyzed for primary and secondary alkaloid content. In 2015, excised leaf was collected to conduct a bioassay with tobacco budworms

(*Heliothis virescens*) to determine preferential feeding differences among varieties. Yield and quality for transgenic lines were similar to traditional K-326 and higher than NC-95 and LAFC-53 in all environments. Nicotine content was 75-80% lower in transgenic varieties when compared to traditional K-326.

AP 22

Nicotiana genomes: beyond tobacco

SIERRO N.S.; BATTEY J.N.D.; OUADI S.; THOMAS J.; LIEDSCHULTE V.; BOVET L.; BROYE H.; LAPARRA H.; VUARNOZ A.; LANG G.; GOEPFERT S.; PEITSCH M.C.; IVANOV N.V.

Philip Morris Products S.A. (part of Philip Morris International group of companies), PMI R&D, Quai Jeanrenaud 5, CH-2000 Neuchâtel, Switzerland

While *Nicotiana tabacum* is likely the most notable species from the Nicotiana genus, various other Nicotiana species are cultivated as crops, grown as ornamental garden plants, or used as model organisms in research. Within Solanaceae, Nicotiana species are peculiar first because although most Solanaceae species are diploids, a high number of Nicotiana species are tetraploids; and second because they have relatively large genomes that are similar in size with Capsicum species and two to three times larger than Solanum and Petunia species.

To date, the genomes of *N. benthamiana*, *N. otophora*, *N. sylvestris*, *N. tabacum* and *N. tomentosiformis* have been sequenced and draft assemblies published, enabling genome-based evolutionary studies of Nicotiana species. With the exception of *N. benthamiana*, all the published Nicotiana genomes are however closely related to *N. tabacum*. To complement these already published genomes we present here new draft genomes for additional Nicotiana species, which we expect will contribute to further our understanding of the diversity and of the impact of polypoidization in the Nicotiana genus.

AP 23

Tobacco genome sequence and its applications to genome-based selection

XU D.; FREDERICK J.; ADAMS A.; PRAMOD S.; KUDITHIPUDI C.; SHEN Y.; LUSSO M.; WAREK U.; STRICKLAND J.

Altria Client Services LLC, Research, Development and Regulatory Affairs, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.

Tobacco (*Nicotiana tabacum* L.), a model organism for research, is also an economically important crop. Tobacco is an allotetraploid derived from interspecific hybridization involving the two diploid species *Nicotiana sylvestris* and *Nicotiana tomentosiformis*. *Nicotiana tabacum*'s genome is complex and its functional genes are often duplicated with multiple homologs. Recently, various organizations have presented draft genomes for three major tobacco types. Here we report a high coverage genome for Burley tobacco and genomic comparisons among Burley, flue-cured, dark, Maryland and Oriental tobaccos. These studies reveal that genetic relationships between these different tobaccos are closely linked but they also have unique genetic identities corresponding to their representative properties. We have also developed a whole genome Axiom® SNP chip and have generated SNP profiles for over 30 additional tobacco lines that serve as important base breeding material. These efforts have provided for rapid recovery of novel traits as well as introgression of traits of interest into elite germplasm. Genes and their functions also provide the basis for understanding tobacco complexity which leads to

novel trait discovery including low constituent tobacco with superior leaf quality. Genome based gene functional analysis will lead to a thorough understanding of the impact that tobacco genes have on agronomic performance and quality traits as well as provide a better understanding of tobacco as a crop, and not just a model organism.

AP 24

Characterization of nitrogen use efficient tobacco for molecular marker development

FREDERICK J.; KUDITHIPUDI C.; XU D.; WAREK U.; STRICKLAND J.

Altria Client Services LLC, Research, Development and Regulatory Affairs, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.

High rates of nitrogen fertilization are required currently for cultivation of Burley tobacco to achieve yields and qualities that are desired. However, Maryland type tobaccos are grown using a nitrogen fertilization rate that is ~60-70% lower than that used typically for Burley tobacco. The objective of this study was to identify possible molecular markers that differentiate nitrogen utilization of Maryland tobacco compared to Burley tobacco. Maryland and Burley tobacco plants were grown in the greenhouse using either 100 ppm nitrogen or 25 ppm nitrogen and were fed continuously in an ebb and flow system. Gene expression was analyzed at a whole genome level by RNA-seq. Metabolite profiling was done using multiple approaches. Profiling of these two tobacco types revealed a difference in the carbon-nitrogen balance and an increase in pools of non-essential nitrogen containing compounds in Burley tobacco at low fertilization rates as compared to Maryland tobacco. Genes that may be responsible for these observations were also differentially regulated when compared. Possible molecular markers were measured in hybrid populations resulting from crosses between Maryland and Burley tobacco to validate their use as markers for nitrogen use efficiency. This study not only revealed possible gene targets that could be modified to improve nitrogen utilization in Burley tobacco, but also identified molecular markers that could be used for selecting nitrogen use efficient lines in breeding programs.

AP 25

Sucker control through biotechnology

XU D.(1); GRUNDMANN L.(2); SHEN Y.(1); NOLL G.(2); YANG J.(1); KUDITHIPUDI C.(1); FREDERICK J.(1); PRUEFER D.(2); WAREK U.(1); STRICKLAND J.(1)

(1) Altria Client Services LLC, Research, Development and Regulatory Affairs, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.
(2) Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Schlossplatz 8, 48143 Muenster, Germany

Tobacco plants exhibit strong apical dominance that effectively inhibits axillary bud development. However, upon shoot apical meristem removal (topping), loss of apical dominance quickly allows axillary buds to grow (suckers) resulting in yield loss and reduced leaf quality. Therefore, tobacco varieties exhibiting inhibited or eliminated sucker growth would reduce production cost, increase yields and improve quality. Molecular profiling of axillary buds before and after topping revealed multiple genes associated with axillary bud development. More than forty axillary bud developmental genes were evaluated for phenotypic impact via up-regulation and/or down-regulation in transgenic tobaccos. Several of these developmental genes produced the desired reduced sucker phenotype. Six associated developmental gene promoters were also tested for axillary bud tissue specificity and efficacy using GUS/GFP reporter genes. An axillary bud tissue specific promoter with high efficacy was fused independently with several selected developmental genes and inhibitory genes and their phenotypic impact was examined.

Over-expression/down regulation of the developmental genes and inhibitory genes with the axillary bud tissue specific promoter resulted in suppression of axillary bud growth after topping. Our data show that combination of an axillary bud tissue specific promoter with a selected group of genes can prevent or delay sucker development after topping in tobacco.

AP 26

Identification of differentially expressed axillary bud specific genes and their promoters

KUDITHIPUDI C.; SHEN Y.; XU D.; FREDERICK J.; YANG J.; WAREK U.; STRICKLAND J.

Altria Client Services LLC, Research, Development and Regulatory Affairs, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.

Tobacco plant exhibits exceptionally strong apical dominance. Molecular signals from shoot apical meristem mediate a hormonal environment that effectively inhibits axillary bud growth. Upon removal of the apical meristem, the signal is lost, enabling the formation of new shoots (or “suckers”) from axillary buds. Sucker growth results in loss of yield and leaf quality. Suckers have been controlled either by manual removal or through application of chemicals such as maleic hydrazide, flumetralin, fatty alcohols and dinitroanaline. However, residues of these chemicals are believed to pose health risks. Moreover, cost associated with these chemicals and labor runs into $70-250 per acre depending on tobacco type. Therefore, development of tobacco traits with limited or no axillary bud growth would result in a reduction of the use of chemical agents and would reduce costs and labor associated with tobacco production. Control of axillary bud outgrowth through conventional breeding, mutation breeding and transgenic approaches has been a major objective for several decades, but successful inhibition has not been achieved. The objective of the present work is to uncover genes that are critical for axillary bud development and identify the axillary bud specific promoters by differential gene expression profiling. A total of 10 different samples including axillary buds before topping, axillary buds after topping (2h, 6h, 24h and 72h), roots before topping, roots after topping (24h and 72h), young leaf at the time of topping and shoot apical meristem were collected, sequenced and analyzed. The top 100 differentially expressed genes were confirmed by real-time PCR and positively selected genes were used to test the efficacy of axillary bud suppression.

AP 27

An alternative solution for sucker control in leaf tobacco production

KANE K.(1); N’GUYEN CHAUME C.(2)

(1) ARVALIS - Institut du végétal, La Tour, 24100 Bergerac, France
(2) JADE International, avenue Ariane, CS 60027, 33693 Mérignac Cedex, France

In France, sucker control is practised on all tobacco crops, currently using two active substances registered for conventional production: 1-decanol and maleic-hydrazide (MH). 1-decanol is used on any tobacco type. An inconvenience is that, according to its assessment, it presents an unfavourable profile for the environment (aquatic and soil faunae). The use of MH, which is registered as a suckericide, should be reduced or avoided in some cases, due to its level of residues or/and the false maturity induced by the substance.

The objective of this study was to find new solutions, more respectful to the environment. Since 2008, we have been conducting trials on the topic. In 2015, a biocontrol product from the

company JADE International, VVH86 (nonanoic acid 680 g/l), was tested in three trials in France, a Burley screening test and two GEP trials (flue-cured and Burley), and showed interesting results.

VVH86 is registered in the Biocontrol List issued by the French Ministry of Agriculture. It is exempt from residue data and disappears from the ground in less than 48 hours. The product application doses were 0.5% in the first application and 1% for other applications (2 and 3 in Burley, flue-cured) to a slurry of 462 l/ha.

The results showed efficiencies and selectivities comparable to conventional solutions. However, the product, at the doses applied in 2015, presented a lower persistence than conventional solutions. The experiments will continue in 2016 to set suitable rates and numbers of treatments for tobacco sucker control.

AP 28

Approaching zero-residue organic tobacco: a possible target with the results of Life+2013 EVERGREEN Project

BARGIACCHI E.(1); MILLI G.(2); MIELE S.(1)

(1) Italian Interuniversity Consortium for Science and Technology of Materials (INSTM), I-50121 Firenze, Italy
(2) Fattoria Autonoma Tabacchi (FAT) & ITT, I-06012 Città di Castello, Italy

There is an increasing interest in zero-residue/organic tobacco, world-wide, both because it is a sustainable market opportunity and because of the fewer number of labelled a.i. due to toxicology and residue problems. Several biocides have also manifested reduced effects on pest and disease for resistance mechanisms and resurgence. To cope with these problems, and develop a new anti-infective approach to crop-pest/disease interrelationship, the EU funded LIFE13 ENV/IT/000461 EVERGREEN project, "*Environmentally friendly biomolecules from agricultural wastes as substitutes of pesticides for plant diseases control*", was started in 2014, with tobacco being one of the reference crops. The research group includes teams of the University of Firenze, Consortium INSTM, CEBAS CSIC (E), ASTRA, and commercial companies. EVERGREEN aims at demonstrating the *in vitro* and *in vivo* efficacy and reliability of polyphenolic-based extracts from agricultural non-food biomass and waste to increase plant resistance to phytopathogenic gram-negative bacteria and nematodes, and replace current agrochemicals and copper salts in agriculture, one of the most toxic biocides. Single fractions and combinations of polyphenols, extracted from sweet chestnut (SC), grapes (GR), olive, and artichoke, were investigated and tested in comparison with ordinary agrochemicals. Their anti-infective mechanisms were studied, using some plant pathogenic bacteria as models; the same was applied to *Meloidogyne* spp. The biomolecules effects on soil, non-target organisms, microrganisms and fertility cycles were also investigated. Organic tobacco was grown at FAT-Città di Castello in 2016 according to a strategy based on EVERGREEN products, with minimum use of copper salts. EVERGREEN has demonstrated the positive effects of some of the tested botanical extracts with hydrolysed and condensed tannins, and their anti-infective and biostimulant actions, prospecting a new, sustainable methodology to face some plant pathogens. Focus is now on zero-copper blue mould, and sucker control as next steps in organic tobacco production.

AP 29

The effects of mycorrhizal fungus on tobacco qualitative and quantitative parameters

GHORBANIAN D.(1); MASOUDI M.(2)

(1) British American Tobacco Pars, No. 2, Saba Blvd., Africa St., Tehran, Iran
(2) Iranian Tobacco Company, Qazvin Ave, Qazvin Square, P.O. Box 1331838734, Tehran 13314, Iran

To study the impact of Mycorrhizal fungus on flue-cured Virginia tobacco (K326) an experiment was carried out in the Golestan Province of Iran in 2014. The experiment was performed as a complete randomised block design with four replications, the factors studied included four different levels of Mycorrhizal Fungus (*Glomus* spp include: *Mosseae*, *Intraradices* and *Etunicatum*) inoculum as follows: 0, 6, 12 and 18 grams with 100 propagules/g. Inoculum was applied at the sowing stage in the float system per cell in seed trays.

The results indicated that Mycorrhizal inoculation had a significant positive effect on the measured characteristics. In the cases of sugar, leaf size, total wet and dry weights of tobacco leaf, there was a significant difference between treatments. In the case of total crop value and the chlorine levels, there were significant differences between treatments. There was no significant change in nicotine content. The maximum tobacco wet and dry weights were 22745 kg/ha and 3218 kg/ha respectively, with 18 g of Mycorrhizal inoculum. By increasing the amount of mycorrhizal inoculum, the sugar and chlorine levels were increased. 18 g of mycorrhizal inoculum made the highest meaningful difference in the sugar level, at 12.8%, compared with 6.15% in the control. Results showed that through increasing the fungi level, the sugar level increases in the leaves showing that the fungi have a positive effect on tobacco. The effect of fungi on the chlorine level was also significant and the highest level of chlorine (mean 5.7%) was achieved with 18 g of mycorrhizal inoculum, and the control treatment had the lowest chlorine level. Considering the total crop value, it can be concluded that with mycorrhizal inoculation, the total crop value increased and the highest increase of 32% per hectare was achieved with 12 g of mycorrhizal inoculum; in other words, using 12 g of mycorrhizal inoculum increased the tobacco dry and wet weights, the leaf quality, the leaf size and the leaf colour. Using mycorrhizal fungus decreased leaf length, with the longest leaf (40.89 cm) achieved by the control treatment and the shortest with 18 g of mycorrhizal treatment.

AP 30

Preliminary investigation of bio-oil compounds from Tombac that kill agricultural pests

KABBARA M.B.(1,2); OUDDANE B.(1); HALWANI J.(3)

(1) Lille 1 University, LASIR Laboratory, UMR CNRS 8516, Physical Chemistry of the Environment Team, 59655 Villeneuve d'Ascq Cedex, France
(2) Régie Libanaise des Tabacs & Tombacs, Tripoli, Lebanon
(3) Lebanese University, Water & Environment Science Laboratory, Public Health Faculty, Tripoli, Lebanon

Currently, scientists are focused on developing new tools to control insect populations, including secondary plant metabolites that show promise for use in plant protection. Secondary plant metabolites cause toxic effects that can be observed at both lethal and sublethal levels, but the most important effect is repellence. These compounds can affect insects at all levels of biological organisation, but their action generally disturbs cellular and physiological processes.

Tombac produces various substances that affect insects belonging to most orders, particularly herbivorous insects and other pests. Many compounds possess insecticidal properties, but they are also classified as molluscides, acaricides, nematocides, fungicides and bactericides.

Preliminary investigation of the effects of bio-oil compounds from whole plant extracts of Tombac were carried out on the beetle *Leptinotarsa decemlineata*, bacterias *Streptomyces scabies* and *Clavibacter michiganensis*, and the fungus *Pythium ultimum*. Testing of the effect of different crude extract on bacterias (*Streptomyces scabies* and *Clavibacter michiganensis*) isolates indicates that the methanol extract is the most bioactive. The test showed that the bioactivities of the crude extracts of the various parts of the Tombac were dependent and the whole plant was the most bioactive. The ethyl acetate fraction of the methanol extract of the whole plant of Tombac has been shown in this work to contain phytochemicals that have shown remarkable repellent activities. The bioactivities against *Streptomyces scabies* and *Clavibacter michiganensis* tested organisms were due to the combined effects of the compounds. Families of terpenoids, flavonoids, alkaloids, saponin, and steroids that were detected in the extracts were identified by GC-MS. The various classes of phytochemicals in the Tombac plant provided the antimicrobial potency of the plant. Only a small percentage of insect species are pests. However, pest species cause significant losses in agricultural and forest crops, and many are vectors of diseases.

In this paper, data will be presented on the sublethal and lethal toxicity caused by pure metabolites and crude extracts obtained from Tombac. Pure substances as well as water and/or alcohol extracts cause lethal and sublethal effects in insects, which is important from an economical point of view. The results of the study will also be discussed and their relevance to plant protection and management shown.

AP 32

Zimbabwean tobacco growers spoilt for choice: TRB continually screens and recommends new greener agrochemicals

GARWE D.; MASUKWEDZA R.; DIMBI S.

Tobacco Research Board, P.O. Box 1909, Harare, Zimbabwe

Due to their detrimental effects on the environment, flora and fauna, many agrochemicals have been banned, phased-out, or their use heavily restricted in recent years. For example, the effective but broad spectrum and purple label crop protection agents such as acephate, monocrotophos, aldicarb and methamidophos, are no longer recommended for use on tobacco even though they are extremely effective. Thus alternatives have had to be sought and availed to growers. In an endeavour to reduce Zimbabwe's tobacco production environmental footprint, the Tobacco Research Board (TRB) has been for decades, actively involved in seeking for and screening of safer and greener agrochemicals for use on tobacco. This is achieved through the Pesticide Approval Scheme Service, a grower advisory and pesticide registration system. This scheme offers assurance that all agrochemicals recommended for use on tobacco are compliant with the principles of Good Agricultural Practices. Thus, the TRB has proactively and consistently been able to provide the Zimbabwean grower with effective and acceptable alternatives for banned or restricted agrochemicals. This paper discusses the range of greener and more compliant agrochemicals that the TRB, in collaboration with a host of international agrochemical companies, has evaluated and recommended for use in controlling insect pests, root-knot nematodes and diseases in tobacco production in Zimbabwe.

AP 33

Characterisation and degradable influence factors of carbendazim residues in tobacco leaf

LI Yiqiang

Tobacco Research Institute, Chinese Academy of Agricultural Sciences, No. 11 Keyuanjingsi Rd, Laoshan District, Qingdao, Shandong 266101, P.R. China

A modified QuEChERS combined with LC-MS/MS method to detect and characterise the pesticide residue of carbendazim and three other benzimidazole fungicides in tobacco leaf was developed. The method has successfully indicated the influence of environmental factors, processing methods and storage conditions on its degradation, and clearly demonstrated the level and formation mechanism of carbendazim residue under reasonable utilisation. The results showed that the standard curve, the quantitative limit and the recoveries conformed to the requirements of the pesticide residue detection method. The theoretical degrading half-life of carbendazim, benomyl and thiophanate-methyl in the fresh tobacco leaf were 6.8, 6.3 and 6.6 days, respectively. The best condition for the degradation of carbendazim was 25 °C and 60% humidity. The processing factors of freeze-dried, editing and curing for the three pesticide residues were 5.11-5.66, 3.95-4.55 and 2.96-3.39, respectively. The half-life of carbendazim in cured tobacco leaf was 224-365 days in normal and constant temperature. The findings of this study also suggested that the degradation rate of carbendazim in the conditions of growing and storage were obviously different, with a rapid and crucial dissipation in the growing period under the the action of active enzyme, and the carbendazim residue level was significantly influenced by the original pesticide concentration and intervals between last pesticide application and harvest. Moreover, the process method, storage conditions and duration also had some impacts on the residue level.

AP 34

Evaluation of non-tobacco labeled herbicides for late season application

WHALEY W.T.; VANN M.C.; FISHER L.R.; WHITLEY D.S.

North Carolina State University, Dept. of Crop Science, Campus Box 7620, Raleigh, NC 27695, U.S.A.

Recently, viable seed from various weed species has been found in tobacco exports from the United States, initiating great concern in foreign markets and a zero tolerance policy. University specialists believe the majority of weed seed contamination is a reuslt of mechanicanized harvest. At present, farmers have a variety of management options, such as cultivation, herbicide application, and hand weeding, to reduce weed pressure and lower seed bank populations for future years. However, the spectrum of herbicide options for tobacco is extremely limited, specifically for post-transplanting application. An evaluation of non-tobacco labeled herbicides for late season application is greatly needed to give farmers alternative strategies in weed management. Research was conducted in 2014 and 2015 in North Carolina to evalute various herbicide programs applied late in each growing season. Treatments were arranged in a randomized complete block design and replicated four times. Eight different herbicides were evaluated, each at two different application timings: before topping and after first harvest. The eight herbicides were as follows: S-metolachlor, sulfentrazone, trifloxysulfuron, fomesafen, glufosinate, mesotrione, linuron, and carfentrazone-ethyl. A ninth herbicide, sethoxydim, was applied after first harvest only and a control plot of sulfentrazone + clomazone (PRE-T only) was included as well. Applications were made with a backpack sprayer containing a twenty inch boom and two Teejet VisiFlo flat spray tip nozzles. Application occurred at a spray volume of 187 L/ha. Spray applications covered the row middles as well as a portion of the tobacco bed. Product rates were based upon Extension recommendations. Following application weed control efficacy, crop injury, leaf yield, quality, value, and chemistry were quantified. In both years, herbicide injury

was greatest in before topping applications of glufosinate (2.75-3.75%), mesotrione (3.50-25.00%), and carfentrazone-ethyl (2.00-2.50%); however, leaf yield was not affected. Palmer amaranth suppression ranged from 80-100% following application for all treatments.

AP 35

Residue degradation of λ-cyhalothrin and fenvalerate during tobacco planting and processing

CHEN Li; HU Bin; PAN Lining; CUI Huapeng; CAI Junlan; JIA Yunzhen; FAN Meijuan; WANG Xiaoyu; LIU Huimin

Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou, Henan 450001, P.R. China

Lambda-cyhalothrin and fenvalerate, two widely used pesticides in tobacco, have been receiving high attention recently in China as their residue is one of the most important factors affecting the quality and safety of tobacco. To find out the key procedures for pesticide residue control, the degradation behaviours of λ-cyhalothrin and fenvalerate in tobacco from planting to processing (involving flue-curing, threshing, redrying and cutting) were studied. Residual field trials, including residue dynamic experiments and final residue experiments, were designed based on the Guideline on Pesticide Residue Trials issued by the Chinese Ministry of Agriculture and conducted in two regions, Linyi in Shandong and Qujing in Yunnan, during 2014. Tobacco processing experiments were carried out after the field trials following the typical flue-cured tobacco processing mode in China. A multi-pesticide residual detection method based on GC-MS/MS was employed to determine the residues of λ-cyhalothrin and fenvalerate in tobacco. The results showed that, the degradation dynamics (from 2 hours to 30 days) of λ-cyhalothrin and fenvalerate in tobacco followed the first-order kinetic equation, however their residues in tobacco leaves possessed different degradation rates in the two experimental regions, with the half-life periods of 16.7 and 17.0 days in Linyi, 8.1 and 8.3 days in Qujing, respectively. The differences in climatic conditions and tobacco growing between the two regions were the main factors resulting in the obvious difference of the half-life period. The degradation of λ-cyhalothrin and fenvalerate in tobacco during processing indicated that their degradation was mainly caused by high temperatures during processing, and their degradation rates during flue-curing were 17.2%-22.1% and 13.5%-18.6%, respectively. Generally, processing did not affect the degradation of pyrethroid residues in tobacco remarkably. The key degradation stage for the two pyrethroids is the tobacco growing stage in the field, which is also the key stage for controlling pesticide residues.

AP 36

Spinosad and cyantraniliprole residues in flue-cured tobacco

INMAN M.D.; VANN M.C.; SEAGROVES R.W.; FISHER L.R.

North Carolina State University, Dept. of Crop Science, Campus Box 7620, Raleigh, NC 27695, U.S.A.

From 2013 to 2015 research was conducted to establish the maximum expected pesticide residue on cured tobacco that would result from a maximum labeled application rate and minimum pre-harvest interval of specific active ingredients. Residues of two insecticides, cyantraniliprole and spinosad, were quantified on flue-cured tobacco produced in six environments in North Carolina during the research cycle. Treatments were applied to flue-cured tobacco grown on research stations near Kinston and Rocky Mount, North Carolina. Tobacco was managed according to North Carolina Cooperative Extension Service recommendations and harvested four times by stalk position. Pesticide residues on cured leaf were determined by

Global Laboratory Services, Inc. in Wilson, North Carolina. Data were analyzed and reported by individual year, location, active ingredient, and stalk position; with primings one and two represented in the “lower” stalk position (Lug + Cutter), priming three represented in the “middle” stalk position (Leaf), and priming four represented in the “upper” stalk position (Tip). Cyantraniliprole residue was below the limit of quantification for all stalk positions in all six environments. Spinosad residue, as either one of or combinations of spinosyn-A and/or spinosyn-D, was only recorded in the lower stalk positions of all Kinston environments. Spinosyn-A residue was consistently greater than spinosyn-D, with the highest means for each being recorded at 1.128 and 0.231 mg/kg, respectively, in the Kinston 2014 environment. Ultimately, it appears that cured leaf residues from either of these active ingredients are likely to be extremely low across a variety of environments relative to other pesticides with established Guidance Residue Limits.

AP 37

Maleic hydrazide (MH) residues resulting from applications at three times of day and three MH rates

MOORE J.M.; LAHUE S.S.

University of Georgia, 2360 Rainwater Rd, Tifton, GA 31793, U.S.A.

The tobacco industry evaluates Maleic hydrazide (MH) residues based on established CORESTA guideline residue levels (GRL) of 80 ppm. Over the last 25 years, changes in application techniques and amounts of MH applied have resulted in lower residue levels. There still appear to be factors in addition to the application rate of the chemical that affect resulting MH residues. The objective of this study was to determine the effects of application time of day and rate on MH residues. Trials were conducted near Tifton, Georgia in 2014 and 2015 to evaluate MH application time of day (7 am, noon, 4 pm) and MH rates (1.25 kg/ha, 2.5 kg/ha, 3.78 kg/ha). MH residues were determined from repeated sampling of green leaves from the fourth leaf from the top of the tobacco plants starting the day after application and following each successive rainfall event. Results of the MH residue samples show a strong correlation between early day application and lower residue levels at each of the MH application rates throughout the harvest period. Residues were reduced by more than half with the first rainfall regardless of the application time and amount applied (Application at 7 am of the three MH rates resulted in next day determined MH levels of 107, 302, and 445 ppm. These were reduced to 39, 81, and 45 ppm respectively for samples taken after 6 mm of rainfall). Successive rainfall events reduced the MH residue level further. These trials confirm that MH residues are directly related to application rate, that rainfall reduced MH residues, that application rates in excess of 1.25 kg/ha often resulted in MH residues in excess of the 80 ppm target, and application early in the day resulted in lower MH residues than at noon or 4 pm.

AP 38

Assessing the genetic diversity of *Nicotiana rustica* accessions using inter-simple sequence repeats

KASHANGURA C.; MUZHINJI N.

Tobacco Research Board, P.O. Box 1909, Harare, Zimbabwe

The use of Zimbabwe tobacco landraces in developing varieties better suited to climate change environments may become critical in the future, thus the need to properly characterise the landraces using molecular based DNA techniques. The occurrence of several landraces with similarity in morphology poses a quandary especially when relying on morphological data which

have been shown to be of limited value for modern systematics due to their inherent simplicity, evolutionary convergence, parallelisms and phenotypic plasticity. Thus, this study sought to determine the genetic relatedness of the Zimbabwe tobacco landraces maintained in the Kutsaga germplasm library for unambiguous use of the accessions in the future. Genetic diversity was determined using seven ISSR markers which showed useful polymorphism in comparison to the rest of the ISSR markers screened. Based on these seven ISSR markers, the *N. rustica* accessions separated into three clusters which were separate from the commercial *N. tabacum* varieties cluster. The *N. rustica* (Nakuru) accession was determined to be genetically distant from the other *N. rustica* accessions as it separated from the other *N. rustica* accessions which may be beneficial in future fitness of *N. rustica* in possibly novel environments brought about by climate change. This study enabled differentiation of the *N. rustica* accessions and elucidated genetic diversity amongst them which can be exploited for potential development of qualitatively improved cultivars.

AP 39

T75: A new Kutsaga drought tolerant tobacco variety

MAGAMA F.; SHAVA J.G.; DIMBI S.

Tobacco Research Board, P.O. Box 1909, Harare, Zimbabwe

Drought is one of the most important environmental stresses in agriculture and efforts have been made by breeders worldwide to maintain crop productivity even under water limiting conditions. Since the inception of the Tobacco Research Board in the 1950s, the Plant Breeding division has aimed to develop high yielding, multi-disease resistant and superior quality tobacco varieties that would be grown in Zimbabwe but marketed on the international market. From 2006, however, in view of climate change, the breeding programme was adapted to include selecting for drought tolerance. Thus, all varieties considered for further advancement henceforth were subjected to thorough drought tolerance screening and this continues to date. As part of the tobacco industry agreed variety release protocol, five limited release varieties T70, T73, T74, T75, and T76 that had satisfied the above breeding objectives, including drought tolerance, were evaluated by growers starting in the 2012/13 season. Gathering information on the performance of varieties under grower management and under different environmental conditions is an extremely important aspect of the release protocol. Field trials were set up over three seasons (2013-2015) at ten sites representative of Zimbabwe's tobacco growing areas. The trials were planted in October and were all rain fed. The last two seasons (2014 and 2015) characterised by a late onset of the rains and long intermittent droughts enabled the varieties to be evaluated for drought tolerance. At each site, in addition to the test varieties, the popular and widely grown varieties K RK26R and K RK66 were included in the plots for comparison. The test varieties had significantly better yields than K RK26R but were comparable to yields of K RK66 in the different environments over the three seasons. However, the variety T75 was clearly ahead of the pack as it exhibited superior performance by yielding the highest and showing the least symptoms of drought stress.

AP 40

Phenotypic plasticity in flue-cured tobacco roots: a strategy for improved drought resilience breeding

SHAMUDZARIRA M.; SHAVA J.G.; MAWIRE B.; MAGAMA F.; DIMBI S.

Tobacco Research Board, P.O. Box 1909, Harare, Zimbabwe

Drought is becoming more prevalent in Zimbabwe where it is affecting yield and sustainable tobacco production. Notably, there has also been an increase in the number of farmers growing

tobacco in marginal and drought prone regions. Different methods are currently in use for screening germplasm for drought tolerance such as screening for osmoprotectants, high chlorophyll content and specialised root architecture. At the Tobacco Research Board, development of varieties capable of extracting water and nutrients more efficiently was considered practical for ameliorating drought conditions. The objective of this experiment was to study the root architecture of recently developed elite varieties for adaptation to drought and marginal conditions to identify root architecture traits that can be used in a comprehensive drought breeding program, the root depth, multi-lateral root development, highly fibrous root systems, root penetration angles and tap root thickness were measured on 15 varieties under dryland conditions. The same varieties were grown under irrigation for comparison. Standard tobacco cultural practices were followed and after the final reaping, stalks were carefully uprooted to ensure the root systems remained intact. All measurements taken were analysed using Genstat version 18. The general trend was that crops grown under dryland conditions had more extensive root systems. Six varieties found to have extensive root systems, showed field resilience and yielded significantly more than those with less extensive root systems under dryland conditions. Although significant differences were observed in root architecture between varieties, no such differences were noted when tobacco was grown under irrigation, these results will be discussed. Interestingly, three of the varieties showing drought resilience shared a common parental (BAZ). BAZ could potentially be used in tobacco drought breeding programmes in Zimbabwe. The knowledge generated from using root architectural traits as selection criteria will inform future breeding strategies for drought tolerance.

AP 41

Investigating the genetics of polygenic soil-borne disease resistance in tobacco

LEWIS R.S.(1); DRAKE-STOWE K.(1); BAKAHER N.(2); GOEPFERT S.(2); PHILIPPON B.(2); MARK R.(2); PETERSON P.(3)

(1) North Carolina State University, Raleigh, NC, U.S.A.
(2) Philip Morris International R&D / Philip Morris Products S.A. (part of Philip Morris International group of companies), Neuchâtel, Switzerland
(3) Clemson University, Florence, NC, U.S.A.

Bacterial wilt and black shank, caused by *Ralstonia solanacearum* and *Phytophthora nicotianae*, respectively, are the most important diseases affecting tobacco production in the United States. An increased understanding of the genetics controlling resistance to these soil-borne diseases, as well as identification of DNA markers associated with genomic regions controlling this resistance, could aid in the development of improved varieties. Flue-cured tobacco cultivar 'K346' exhibits high levels of resistance to both diseases. In an effort to understand the genetics of soil-borne resistance in this cultivar, we developed a recombinant inbred line (RIL) mapping population consisting of 186 lines derived from a cross between K346 and disease-susceptible tobacco accession 'TI1068'. The population was genotyped with more than 300 microsatellite markers and evaluated for two years for field resistance to both black shank and bacterial wilt. Several genomic regions of K346 origin were found to affect resistance to both pathogens, a finding that may at least partially explain previously observed correlations between resistance to black shank and bacterial wilt among current cultivars and within breeding populations. Quantitative trait loci (QTLs) discovered to affect disease resistance in K346 were compared to those previously identified for 'Florida 301' and 'Beinhart 1000'.

AP 42

Selection of elite lines of flue-cured tobacco based on electron-beam irradiation

YANG Huijuan(1); ZHAO Li(1); SHI Hongzhi(1); ZHAO Shimin(2); LI Zhaomin(2); YANG Qinmin(2)

(1) Henan Agricultural University, Tobacco Cultivation Key Laboratory of China Tobacco, 95 Wenhua Road, Zhengzhou 450002, P.R. China
(2) Luoyang Tobacco Company, Luoyang 471000, P.R. China

In order to screen elite flue-cured tobacco germplasm, the mutagenesis method of electron-beam irradiation was employed to select an elite line among three flue-cured tobacco lines: Songyan A (A), Songyan B (B) and Songyan C (C). Dosage of 500 Gy was used as the proper dosage of screening. The field performance of the three original lines and irradiation mutagenesis lines (M0) which were named A500, B500 and C500 were also evaluated and some individual plants were selected as M1 generation seeds sources. The results showed that: (1) Quality of cured leaf from line A is excellent among the three original flue-cured tobacco lines. The equilibrium moisture, potassium, carotenoid and total sugar content in line A are much higher than the other two lines. It also has a lower protein content and moderate nicotine content. Original line A bears the best physical properties, chemical components and aroma constituents; (2) The seeds mutated by the radiation dosage of 500 Gy (A500, B500 and C500) can germinate normally. Percentage of geminated A500 seeds was higher than 80% while germination percentage of B500 and C500 seeds were only about 50%; (3) The mutated seed lines were planted in the fields of Songxian, Luoyang, which are normally dry and here the A500 line showed a good field performance compared with B500 and C500; (4) During the M1 population of A500, B500 and C500, disease-resistant M1 generation individual plants were selected. In total, sixty M1 plants from A500, sixteen from B500 and twenty-two from C500 were selected, and finally five M1 individual plants from A500 and three M1 individual plants from C500 survived the black shank disease. Seeds germination tests on M1 generation showed that M1 lines from A500 germinated at a higher percentage among all the M1 lines whether under normal conditions or drought conditions. These findings suggested that electron-beam irradiation is an effective way to screen for elite flue-cured tobacco germination lines.

AP 43

The pattern of benzo[a]pyrene and tobacco-specific nitrosamine accumulation in fire-cured tobacco: a comparison of two barn types

JACK A.M.; BAILEY W.A.; SCHOERGENDORFER A.; JI H.; WU Y.

University of Kentucky, KTRDC, 1401 University Drive, 202B KTRDC Bldg., Lexington, KY 40546, U.S.A.

A two-year experiment was done to study the pattern of accumulation of benzo[a]pyrene (B[a]P) and tobacco specific nitrosamines (TSNAs) during fire-curing, and to establish whether B[a]P and TSNAs can be reduced by modifying the firing regime. The 2012 study was done in a traditional fire-curing barn, with five firings, over a total of 25 days. B[a]P at takedown was 262 ppb, and total TSNAs were 5.4 ppm. The study was repeated in 2015, in a new, “tighter” barn, in which it is possible to achieve a satisfactory finish with fewer and shorter firings; a regime which might be expected to result in lower TSNAs and B[a]P. There were three firings, over a total of 18 days. B[a]P at takedown was 489 ppb, and total TSNAs were 8.5 ppm; much higher than in the 2012 study cured in the older barn with more firings. These results were unexpected, because the decrease in firing in the 2015 study would be expected to result in lower B[a]P and TSNAs. Seasonal differences can have a considerable effect on TSNA levels, but are unlikely to affect B[a]P levels. We are investigating possible causes of these anomalous results.

AP 44

Establishing a sampling protocol to estimate tobacco specific nitrosamines in growers' bales: results from the first year of a two year study

FISHER C.R.(1); McQUERRY K.(2)

(1) University of Kentucky, Plant & Soil Sciences, Plant Sciences Building, Lexington, KY 40546, U.S.A.
(2) University of Kentucky, Department of Statistics, Multidisciplinary Science Building, Lexington, KY 40536, U.S.A.

The CORESTA Sub-Group "TSNA in Air-Cured and Fire-Cured Tobacco" is drafting a protocol for the procedure of sampling bales of tobacco presented for sale by the grower. At the Sub-Group meeting in 2014, several queries were raised including the number of cores required from any single bale, the total quantity of leaf suggested and the reliability of the data from this sample in representing the overall tobacco specific nitrosamine (TSNA) concentration of the bale. It was proposed that the sampling protocol should be tested and modified to best satisfy the needs of the industry. Six core samples were taken from each of six big (220–270 kg) bales. Three methods were used to sample each of six small (25–35 kg) straight laid bales: four core samples from each of three positions along the length of the leaf, four grab samples of about 40 leaves each from different positions within the bale, and one sample of 160 random individual leaves from throughout the bale. The thicker sections of midrib were separated from the lamina to simulate the midrib size removed during threshing. The individual leaf samples from the small bales were then split into four separate samples. The samples were analysed for TSNA content. The mean total TSNA in each of the big bales ranged from 1.4 to 42 µg g^{-1}, and from 1.4 to 14 µg g^{-1} in the small bales. Data from this first year of the study suggest that it may be possible to estimate the TSNA of the lamina in small straight laid bales by taking as few as four core samples, with a total weight of 300 to 400 g, from a specific position along the length of the leaf as it is laid in the bale, and analysing unseparated lamina and midrib.

AP 45

Interaction effect of storage temperature and leaf moisture content on TSNA formation of Burley tobacco during leaf storage

SHI Hongzhi(1); WANG Jun(1); SUN Wenshu(1); ZHOU Jun(2); JIN Tong(1); YANG Huijuan(1);
BAI Ruoshi(2); MA Yanjun(2)

(1) Henan Agricultural University, Tobacco Cultivation Key Laboratory of China Tobacco, 95 Wenhua Road, Zhengzhou 450002, P.R. China
(2) Beijing Cigarette Factory of Shanghai Tobacco Group, Beijing 100024, P.R. China

The formation of tobacco-specific N-nitrosamines (TSNA) during leaf storage is substantial and is influenced by factors such as temperature, leaf moisture and nitrate content. The controlled experiments were conducted in this study to investigate the interaction relationship and effect on TSNA formation during leaf storage in incubation chambers. Leaf moisture was set at three levels (7.71%, 15.30% and 22.10%), which were achieved by balancing leaf moisture with solutions of potassium carbonate of different saturation degrees in enclosed cubes. Leaf samples with different moistures were then stored at five different storage temperatures (10 °C, 20 °C, 30 °C, 40 °C and 50 °C) for 15 days. The results showed that both storage temperature and leaf moisture content had significant effects on TSNA formation and they had significant interaction effects. Total TSNA content of low moisture tobacco (7.71%) increased from 0.8 µg/g to 8.9 µg/g as storage temperature increased from 10 °C to 50 °C, while that of high moisture tobacco (22.10%) increased from 1.7 µg/g to 2.2 µg/g within the same temperature range. Analysis showed that storage temperature was the principle contributor to total TSNA variation with the contribution percentage of 56.1%, followed by leaf moisture content (25.6%) and interaction

effect (18.2%). The two factor models were established to reflect the response of individual and total TSNA formation to storage temperature and leaf moisture with the determination coefficients of 96.0%, 95.5%, 96.5%, 93.2% and 96.1% for NAT, NNN, NAB, NNK and total TSNA, respectively. Based on the results and analysis, it was suggested that storage temperature be controlled lower than 25.5 °C and leaf moisture be at moderately higher level as long as not exceeding 18% so as to effectively inhibit the TSNA formation of storing tobacco leaves.

AP 46

Effect of fertilisation with nitrogen, with or without the application of endomycorrhizae, in planting on the yield and quality of flue-cured tobacco

TURŠIC I.(1); SLEZAK R.(2); RACZ A.(3); ŽALAC S.(4)

(1) Tobacco Institute Zagreb, Svetosimunska cesta 25, 10000 Zagreb, Croatia
(2) Bio-Buducnost d.o.o., Zagreb, Croatia
(3) University of Applied Health Sciences, Croatia
(4) Agrokontrola d.o.o., Zagreb, Croatia

The effect of fertilisation with different amounts of nitrogen and the application of endomycorrhizae in planting on the yield and quality of tobacco were investigated in the experimental field of the Faculty of Agriculture in Zagreb. Studies were performed in two field experiments with four treatments.

In the first experiment 20, 40 and 60 kg of calcium ammonium nitrate were used beside a non-fertilised variety. Fertilisation with phosphorus and potassium was uniform for all treatments and amounted to 60 kg P_2O_5 and 160 kg K_2O.

In the second experiment beside the same nitrogen fertilisation (0, 20, 40, 60 kg NH_4NO_3 ha^{-1}) tobacco seedlings were treated with special vaccine MYKOFLOR® (mycorrhizal mycelia). Tobacco was harvested manually, topped at the beginning of flowering and harvested six times. The content of nicotine and sugar was determined in samples taken from the third harvest. The results obtained were processed by analysis of variance.

Tobacco treated with MYKOFLOR® had significantly higher yield and better quality. In 2015, a dry year, tobacco treated with MYKOFLOR® had stronger developed root system and a greater possibility of taking up water and dissolved macro and micro elements from the soil.

Untreated tobacco had 25-50% lower yield and lower quality of the dried leaf depending on the level of nitrogen fertilisation. In the very arid year of 2015, higher nitrogen fertilisation of tobacco without the application of mycorrhiza increased the nicotine content and reduced sugar content, which greatly affected the quality of flue-cured tobacco.

AP 47

The effects of different planting methods on tobacco quality, yield and yield parameters

CELEN A.E.; EKREN S.; KOPARAN V.; ILKER E.

Ege University, Agriculture Faculty, Department of Field Crops, Bornova-Izmir, Turkey

Tobacco still has great economic importance in the world. Turkey produced 90.000 tonnes of the world's total tobacco production of 7.3 million tonnes. According to 2013 statistics, 50.877 kg of tobacco, which is more than 50% of the total production of Turkey, were obtained from the Aegean region of Anatolia. A field study on Izmir-Ozbas type tobacco (*Nicotiana tabacum* L.) was carried out to determine the effects of different planting methods (traditional, double cross and

double opposite planting) in 2014 in Denizli Province in Turkey. In the trial, the experimental design was randomised complete block design with three replications. The effects of different planting methods on tobacco plant height (cm), leaf weight (g), leaf width (cm), leaf length (cm), number of the leaves (per/plant), yield (kg/ha^{-1}) and visual quality were evaluated. According to the results, the highest leaf weight, leaf length and leaf width were determined in traditional planting methods. However, the highest number of leaves and highest yield was found in double opposite planting methods and the highest plant height was determined in double cross planting methods. When the visual quality values were assessed, American Grad tobacco rate in double opposite method was higher than the other two methods. It was found that the double opposite planting method was better than the traditional and double cross planting methods.

AP 48

TALEN-mediated mutagenesis of *NteIF4E1a* leads to potato virus Y (PVY) resistance in flue-cured tobacco (*Nicotiana tabacum* cv. K326)

YANG Dahai(1,2,3); YAO Heng(1,2,3); XIE He(1,2,3); BAI Ge(1,2,3); CHEN Xuejun(1,2,3); LI Yongping(1,2,3)

(1) Tobacco Breeding and Biotechnology Research Center, Yunnan Academy of Tobacco Agricultural Sciences, Kunming 650021, P.R. China
(2) Key Laboratory of Tobacco Biotechnological Breeding, Kunming 650021, P.R. China
(3) National Tobacco Genetic Engineering Research Center, Kunming 650021, P.R. China

Potato virus Y (PVY) infection causes severe yield reduction and quality decline of cultivated tobacco (*Nicotiana tabacum*) leaves, and results in large economic losses. In tobacco production, effective control of PVY is urgently needed. Breeding PVY-resistant tobacco varieties is a cost-effective and environmentally friendly way to control PVY disease. Therefore, cloning PVY-resistance genes in tobacco and studying the molecular mechanism of PVY resistance are important for breeding PVY-resistant tobacco varieties. In order to study important PVY-resistance genes in tobacco, five *eIF4E* or *eIF(iso)4E* gene family members were cloned. TALEN constructs targeting the first exon of *NteIF4E1a* gene were constructed. These constructs were transformed into tobacco cultivar K326 via Agrobacterium-mediated transformation. After identifying the successfully transformed tobacco plants, sequencing results of the first exon of *NteIF4E1a* revealed that *NteIF4E1a* had been successfully edited. Two PVY strains (PVY^{N} and PVY^{NTN}) were inoculated into the seedlings of tissue culture propagation from *NteIF4E1a*-edited K326 tobacco. At fourteen and 21 days post inoculation (dpi) of PVY, results from ELISA detection of PVY showed that the editing (mutation) of *NteIF4E1a* in K326 improved its resistance to PVY in varying degrees, suggesting that *NteIF4E1a* is a critical gene for tobacco resistance to PVY.

AP 49

Construction and genetic evaluation of chromosome segment substitution lines in tobacco (*Nicotiana tabacum* L.)

TONG Zhijun; JIAO; Fangchan; CHEN Xuejun; XIAO Bingguang

Yunnan Academy of Tobacco Agricultural Sciences of CNTC, Key Laboratory of Tobacco Biotechnological Breeding, National Tobacco Genetic Engineering Research Center, Kunming 650021, P.R. China

A set of chromosome segment substitution lines (CSSLs) of tobacco (*Nicotiana tabacum* L.) was first developed by molecular marker assisted selection (MAS) and successive backcrossing with Y3, the flue-cured tobacco germplasm with comprehensive traits as the recipient parent, and two common tobacco cultivars Beinhart1000-1 and K326 as the donor parents. The cigar tobacco

cultivar Beinhart1000-1 carries a variety of resistance traits including black shank (both race 0 and race 1) and brown spot resistance, while the flue-cured tobacco K326 was a commercial cultivar with high quality. In 256 CSSLs, a total of 377 substituted segments derived from donor Beinhart1000-1 and K326 in the genetic background of Y3 were distributed on 24 linkage groups. Each CSSL contains only 1–5 substituted segments and length of the substituted segments ranged from 0.05 to 36.88 cM with an average of 7.75 cM. The total length of the overlapped substituted segments was 2922.57 cM, which was 2.61 times of the whole tobacco genome. The genome covered length was 1114.32 cM, with a covered ratio of 99.45% of the recurrent tobacco genome. The CSSLs constructed in this study are excellent genetic materials for gene mapping, quantitative trait locus (QTL) analysis of quantitative traits, developing varieties by marker assisted selection in *Nicotiana tabacum* L.

AP 50

Reducing production costs of French Burley tobacco by mechanisation of stripping with Cured Plant Segmenting/Separator System CP3S

GOUDOUNECHE J.L.(1); WELLS L.G.(2)

(1) ARVALIS - Institut du végétal, Service Génétique Physiologie et Protection des Plantes, La Tour, 24100 Bergerac, France
(2) University of Kentucky, Biosystems and Agricultural Engineering, 128 C.E. Barnhart Bldg, Lexington, KY 40546-0276, U.S.A.

In 2011 ARVALIS and the University of Kentucky started a partnership in order to offer cheaper means of production and allow specialisation of French tobacco farmers.

The first steps were completed between 2011 and 2014 as follows:

- Adaptation of French production techniques to the harvesting process with self-propelled GCHI 'Gold Standard' Burley tobacco harvesting system: 5 hours to harvest 1 hectare with 2 workers (20 000 plants).
- Trials on curing racks in southern France: 7 weeks for curing was needed when harvesting in August.

The purpose of this study was to test the Cured Plant Segmenting/Separator System CP3S designed by G.B Day V, T.D. Smith and L.G. Wells (University of Kentucky) and then perform the threshing of the tobacco material obtained.

The machine which cuts stalks and leaves at the same time, and then separates the stalk segments from leaf segments, allows to strip 4800 plants per hour with 6-7 persons, meaning 35 labour hours per hectare, compared to 200 hours per hectare usually required.

The resulting raw material being no longer quite the same, the stripped tobacco leaves were imported to France to be threshed in the transformation factory of France Tobacco in Sarlat. The results were positive as regards the rib rates of the final product: 66.10% of strips compared to 64.52% (which was the average in 2008-2013), but slightly penalised in terms of segment size: the percentage of strips over ½ inch was 7% lower than usual (65.91%). This rate could be increased if the tobacco materiel was dryer before being introduced into the threshing line. This innovative stripping system gives a modified final product (leaf segments instead of whole leaves), which seems to suit the manufacturers, while providing interesting prospects for the competitivity of tobacco farmers in France.

AP 51

Potato virus Y (PVY) resistance in tobacco: identification of an alternative source of resistance against PVY resistant breaking variant

JULIO E.(1); COTUCHEAU J.(1); DECORPS C.(1); VOLPATTI R.(1); MICHEL V.(2); GERMAN RETANA S.(2); DECROCQ V.(2); CANDRESSE T.(2); DORLHAC DE BORNE F.(1)

(1) Imperial Tobacco, La Tour, 24100 Bergerac, France
(2) UMR 1332 BFP, INRA, Univ Bordeaux, Campus INRA, 71 Av. Edouard Bourlaux, CS20032, 33882 Villenave d'Ornon Cedex, France

Potato virus Y (PVY) is one of the most damaging viruses on tobacco worldwide.

This virus is responsible for mosaic or necrotic symptoms depending on the PVY strain. Aggressive PVY necrotic strains can lead to considerable yield losses.

A large deletion conferring resistance to PVY, the “va” gene, has been recently characterised in tobacco (Julio et al., 2014). It has been shown that the loss of a eukaryotic translation initiation factor 4E copy (*eIF4E*) results in recessive resistance to PVY. However, PVY isolates overcoming the *va* gene have been observed in several countries. These resistance-breaking PVY isolates induce necrotic symptoms in the Virgin A mutant (VAM) resistant cultivar, the main source used to transfer the *va* resistance into tobacco varieties.

Phenotyping tests were conducted on a large collection of 162 varieties from the Imperial Tobacco collection. The absence of a functional *eIF4E* was characterised by Polymerase Chain Reaction (PCR) and PVY resistance was estimated by symptom survey and ELISA tests. The screening was performed with different PVY necrotic strains, including one able to overcome the *va* gene.

Ten cultivars displaying resistance to PVY while expressing a functional *eIF4E-Va* gene were identified, suggesting that the resistance observed does not rely on *va* in these cultivars. ELISA assays showed that PVY isolates can infect the ten cultivars, although they do not induce necrotic symptoms. Therefore, a different mechanism, involving tolerance to necrosis, appeared to be involved.

Fine mapping on a F2 segregating population confirmed that this tolerance trait is inherited as a single recessive gene on chromosome 13. Anchoring the linkage map to the tobacco genome physical map allowed the identification of a candidate gene with a Single Nucleotide Polymorphism (SNP) in eight of these cultivars. This gene could be used in future breeding programmes to minimise the impact of PVY resistance-breaking strains able to overcome the *va* mediated-resistance and to limit crop losses.

AP 52

A breakthrough in breeding for PVY resistance in elite flue-cured tobacco parentals in Zimbabwe

MAGAMA F.; KASHANGURA C.; MUFUNDA F.; SIGOBODHLA T.; DIMBI S.

Tobacco Research Board, P.O. Box 1909, Harare, Zimbabwe

Potato virus Y (PVY) is one of the more economically important viruses of tobacco (*Nicotiana tabacum* L.) in Zimbabwe. Previously, this debilitating disease was controlled by cultural practices which are no longer being strictly adhered to resulting in its upsurge. This situation is compounded by the unavailability of PVY resistant varieties in Zimbabwe. The use of genetic resistance to PVY is a promising strategy that confers effective protection, is cost effective and environmentally benign. A backcross breeding programme was initiated using TB4 (dominant resistance gene) as the donor parent and four elite but PVY susceptible flue-cured parentals (XS,

ONC, XZ and K326) as recurrent parents. Six elite and popular cultivars (KRK26, T72, T73, T74, T75 and T76) were used as susceptible controls and Virgin A Mutant (VAM) tobacco line as a resistant control. PVY inoculum was routinely prepared from 20 different plants exhibiting typical PVY symptoms and used to screen progenies. Additionally, progenies were screened using a polymerase chain reaction (PCR) with primers designed to amplify a plant endogenous eukaryotic translation initiation factor gene (*eIF4E*). After six backcross generations, the lines being developed for PVY resistance had between 3-4% PVY incidence compared to 74.7-99% incidence of susceptible controls. PVY strain distribution was between 5-10% and 2-5% mosaic and necrotic strains, respectively, while for the susceptible controls strain distribution approximated 50% for each strain in field tests under artificial inoculation. The VAM resistant line had PVY incidence and strain distribution of that of developed PVY resistant lines. Of the four developed PVY resistant lines, two lack the *eIF4E* gene while the other two have this gene present. Resistance to PVY in these developed lines may, therefore, be mediated through the absence or mutated form of the *eIF4E* gene. The bred PVY resistant lines will be used to constitute hybrids with some level of resistance to common PVY strains in Zimbabwe.

AP 53

A resistance-breaking potyvirus uses a eukaryotic translation initiation factor different from that of a non-breaking virus: evidence obtained from interaction of potato virus Y and *Nicotiana tabacum*

TAKAKURA Y.; SHINJO A.; UDAGAWA H.; KOGA K.

Japan Tobacco Inc., Leaf Tobacco Research Center, 1900 Idei, Oyama, Tochigi 323-0808, Japan

Success of the viral infection cycle depends on complex interplay between components encoded by viral and host genomes. Potyviruses use host eukaryotic translation initiation factors (eIFs) for their infection cycle. Many reports have described that impaired eIFs in host plants confer enhanced resistance (reduced susceptibility) to potyviruses. However, no eIF that interacts with a host resistance-breaking (RB) potyvirus has ever been reported. In *Nicotiana tabacum*, Virgin A Mutant (VAM) has been used as a genetic resource for resistance to potato virus Y (PVY). The resistance is conferred by a recessive locus “va” that has been shown recently to result from the loss of *eIF4E-S* gene. In recent years, a strain of PVY that breaks the VAM resistance has been reported worldwide. Results of this study show that an RB strain of potato virus Y (PVY-RB) uses *eIF(iso)4E*, although PVY uses *eIF4E* in *N. tabacum*. Therefore, an isoform of eIF plays a key role in the emergence of resistance-breaking PVY. The loss-of-function of *eIF(iso)4E-T* gene conferred enhanced resistance to PVY-RB, although that of *eIF(iso)4E-S* gene did not. This homeologous gene-specific resistance implies that PVY and PVY-RB recruit each specific eIF in *N. tabacum* for their infection cycle. To our knowledge, this report is the first demonstrating that a host resistance-breaking potyvirus uses a eukaryotic translation initiation factor different from that of a non-breaking virus.

AP 54

The regulation of autophagy of susceptible *Nicotiana tabacum* under tobacco mosaic virus infection

YANG Jinguang(1); LIU Xaingfu(1); LIU Wei(1); QIAN Yumei(1); ZHANG Jing(2); LI Wei(2); DONG Shifei(2); WANG Yaofeng(3); HUANG Mingdi(4); WANG Fenglong(1)

(1) Tobacco Research Institute, Chinese Academy of Agricultural Sciences, Key Laboratory of Tobacco Pest Monitoring Controlling & Integrated Management, Qingdao 266101, P.R. China
(2) Hongyun Honghe Tobacco (Group) Co., Ltd., Kunming 650231, P.R. China
(3) Qingyang Branch of Gansu Province Tobacco Company, Xifeng 745000, P.R. China
(4) Longnan Tobacco Company, Gansu Tobacco Cooperation, Wudu 746000, P.R. China

Autophagy (or self-eating) is an important conserved process by which eukaryotic cells recycle intracellular components. This study was undertaken to elucidate whether tobacco mosaic virus (TMV) infection on tobacco plants induced autophagy. TMV-U1 and its susceptible host *Nicotiana tabacum* cv. Blight yellow was used in this study. Transmission electron microscope (TEM), monodansylcadaverin (MDC) staining, an autophagosome marker enhanced cyan fluorescent protein (ECFP)–tagged tobacco ATG8f (ECFP-ATG8f), real-time fluorescent quantitative (qRT-PCR) and real-time fluorescent quantitative (qRT-PCR) assay were used to investigate the formation of autophagic structures, acidic compartments, and the transcript levels of autophagy related genes, including ATG3, ATG4, ATG5, ATG6, ATG7, ATG8a and ATG18a, in tobacco plants infected by TMV, respectively. A number of autolysosome-like structures were observed in the cytoplasm and vacuole of tissue in 72 hpi by TEM. Strong punctate MDC-stained autolysosomes in the cells were observed in the cells in 72 hpi. The expression levels of all these ATG genes, except for ATG5, were increased at 48 hours post inoculation (hpi), and peaked at 72 hpi. These results suggest that TMV infection induced cell autophagy of the susceptible host tobacco.

AP 55

Colonisation of *Ralstonia solanacearum* on tobacco roots and factors affecting virulence

LI Xiang; LIU Yanxia

Guizhou Academy of Tobacco Science of CNTC, Longtanba Road 29, Guanshanhu District, Guiyang, Guizhou, 550081, P.R. China

Tobacco bacterial wilt, caused by *Ralstonia solanacearum* (*R. solanacearum*), is one of the most serious diseases affecting tobacco cultivation worldwide. To determine the colonisation and virulence factors affecting *R. solanacearum*, we applied green fluorescent protein labelling (GFP; GFP-Rs) to monitor the location and survival dynamics of *R. solanacearum* under different conditions. The population of *R. solanacearum* in the rhizosphere and bulk soil was tested using real-time polymerase chain reaction (qPCR). The response surface methodology (RSM) was used to evaluate the pathogenic threshold concentration of virulent *R. solanacearum*, the optimum temperature and humidity to induce tobacco bacterial wilt, and the effect of *R. solanacearum* on tobacco growth. The results showed that a virulent strain of *R. solanacearum* was isolated from the soil in the Guizhou Province. The GFP-Rs was densely colonised in the root tips and root hairs, and intermittent cells were observed in the root elongation zone or at the point of emerging lateral roots. The GFP-Rs population in the rhizosphere soil was 1.15, 1.33 and 1.42 times higher than the bulk soil at 10, 15 and 20 days after transplantation, respectively. Higher colonisation of *R. solanacearum* was observed on the root surface and in the rhizosphere soil compared with the bulk soil. The highest incidence of wilt was 91.13%, which occurred when the population of *R. solanacearum* reached $10^{6.82}$ CFU/g in the soil, the environmental temperature was 30.55 °C, and the humidity was above 81.42%. In conclusion, increased colonisation of the GFP-Rs was observed in the rhizosphere soil compared to the bulk soil, which was related to the population of

the pathogen, the environmental temperature and the humidity in the soil. These three conditions determined whether the bacteria would induce tobacco wilt. This is the first study to investigate factors affecting the virulence of a tobacco wilt bacterial pathogen, which is important for conducting a field diagnosis and biocontrol of tobacco bacterial wilt.

AP 56

Heat generation from common Eucalyptus species used for tobacco curing in Zimbabwe

MUNANGA W.

Tobacco Research Board, P.O. Box 1909, Harare, Zimbabwe

Tobacco curing has been labelled as a major contributor to deforestation in Zimbabwe. This problem arose as a result of the drastic increase in the number of small scale tobacco growers from a mere 1 500 in 1999 to well over 87 000 in 2015. Most of the small scale tobacco growers rely on indigenous wood species for tobacco curing, which exerts pressure on forests. In an effort to promote sustainable tobacco production, several strategies by the tobacco industry players such as the use of alternative fuels, development of energy efficient barns and the Wood Energy programme have been put in place. Amongst these interventions, there has been a major drive to encourage small scale growers to establish their own wood lots for tobacco curing. Fast growing Eucalyptus species such as *E. grandis*, *E. cloeziana*, *E. camaldulensis* and *E. tereticornis* have been the most popularly grown in Zimbabwe. However, despite their use in tobacco curing, no work has been conducted to ascertain the heat generation efficiency of these species. This will assist growers in selecting suitable species to establish. The objective of this study was, therefore, to evaluate and avail information on growth, biomass energy and heat value of these four species. The source of wood was a 4 year eucalyptus plantation where all four species had been established. Before the start of the curing trials, the calorific value of each species was determined by using a bomb calorimeter. The curing trials were conducted in 400 clip capacity rocket barns and all wood used was first weighed before use. Results show that all species gave good cures after 7 days. However, *E. cloeziana* generated the most heat followed by *E. tereticornis* and are thus a cost effective option. The implications of these results are discussed.

AP 57

A solar energy collector integrated with central heating supply for tobacco curing

WANG Jianan(1,2); YANG Gaihe(2)

(1) Henan Agricultural University, 95 Wenhua Road, Zhengzhou 450002, P.R. China
(2) Northwest A&F University, Yangling 712100, Shaanxi, P.R. China

As a supplementary heat source to the central heat supply of the group of 20 bulk curing barns, solar collectors were constructed on roofs of barns with a 50 m^3 hot water storage tank to provide heating for tobacco curing. The heat collectors made of vacuum tube were connected to the storage tank by pipes and pumps, and the area of heat collecting totalled 396 m^2. To investigate its performance, the solar hot water system was utilised to provide tobacco curing heat from 5 July-25 September during the year 2014-2015. The daily conversion efficiency of solar energy to hot water energy ranged within 65-67%. From 10 am to 2 pm per day and with sunny or partly cloudy weather, hot water with more than 75 °C could be derived from hot water storage tank to bulk curing barns. In the curing season, the curing energy provided by the solar energy in one curing season accounted for 17.66 of the total energy consumption that could be supported by the boiler hot water heating system. They can also be used for drying mushrooms and other

products during the off-season. The study provides a strategy for saving energy and environmental conservation in the construction of large bulk curing barn groups by integrating solar hot water with central heaters.

AP 58

A first French tobacco energy and greenhouse gas assessment with the EGES® method

FONTAINE B.; TAILLEUR A.; WILLMANN S.

ARVALIS - Institut du végétal, La Tour, 24100 Bergerac, France

The recent COP 21 agreement (Paris – December 2015) has, once more, highlighted climatic and global warming issues. Statistics revealed that, in France, agriculture contributes to 21% of the national total amount of greenhouse gas (GHG) emissions (2011). What about the tobacco crop impacts on GHG?

France Tabac ordered a study conducted by ARVALIS (2013) to have an idea of the tobacco crop contribution towards GHG. EGES® is a tool based on the life cycle analysis method to calculate the balance between inputs (fertilisers, chemicals, fuel, etc.) and output flows (yields) estimated on tobacco fields and the whole crop rotation. The study was conducted on two representative farms (air-cured Burley and flue-cured Virginia) and compared to a soft wheat reference. All data was converted into CO_2 tonne equivalency using international and standardised databases.

The first results show that:

- on the field scale, one hectare of flue-cured tobacco has the same footprint as soft wheat, whereas Burley emissions are twice as high, due to fertiliser amount and to nitrogen demineralisation, which is an important source of N_2O, a gas with a global warming potential 300 times greater than CO_2.
- if the curing process is added, the flue-cured tobacco footprint is widely penalised due to the fossil gas used. In this case, the curing process has a higher impact than the field one. At the same time, the air-curing process does not contribute to GHG emissions, and finally, explains why Burley has a total impact lower than the flue-cured tobacco.

Thanks to these results, France Tabac and ARVALIS are able to focus on the main GHG sources to implement trials, training or action plans to improve critical crop operations.

AP 59

Energy saving and renewable energy production for a more sustainable tobacco crop

MILLI G.(1); BARGIACCHI E.(2); MIELE S.(2)

(1) Fattoria Autonoma Tabacchi (FAT) & ITT, I-06012 Città di Castello, Italy
(2) Italian Interuniversity Consortium for Science and Technology of Materials (INSTM), I-50121 Firenze, Italy

Energy costs of tobacco, especially for curing and threshing, are a major expense and a potential threat to crop sustainability. Since 2006, an intensive program of renewable energy has been under development both at FAT, and the network of OPTA Cooperatives. At the same time, every energy-consuming operation through the crop production chain was revised, to maximise efficiency. The aim of this programme was not only to reduce energy waste and produce non-fossil energy, but also to make growers more conscious of the importance of being energy-

efficient, becoming themselves energy suppliers. Over the years, the threshing line and all the curing barns have been upgraded with better insulation and computer-assisted programs, not mentioning the use of more energy efficient tractors and self-propelled equipment in the fields. Photovoltaic plants were installed, for the production of power (2,860,000 kW/y for 8 units, each one with 45 barns, and the factory). Two anaerobic digestion plants, cogenerate power (15,760,000 kW/y) and heat, the latter for running 34 barns. Three wood combustion plants produce heat for 162 barns and a small municipality. Growers were directly involved in production of silage crops for the anaerobic digestion plants, and collection of their wood from coppice and forest maintenance programmes, with more opportunities of income. Most of the farmers also installed photovoltaic and small combustion plants. Presently >65% of the yearly energy consumption of tobacco derives from renewable energy. However, this is a starting point for the pursuit of more efficient new technologies that lie ahead: combined charcoal-power production, biomass gasification, HydroThermal Liquefaction, and so on.

AP 60

Application of biomass moulding fuel to flue-cured tobacco furnaces: efficiency and cost effectiveness

REN Tianbao(1); YANG Chao(2); WANG Jianan(1); CHEN Yiyin(1); WANG Huanhuan(1); LIU Guoshun(1)

(1) Henan Agricultural University, China National Tobacco Cultivation & Physiology & Biochemistry Research Centre, Zhengzhou 450002, P.R. China
(2) Chongqing Tobacco Science Research Institute, Chongqing 404100, P.R. China

Biomass moulding fuel (BMF) is an environmentally friendly and renewable energy source. We studied an independently-developed automatic flue-cured tobacco furnace, employing a biomass fuel source and studied the effect of BMF on tobacco flue-curing. We found that 1 kg of dried tobacco required 1.2 kg of BMF (heating value = 3550 kcal/kg). The energy input cost was 1.43 RMB (fuel and electricity) and the labour cost was reduced by 75%, a reduction of 18.6% compared to the cost of burning coal. With respect to the environmental impact, the average emission concentration of smoke in the exhaust gas from the furnace was 16.2 mg/m^3, SO_2 was 13.6 mg/m^3, and NOx was 2.3 mg/m^3. Ringelmann blackness was less than 1. Compared to burning coal, all emissions were very low, demonstrating that the BMF furnace saved energy and reduced emissions compared to coal. In addition, the quality of cured tobacco and economic index were significantly improved.

AP 61

Lower leaf removal to reduce lower stalk grades of flue-cured tobacco

FISHER L.R.(1); VANN M.C.(1); INMAN M.D.(1); BROWN A.B.(2)

(1) North Carolina State University, Dept. of Crop Science, Campus Box 7620, Raleigh, NC 27695, U.S.A.
(2) North Carolina State University, Department of Agriculture & Resource Economics, Raleigh, NC 27695, U.S.A.

At present, interest has been expressed by the tobacco industry in regards to lower leaf removal programs designed to eliminate lower stalk grades (Priming and Lug) in flue-cured tobacco. Research was conducted in North Carolina to evaluate removal of zero, four, or eight leaves at topping and the effect on yield, quality, leaf chemistry, crop throw, and value per acre. Removal of four leaves resulted in minimal loss of yield (3-12%) and value, but did not consistently eliminate Priming (P) and Lug (X) grades. Alternatively, removal of eight leaves consistently reduced yield (21-30%) and value but generally did eliminate P and X grades. Leaf removal had

no effect on leaf chemistry. In addition, economic analyses were conducted to account for all leaf removal scenarios. Profit declined in both the four leaf and eight leaf removal systems by 1,516 and 2,442 USD per hectare, respectively. Research in 2016 and 2017 will evaluate agronomic practices, such as nitrogen application following leaf removal and leaf removal timing, to improve yield when the bottom eight leaves are removed from each plant.

AP 62

Effect of curing conditions on amino acids induction among flue-cured and Burley leaf

NAKAYAMA H.; NAGAI A.

Japan Tobacco Inc., Tobacco Science Research Center, 6-2 Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan

A comprehensive understanding of changes which are induced in the composition of amino acids in tobacco leaves during curing is indispensable for the quality control of tobacco leaves, since amino acids are amongst the key components relating to the aroma/taste of cigarette smoke. This study is intended to elucidate the effect of curing conditions and tobacco leaf types on the dynamic profiles of amino acids induced during curing.

Flue-cured Virginia (FCV) and Burley (BLY) were cured with two methods, a JT typical barn or a modified barn, in which the temperature and humidity of air were each kept at a constant value (38 °C and 85%R.H.), the same as FCV yellowing conditions. A proportion of the cured leaves was taken out of the barn at fixed intervals during the curing to measure their amino acid profiles. Amino acids were extracted with H_2O/Methanol solution, and this was followed by a quantitative analysis with a CE-ESI-MS system.

Induction patterns of amino acids were dependent on curing conditions, and amino acid profiles showed significant differences between FCV and BLY type leaves, even though these samples were treated under the same curing conditions. Proline and asparagine are known to be high volume components of the cured FCV and BLY type leaves, respectively. Dynamic ranges of these amino acids were completely different between FCV and BLY, while the induction patterns of both amino acids had similar tendencies throughout the curing process. The other metabolites and/or leaf types, such as Oriental leaf, should be analysed in the future to support a more precise interpretation of amino acid induction.

AP 63

After-ripening improves germination performance in Zimbabwean flue-cured tobacco varieties

RUKUNI D.; MUDZENGI T.D.

Tobacco Research Board, P.O. Box 1909, Harare, Zimbabwe

Optimum seed germination is essential for the establishment of a successful tobacco transplant nursery. However, fresh tobacco seeds are dormant and require a period of warm dry storage (after-ripening) to alleviate dormancy and elicit maximum germination. After-ripening is known to improve germination in seeds with non-deep physiological dormancy such as tobacco seeds, but the period required for dormancy to diminish can be variety dependent. Also, in other seeds such as tomato, hydrolase enzyme activity is involved in the weakening of the endosperm, a phenomenon that facilitates germination. This study was carried out to determine the after-ripening duration for five Zimbabwean flue-cured tobacco varieties (K RK26R, K RK29, K RK66,

T71 and T72) and also, to ascertain if this was linked to increased enzyme (Class 1 ß-1, 3-glucanase (ßGlu I)) activity in the endosperm. Fresh seeds were stored at either 30 or 5 °C and germination tested every four weeks for 26 weeks. Germination stimulation methods such as dry heat treatment (DHT), gibberellic acid (GA) or hydrogen peroxide were tested for their effect on germination. Seeds were germinated under light/dark conditions at 30 °C. For enzyme activity assays, ßGlu I activity was determined in T71 and K RK26R seeds using spectrophotometry at 8 weeks of storage. Results showed that storage of seeds at 30 °C hastened after-ripening more than when seeds were stored at 5 °C. Gibberellic acid further improved germination for seeds stored at both temperatures, and dry heat treatment slightly improved germination at all times. ßGlu I activity was high in treatments that produced improved germination, such as GA, signalling the overcoming of dormancy. Storage of seeds at 30 °C for at least 6 months is therefore recommended to accelerate after-ripening in tobacco seeds. Additionally, GA and DHT can be used in seed priming to enhance germination in partially after-ripened seeds.

AP 64

Moisture effect to microbial diversity in aging tobacco leaves

MOCHIZUKI D.; TAGUCHI H.; NAGAI A.

Japan Tobacco Inc., Tobacco Science Research Center, 6-2, Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan

The aging process, long-term leaf preservation before cigarette production, improves the aroma and taste of non-aged tobacco leaves. To understand the reaction mechanism of the chemical change in the aging process, various kinds of studies have been conducted. Research in recent years has shown that the aging process affects the microbial community in leaves, and implies that microbes are related to the chemical changes. In this study, to understand the effect of moisture on the microbial community during the aging process, the microbial diversity of leaves conditioned at low and high moisture levels was analysed.

Flue-cured Virginia (FCV), cultivated and cured in Japan, was used for this test. The leaves were conditioned under low (40%RH) or high humidity (87%RH) at 22 °C for 48 h, which adjusted them to a moisture content of 6.7% or 23.7%, respectively. The leaves were then aged at 22 °C for 6 months. The changes in bacterial count and community in each leaf before and after aging were evaluated by enzymatic total viable count (TVC) testing and metagenomic analysis with a Next Generation Sequencer.

While no differences in bacterial count and community were found in low-moisture leaves on TVC testing, significant differences were found in high-moisture leaves when aged leaf was compared with non-aged leaf. Metagenomic analysis also showed that the bacterial community in high-moisture leaves was clearly different between aged and non-aged leaf. Bacterial species detected in the high-moisture leaves were approximately twice as many in the aged leaf as those in the non-aged leaf.

It was therefore concluded that high-moisture conditions affect the microbial community in aging leaves more than low-moisture conditions do.

AP 65

Management of Orobanche in flue-cured tobacco in India

REDDY B.S.R.; SWAMY S.; MANI M.

ITC Limited - Agri Business Division-ILTD, Research Department, Rajahmundry 533103, Andhra Pradesh, India

Orobanche (*Orobanche cernua* Loefl.) is a serious root parasite infesting tobacco roots in Northern Light Soil (NLS), Traditional and Mysore tobacco growing regions of India. The yield losses were ranging from 30-70% and plants show the symptoms of paleness with necrotic leaf tips and lamina.

The management practices in India include hand removal of Orobanche stems. Trials were conducted to identify better options for the control of Orobanche. Randomised block design experiments were conducted using amino acid as root dip and post emergence sprays of herbicides for the management of Orobanche in flue-cured Virginia (FCV) tobacco growing areas. The treatments include Methionine as seedling root dip, Imazethapyr (10% SL), paraquat dichloride (24sl) 0.5% and eco-friendly treatment of salt solution and vinegar (5%) as post emergence sprays at 55-70 days after planting. Seedling root dip for 15 minutes in methionine (3 mM solution) before planting has reduced the incidence of Orobanche by 73%. Imazethapyr (10% SL) at 0.15% spray concentration applied at 50-70 days after planting reduced the recurrence of Orobanche stem number by 79.2%. Growing gingelly with a seed rate of 7.5 kg/ha reduced the incidence of Orobanche by 81%. Paraquat dichloride (24sl) and vinegar (5%) + salt solution killed the Orobanche plant within 24 hrs of spray treatment.

The integrated Orobanche management approach includes: a) crop rotation with gingelly; b) seedling root dip with amino acid methionine; c) post emergence spray application of herbicides paraquat dichloride & imazethapyr; d) post emergence spray application of vinegar + salt solution.

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POSTERS

APPOST 01

Polyphenols metabolism is regulated by phytochrome B gene *NtPHYB1* in tobacco leaf

ZHAO Jiehong; LIU Yanxia

Guizhou Academy of Tobacco Science of CNTC, Longtanba Road 29, Guanshanhu District, Guiyang, Guizhou, 550081, P.R. China

Plant polyphenols play important roles in defense against pathogens, promoting production quality, and improving human health. Light is an important external environmental factor that greatly impacts on the polyphenols metabolism, whereas the impact mechanism remains unknown. In order to investigate whether light receptors participate in the regulation of polyphenols metabolism in tobacco (*Nicotiana tabacum*) leaf, a *Phytochrome B* homolog, *NtPHYB1*, was isolated from *N. tabacum* cv. K326, and its function on polyphenols metabolism was carried out by over expression and RNAi approaches. Consistent and complemental results indicated that *NtPHYB1* is involved in polyphenols metabolism in tobacco leaves, and the high level of *NtPHYB1* transcripts is in favour of accumulation of chlorogenic acid and its isomers, all of which are key components of tobacco polyphenols. To understand the regulation mechanism of *NtPHYB1* in the secondary metabolism pathway, transcriptome analysis was carried out. Compared with WT, 1665 and 1421 genes are found differentially-expressed in *NtPHYB1-GFP* and *NtPHYB1*-RNAi transgenic lines respectively. Among them, about 30 genes were related to phenylpropanoid biosynthesis, which is the pathway for polyphenols biosynthesis. Further evidence from quantitative RT-PCR confirmed that *NtPHYB1* may control polyphenol metabolism through regulating the transcription of *PAL4* (*phenylalanine ammonialyase 4*), *4CL1* (*4-coumarate: coenzyme A ligase 1*) and *COMT* (*caffeic acid 3-O-methyltransferase*) genes. Our results indicated that phytochrome B is involved in regulating polyphenol metabolism in tobacco leaves. This perhaps provides a novel clue on the regulation mechanism and a strategy to improve the polyphenols accumulation.

APPOST 03

Molecular cloning and expression analysis of *NteIF2α* in response to stress in *Nicotiana tabacum*

ZHAO Qi; LI Sibin; LI Ning; GUO Hao; LONG Yue; CUI Hong; YANG Yonxia; JIA Hongfang; ZHANG Hongying; ZHANG Songtao

Henan Agricultural University, College of Tobacco Science, Tobacco Cultivation Key Laboratory of China Tobacco, Zhengzhou 450002, P.R. China

eIF2α (a subunit eukaryotic translation initiation factor 2) plays an important role in the regulation of protein synthesis. The phosphorylation of *eIF2α* could lead to down-regulate global protein synthesis. To investigate the function of *eIF2α* and explore its roles in plant stress response, the ORF (Opening Read Frame) of *NteIF2α* was cloned from *Nicotiana tabacum* L cv. K326 by RT-PCR and the mRNA expression level in different tissues and stress conditions were analysed by qRT-PCR method. Our results showed that there were two copies of *NteIF2α* in *N. tabacum* K326, *NteIF2α-1* (GenBank accession number: KR184725) and *NteIF2α-2* (GenBank accession number: KR184726). The nucleotide sequences shows 98.3% similarity, while the protein sequences show 99.7% similarity to each other. Also, *NteIF2α* contains the conserved domain of *eIF2α* which includes kinase interaction site, phosphorylation site and *eIF2B* interaction site. Further, phylogenetic analysis revealed that *NteIF2α-1* and *NteIF2α-2* might be derived from its progenitor *Nicotiana sylvestris* and *Nicotiana tomentosiformis*, respectively. Twenty-four phosphorylation sites were predicted in the protein sequence. Among them, the Ser56 which was the phosphorylated site of protein kinases was highly conserved from animals to plants. In addition, the expression pattern was detected in different organs of *N. tabacum* K326. The results showed *NteIF2α-1* and *NteIF2α-2* were expressed in all organs examined and the expression profiles in *N. tabacum* displayed differently, because the expression of *NteIF2α-2* was higher than that of *NteIF2α-1*, which indicates the function of the *eIF2α* family members were different in the organs. Finally, the activation of *NteIF2α* by SA (salicylic acid) and MeJA (methyl jasmonate) suggests that *NteIF2α* participates in the plant defense response to insects. Our results provide an important basis for further studying the function of *eIF2α* and its roles in plant stress response.

APPOST 07

Effects of topping and spraying exogenous hormone on tobacco gene AN

WANG Jing; YANG Huijuan; SHI Hongzhi; LIU Guoshun

Henan Agricultural University, College of Tobacco Science, Key Laboratory for Cultivation of Tobacco Industry, Zhengzhou 450002, P.R. China

To solve the problem of the narrower and thicker upper-tobacco leaves in southwest tobacco planting areasin China, the influence of topping and spraying exogenous hormones (GA and NAA) on agronomic traits and mRNA expression levels of S type (NtAN-S) and T type (NtAN-T) of the Angustifolia (AN) gene subfamily related with the leaf width were studied in K326 and Yunyan 97 upper leaves during different periodd. Firstly, the length and width of the fourth leaf from topping in different treatments were measured on the day of topping and 5, 13 and 20 days after topping. Secondly, based on the cloned tobacco AN genes sequence in our previous study and the internal reference gene actin sequence on lane, primers of AN genes and internal reference actin gene were designed for fluorescent quantitation PCR (real-time PCR). The relative expression quantity of different treated upper leaf AN genes at topping and 2, 6, 9, 24 and 36 hours, and 2, 3, 5 and 7 days after topping were measured by real-time PCR. In comparison with untopped tobacco, topping promoted the extension of the K326 and Yunyan 97 upper leaves, but the extension capability was limited. The leaf width of these two tobacco varieties significantly increased by spraying exogenous hormones GA and NAA. For Yunyan 97, the AN genes expression of upper leaves was promoted by topping and reached a peak in two days after topping. The maximum AN genes expression quantity appeared in advance by

spraying the exogenous hormones GA and NAA and occured nine hours and 24 hours after topping, respectively. For K326, the AN genes expression of upper leaves was also promoted by topping and reached a peak at 36 hours after topping. For K326 variety, topping also promoted the expression of upper leaves' AN gene, and reached a peak 36 hours after topping. The maximum AN genes expression quantity was further promoted by spraying the exogenous hormones GA and NAA. These research results showed that the AN genes could regulate the shape of upper tobacco leaves and this regulation varied according to the tobacco varieties.

APPOST 08

Agronomic performance of cytoplasmic male sterile forms of flue-cured tobacco

CZUBACKA A.; DEPTA A.; DOROSZEWSKA T.

Institute of Soil Science and Plant Cultivation - State Research Institute, Department of Plant Breeding and Biotechnology, ul. Krancowa 8, 24 100 Pulawy, Poland

Cytoplasmic male sterility (cms) in many crops is the basis for the production of commercial F_1 hybrids. This is obtained by the combination of the nucleus of cultivated species with the cytoplasm of a wild species belonging to the same genus. The reason for the loss of fertility is the incompatibility of nuclear and cytoplasmic genes. Within the genus *Nicotiana* several species have been found to be carriers of cms genes which become active as a result of the interaction of the *Nicotiana tabacum* nucleus with the cytoplasm of a wild species. Because of male sterility, there is no need to remove male organs to prevent self-pollination. Moreover, the distribution of cytoplasmic male sterile forms allows protection of the breeder's interests.

However, the presence of cytoplasm originated from other species influences not only fertility so alloplasmatic forms can differ from an initial cultivar in terms of a number of features.

Therefore, we compared the main agronomic traits of nine alloplasmic forms of the flue-cured cultivar Wiślica, which is popular in breeding programmes. The experiment was conducted under field conditions.

Morphological evaluation included such traits as: plant habit; plant height; shape, colour and size of leaves, shape and colour of flowers and inflorescences, and shape of seed bags. The earliness of the alloplasmic forms and the ability to develop suckers were also taken into consideration.

The results showed that there are some differences among cms forms. Individuals Wiślica cms *amplexicaulis* were approximately 20 cm higher than Wiślica cms *raimondii*. However, the latter ones had relatively more numerous leaves and long flowers. In turn, the plants with *N. goodspeedii* cytoplasm were characterised by large leaves while those with cytoplasm originated from *N. undulata* had smaller leaves. We observed also differences in the development of suckers. However, the plant habits were similar for studied cms forms.

The results have provided information which may be helpful in planning breeding programmes to obtain cms forms.

APPOST 09

Performance of new breeding lines of flue-cured tobacco and their test-hybrids for economic traits

BUDIMIR A.(1); ŠARCEVIC H.(2); KOŽIĆ K.(1)

(1) Hrvatski duhani d.d. Virovitica (Croatian Tobaccos Inc.) / British American Tobacco (BAT), Osjecka 2, 33000 Virovitica, Croatia
(2) University of Zagreb, Faculty of Agriculture, Svetošimunska 25, 10000 Zagreb, Croatia

During the 2014 and 2015 growing seasons a field trial with ten new breeding lines of flue-cured tobacco, their test-hybrids with two line testers, and two controls (the hybrid cultivar DH17 and the line cultivar NC55) was conducted in five environments (year-location combinations) in Croatia. The cultural practice applied was as recommended for commercial flue-cured tobacco production. After harvesting and curing, leaf yield (kg/ha^{-1}), price (\$/kg^{-1}) and value (\$/ha^{-1}) were determined. Analysis of variance across environments revealed significant differences among genotypes for all traits. Compared to the corresponding breeding lines four, 15 and 10 test-hybrids had significantly higher yield, price and value, respectively. Mean yield of five breeding lines and 12 test-hybrids were significantly higher compared to the mean yield of the higher yielding control DH17. All breeding lines and nine test-hybrids had significantly lower price as compared to the better control NC55, whereas the price of the remaining eleven test-hybrids was not significantly different from the control. Mean value of six breeding lines and 17 test-hybrids was significantly higher compared to the mean value of the better performing control DH17. The value of the best four hybrids was 11-16% higher than the value of the better control.

APPOST 10

Breeding for disease resistant dark-fire-cured tobacco in Malawi

CHAMANGO A.M.Z.; GOMONDA R.W.J.; MAINJENI C.E.D.; MSANGOSOKO K.R.; KHUMBANYIWA A.G.; KASANGA T.

Agricultural Research and Extension Trust (ARET), Private Bag 9, Lilongwe, Malawi

Dark fire-cured tobacco (DFC) is the third most important tobacco type grown in Malawi after Burley and flue-cured. With Malawi Western, MW 86-57 and AWL 28 as the leading approved DFC varieties for production in Malawi, the local and regional requirements are well supplied despite the genetic deficiencies that come with them. Such genetic deficiencies include lack of resistance to root-knot nematodes (*Meloidogyne* sp.), fusarium wilt (*Fusarium oxysporum*), bacterial wilt (*Ralstonia solanacerum*), angular leaf spot (*Pseudomonas syringae* pv *tabaci*) and alternaria brown leaf spot (*Alternaria alternata*). In a country where farm-served seed is a common practice, including that of open pollinated tobacco varieties, development of male sterile varieties has become urgent in order to comply with good agricultural practices (GAP) and ensure seed integrity and traceability by discouraging tobacco seed recycling. Consequently, a breeding programme involving the transfer of resistant genes from the flue-cured and Burley environments through backcross breeding started in the early 1990s in order to develop multiple disease resistant parental breeding lines that would later facilitate the development of the first group of male sterile Malawi DFC hybrids with resistance to nematodes, Fusarium wilt, Bacterial wilt, Angular leaf spot and Alternaria brown leaf spot. This report summarises data of the recent development of the DFC tobacco improvement programme by assessing disease ratings, leaf yields and quality of the first promising group of the hybrids to emerge from this long term conventional breeding. Recent results showed that two of the several hybrids have expressed good resistance, albeit at varied levels, to nematodes, bacterial wilt, fusarium wilt, angular leaf spot and alternaria. Further breeding is continuing to build on the genetic gains that earlier breeding efforts have achieved so that more superior parental lines are harnessed for development of even better hybrids in the near future for use by growers in Malawi.

APPOST 12

Evaluation of foliar fertilisers for tobacco production in Zimbabwe

CHINAMO D.; MATOKWE M.; RUKUNI D.

Tobacco Research Board, P.O. Box 1909, Harare, Zimbabwe

Adequate fertilisation is critical in producing healthy seedlings and a high yielding, quality field crop. Soil applied fertilisers are the most commonly used in tobacco production while water applied soluble fertilisers are used in the float system. However, in the last decade, there has been an influx into Zimbabwe of a wide range of foliar fertiliser products. These products originally targeted for use in horticultural crops have been extensively marketed for use in commercial tobacco production. To protect tobacco growers and in fulfilment of the Tobacco Research Board's Pesticide Approval Scheme Service, all foliar fertiliser products have to be evaluated before use. The main objective of these trials was to evaluate the suitability of foliar fertilisers for transplant production and improvement of leaf yield and quality. Since 2008, a total of 12 foliar products were evaluated both in the seedbed and in the field. Results in transplant production showed that seedling growth, development and dry matter accumulation were significantly lower when foliar fertilisers were solely used than the standard fertiliser programme. Nitrogen and phosphorous deficiency symptoms were evident, and seedling dieback was observed for some foliar fertilisers due to extreme nutrient deficiency. When foliar fertilisers were used as supplements, transplant production costs escalated but with no significant improvements in transplant quality. In the field, foliar products were also ineffective. This paper discusses in detail the range of foliar products tested and the results obtained.

APPOST 14

Addressing calcium deficiency in flue-cured tobacco

VANN M.C.; FISHER L.R.; INMAN M.D.; WHALEY W.T.

North Carolina State University, Dept. of Crop Science, Campus Box 7620, Raleigh, NC 27695, U.S.A.

With the exception of potassium and nitrogen, calcium (Ca) accumulation by flue-cured tobacco is greater than all other nutrients. Despite such high demand, Ca application is not recommended to well-limed soils. In 2015, Ca deficient tobacco was visually and analytically detected across much of North Carolina. Research efforts to correct Ca deficiency have resulted in little or no level of confirmed success. To evaluate the effect of foliar applied liquid Ca, research was initiated in 2015 at a single field site in the Coastal Plain of North Carolina. Ten percent liquid Ca was applied through a solution volume of 187 L/ha at 5.6, 11.2, and 22.4 kg Ca/ha. The same material was also applied at 11.2, 22.4, 33.6, 44.8, and 56.0 kg Ca/ha through a solution volume of 468 L/ha. An additional treatment of 0 kg Ca/ha was included as a control. All applications were delivered five days after topping. Plant tissue and soil samples were collected prior to topping to establish baseline estimates for Ca availability and plant utilization. Additional tissue samples were collected one and three weeks after treatment. Tobacco yield from upper stalk positions and leaf quality were quantified by following final harvest. Calcium content increased with Ca application rate; however, yield was not increased beyond that in the non-treated control. It is theorized that increased Ca content was a result of Ca crystallizing on the surface of treated leaves. Additionally, significant injury was observed in all Ca treated plots, indicating that the salt indices of the various treatments were too great for tobacco to withstand. Lastly, the cost of the chosen Ca material was prohibitive as material cost per application ranged from 104 to 1,041 USD/ha. Ultimately, producers should utilize Ca reserves within the soil profile and practice early topping to promote suitable root growth for nutrient utilization.

APPOST 16

Effects of biochar on soil quality and tobacco growth during four years of consecutive application

LI Jiangzhou(1); JI Sigui(1); ZHANG Limeng(1); QIAO Zhixin(1); ZHANG Qingzhong(2); DAI Kuai(1); JIAO Yongge(1); SHA Chuanping(1)

(1) Yunnan Tobacco Company, Yuxi Branch, Fenghuang Road No. 102, Yuxi City, 653100, P.R. China
(2) Institute of Environment and Sustainable Development in Agriculture, Chinese Academy of Agricultural Sciences, Zhongguancun South Street No. 12, Haidian District, Beijing 100081, P.R. China

Background: To understand the effects of biochar application on the quality of crop products as well as how biochar tolerance in different soils can benefit the wide use of biochar.

Methods: In this study, soil physical-chemical properties, yields and output values of flue-cured tobacco leaves were measured during four years of consecutive biochar application at a rate of 0, 15, and 30 t ha^{-1} $year^{-1}$ at two sites with different soil fertility. Chemical properties relevant to quality of tobacco were measured in the fourth year.

Results: The results showed that in the fourth year soil pH was increased by approximately 0.5-0.6 units, while soil bulk density was significantly decreased. Soil organic carbon (SOC) content was significantly increased by 77.7% and 46.7% in Huaning soil with low fertility and Tonghai soil with high fertility, respectively. The increase of SOC contents are attributed mainly to the aggregate-occluded particulate organic matter (oPOM), indicating that biochar-C has been intensively protected by aggregates. Soil mineralised nitrogen (N), available phosphorus (P) and available potassium (K) contents were significantly increased by biochar application, while soil apparent K loss was significantly increased. Apparent N loss was decreased when the cumulative amount of biochar was less than 60 and 30 t ha^{-1} in Huaning soil and Tonghai soil, respectively. However, the value was increased when the cumulative amount of biochar exceeded the two thresholds. Biochar application increased the yield, output value and chemical component contents of flue-cured tobacco, with the maximum increments of yield by 5.1-12.9% and output value by 12.6-19.7% at a cumulative amount of 60 t ha^{-1} biochar. In addition, the quality of tobacco reached the optimal point at this cumulative amount. The low fertility soil responded to biochar more rapidly and showed higher tolerance than the high fertility soil.

Conclusions: In summary, the cumulative biochar amount of 60 and 30 t ha^{-1} appears to be the optimal choice for tobacco production in Huaning soil and Tonghai soil, respectively. When biochar is applied at a considerable high rate, chemical K fertiliser should be reduced accordingly to avoid the risk of non-point source pollution.

APPOST 21

Effective range of a pheromone trap and its installation interval

SASAKI R.; KAGAMI C.

Fuji Flavor Co., Ltd., Ecomone Div., 3-5-8, Midorigaoka, Hamura, Tokyo 205-8503, Japan

The tobacco beetle (*Lasioderma serricorne*) is a notorious pest in stored tobacco. Pheromone traps have been used as a monitoring tool to prevent infestation with the beetles. They should be placed in accordance with a certain installation method to correctly grasp the situation of a site, factory or warehouse, and a method for each pheromone trap is usually fixed by each manufacturer of pheromone traps. An interval between traps is one of the important parameters related to trap installation. A recommended interval of NEW SERRICO, a pheromone trap for the tobacco beetle, is 10 m. However, traps are not always placed at 10 m intervals at a site. The purpose of this study was to clarify influence of intervals on trap efficacy.

NEW SERRICOs were placed at 10 m intervals in a warehouse (20 × 20 m). Three hundred male beetles were released from one point in a room. The catches were counted one week after release. This experiment was replicated three times. A series of the experiments was conducted for traps at 5 m and 20 m intervals. All released experiments were carried out from July to September.

Total catches of all traps at 5 m intervals was the maximum, because the number of traps in a warehouse was the most. But average catches per trap was not different among three placement patterns. It indicated that efficacy per trap is constant regardless of the number of traps in a room.

The results suggest that it is possible to adjust an installation interval with a focus on 10 m depending on the situations of factories or warehouses; nevertheless the interval of 10 m is still recommended, considering that the infestation source is first detected from monitoring data.

APPOST 22

Efficacy of vacuum packaging (VP) and modified atmosphere packaging (MAP) for the control of the tobacco beetle (*Lasioderma serricorne*) in stored tobacco

MASUKWEDZA R.; RUZANE R.; DIMBI S.

Tobacco Research Board, P.O. Box 1909, Harare, Zimbabwe

The tobacco beetle, *Lasioderma serricorne*, is an economically important postharvest pest of tobacco and annually, raw and manufactured tobacco products are lost to this storage pest. In Zimbabwe, the management of established beetle infestations is largely dependent on the use of fumigants, namely aluminium and magnesium phosphide tablets. However, due to the toxicity of fumigant insecticides to both users and the environment, it is important to search for and avail safer and environmentally friendly options. The use of modified atmosphere packaging (MAP) which entails the removal and/or replacement of the atmosphere surrounding the product before sealing in vapour-barrier materials has not been evaluated on tobacco in Zimbabwe. This study sought to investigate the efficacy of MAP in the form of vacuum packaging (VP) and gas replacement for the management of the tobacco beetle in stored tobacco. The VacQPack System (Netherlands) was used. Three treatments T1 (normal packaging), T2 (vacuum packaging) and T3 (a combination of VP and carbon dioxide gas replacement where oxygen was reduced to less than 5% and replaced with three carbon dioxide flushes) were evaluated. Ten adult beetles were placed in each package before treatment application. Thereafter, the packages were stored at three temperature regimes of 25 °C, 28 °C and 30 °C. Beetle mortality was then assessed at day 1 and day 7 after treatment. Results showed that there was no interaction between temperature, packaging and time. However, significant differences were observed in adult beetle mortality where significantly higher mortality was observed in the Vacuum packaging with carbon dioxide flush treatment (95%) as compared to the normal (7%) and vacuum packaging only treatments (78%). Thus both vacuum packaging and modified atmospheres are viable methods of controlling the tobacco beetle in stored tobacco.

APPOST 23

Influence of PVY infection on chemical composition of tobacco

PRZYBYS M.; CZUBACKA A.

Institute of Soil Science and Plant Cultivation - State Research Institute, ul. Krancowa 8, 24 100 Pulawy, Poland

Potato virus Y (PVY) is the type member of the genus *Potyvirus* in family Potyviridae. PVY has a worldwide distribution and is one of the most economically important viruses in tobacco. PVY causes limitation of assimilation, gas exchange, inhibition of water and mineral salts transport to leaf tissues. It affects changes in cell metabolism, resulting in changes in the chemical composition of tobacco, and finally it affects the taste and aroma of cigarettes. The objective of this study was to determine changes in content of nicotine, nornicotine, proteins and reducing carbohydrates in tobacco infected with the PVY. *Nicotiana tabacum* 'Xanthi' plants were grown in a temperature- and humidity-controlled growth-chamber. In the growth-chamber the temperature was 20 °C. Day length was 16h with fluorescent light. Fourteen days after artificial inoculation individual plants were sampled and analysed for alkaloids, proteins and reducing carbohydrates. The control plants were not infected. The artificial inoculation of the plants caused a decrease in average nicotine and nornicotine content by 71% and 9%, respectively. At the same time the conversion of nicotine to nornicotine increased by 47%. The average content of true protein in tobacco leaves 14 days after inoculation decreased to 10.83%, while the sugar content increased by 8% to 15.95% of dry matter. It was found that the virus infection has a significant impact on the chemical composition of tobacco.

APPOST 24

Tobacco breeding for TSWV resistance using RTSW-al factor derived from cultivar Polalta

TROJAK-GOLUCH A.(1); KORBECKA-GLINKA G.(1).; GOEPFERT S.(2); DOROSZEWSKA T.(1).; BERBEC A.(1)

(1) Institute of Soil Science and Plant Cultivation - State Research Institute, Department of Plant Breeding and Biotechnology, ul. Krancowa 8, 24 100 Pulawy, Poland
(2) Philip Morris Products S.A. (part of Philip Morris International group of companies), Quai Jeanrenaud 3, 2000 Neuchâtel, Switzerland

Tomato spotted wilt, caused by the tomato spotted wilt virus (TSWV), causes significant economic losses in the cultivation of tobacco worldwide. The best approach to the management of the disease is growing resistant cultivars. Within *N. tabacum*, the only source of resistance to TSWV is the Polish cultivar Polalta with resistance derived from the wild species *N. alata*. This variety is not cultivated due to poor functional quality. In addition, the F1 progeny obtained by crossing Polalta with another genotype of tobacco reveals malformations of leaves and morphological tumors, which may result from an interaction between tobacco genome and wild species introgressions. A breeding programme, aiming at developing a high quality tobacco cultivar resistant to TSWV, was started at the Institute of Soil Science and Plant Cultivation (Pulawy, Poland). It resulted in obtaining PW-834, PW-900 lines and then double haploid lines: (WGLxPW-834) DH, (WGLxPW-900) DH. Both, PW and DH lines carry a TSWV resistance gene (RTSW-al) derived from Polalta. DH lines show a significantly decreased expression of malformations compared to Polalta and PW lines. Average nicotine content varied among DH lines from 2.49 to 3.33%, while it equalled 3.54% for Polalta. Sugar content varied between 12.03 and 18.63% (Polalta: 13.50%). The next aim of this research was to compare the morphology of F1 hybrids of 14 DH lines in order to obtain the best material for further breeding. Each of the 14 DH lines was crossed with the Polish flue-cured cultivar WAC 121 D7, recognised for the high quality of the leaves. The F1 hybrids were grown in a one-year field experiment set up using a randomised complete block design with three replications. Plants were rated for morphological

deformation using a seven-class rating system. The F1 offspring producing the smallest degree of deformations were self-pollinated to produce F2 seeds.

APPOST 25

Resistance-risk assessment of *Phytophthora nicotianae* to dimethomorph and fungicide combination development

MU Wenjun; XUE Chaoqun; XI Jiaqin; HU Liwei; LIU Fang; SONG Jizhen

Zhengzhou Tobacco Research Institute of CNTC, No. 2 Fengyang Street, High-tech Industrial Development Zone, Zhengzhou, Henan 450001, P.R. China

Dimethomorph is a carboxylic acid amide (CAA) fungicide with high activity against the tobacco pathogen *Phytophthora nicotianae*. We investigated the risk that *P. nicotianae* can develop resistance to dimethomorph and screened the fungicide mixture containing dimethomorph. The sensitivities of 60 *P. nicotianae* isolates collected in 2014~2015 was determined. The EC_{50} value had a unimodal frequency distribution with a mean of 0.3502 µg/mL. There was no significant shift in sensitivity of isolates which were collected in different years and from different locations. These data can be used for resistance monitoring in fields. Eight laboratory mutants, classified into two types, were generated via exposure of sensitive isolates to dimethomorph-amended media. Four mutants with a 2~5 fold resistance factor were low to medium resistance and four mutants had high resistance to dimethomorph with a more than 250-fold resistance factor. The resistant mutants had no less fitness than the sensitive isolates, which means the mutants had the ability to become a dominant subpopulation. The results suggest that the risk of *P. nicotianae* resistance to dimethomorph could be low to moderate. Cross resistance was found between dimethomorph and flumorph, mandipropamid, but not between dimethomorph or metalaxyl, fluazinam, fluopicolide. To control tobacco black shank disease caused by *P. nicotianae*, 76% wettable powder of the fungicide combination containing dimethomorph and metalaxyl was screened and the mixture showed 80.32%~84.00% field control efficacy against the disease. This research could supply more alternative fungicides for tobacco black shank disease control and provide technical support for dimethomorph resistance management.

APPOST 26

Crop compliance: crop protection agents testing programme within purchases

BODE L.M.; EVANS W.; INTORP M.

Reemtsma Cigarettenfabriken GmbH (an Imperial Brands PLC Company), Albert-Einstein-Ring 7, D-22761 Hamburg, Germany

In order to ensure the compliance of our products, leaf grade purchases are monitored for the presence of any residual crop protection agents (CPA). CPA residue levels are first assessed for compliance against limits set by regulatory authorities and secondly evaluated against Guidance Residue Levels (GRL) based on Good Agricultural Practice (GAP) recommended and published by the CORESTA Agrochemical Advisory Committee (ACAC).

The objective of this investigation was to monitor residue results from multiple measured crop purchase samples intended to be used for cigarette production. Around 250 samples in all were measured from locations across the world (i.e. drawn from 22 countries, all continents represented, for crop years 2012-2015).

Additionally, an independent sampling scheme covering 40 purchase orders was performed in order to record and evaluate the distribution of CPA residue levels within the purchases. The purchase lots ranged from 77 to 576 tonnes.

Notable variation between samples was found: e.g. a variation in levels of Maleic Hydrazide of up to 8 fold within one sample set (3 and 25 ppm), and a fourfold difference in Butralin (0.6 and 2.3 ppm) between two samples was found, but all quantified values were far below the GRL. Also the CPA residue pattern shows a high variation between the comparable sample sets.

Nevertheless, the current sampling procedure should be adequate to ensure crop compliance since the differences in residue values within the purchases were detected far below current GRLs. However, if new regulation limits were to be set below these guidance limits then the sampling procedure might need to be adjusted.

APPOST 27

Potential of Jatropha seedcake for control of root-knot nematodes (*Meloidogyne* spp.) in tobacco

MAINJENI C.E.D.; DUMBO E.L.C.; KWENDA H.A.B; MTONGA Y.P.

Agricultural Research and Extension Trust (ARET), Private Bag 9, Lilongwe, Malawi

Jatropha seedcake is reported to be efficient in the control of root-knot nematodes (*Meloidogyne* spp.) in crops such as tomato. In this study, five application rates of Jatropha seedcake were explored for control of nematodes in tobacco seedbeds. Plots received Jatropha seedcake at the rates of 0.75 kg/m^2, 1.5 kg/m^2 and at 3.0 kg/m^2. There was also a standard chemical control, Herbifume (a.i. Metham sodium) at the rate of 100 ml/m^2 while another plot had neither Jatropha or Herbifume as a Nil control. The trial was a randomised complete block design with four replicates at two locations in Central Malawi for two seasons from 2014 to 2016. Results showed that at both locations Jatropha treatments reduced nematode root galls by 44% at eight weeks after germination compared to the non-treated plot. On the other hand, Herbifume was 50% more efficient in controlling galling than the Jatropha treatments but in the latter, the seedlings also exploited the nutritive benefits of the cake to stay relatively healthy, vigorous and strong. It was therefore the finding of this study that Jatropha seedcake at the rate of 1.5 kg/m^2 effectively controlled root galling. It is possible to reduce dependence on costly synthetic chemicals to more eco-friendly bio-sources such as those from Jatropha seedcake for the benefit of Malawi tobacco farmers for increased productivity and reduced cost of production.

APPOST 39

Tobacco production in Macedonia

MICESKA G.; DIMITRIESKI M.

University “St. Kliment Ohridski” - Bitola, Scientific Tobacco Institute - Prilep, Kicevska bb., 7500 Prilep, Republic of Macedonia

Tobacco production has a very important place in the economy of Macedonia due to both economic and social reasons. Tobacco cultivars grown in the Republic of Macedonia are of Oriental origin, mainly of the types Prilep, Jaka and Basma. With a share of 3%, Macedonia is positioned among the eight major tobacco producing countries in the world. Of the total arable land in the country tobacco occupies 3,4% in the area under energy crops around 81.1% of the total area. The average area under tobacco cultivation in Macedonia in the period 2012-2015 was 16.962 ha. With an average yield of 1.270 kg/ha, the total production for the same period was 21.662 tons of Oriental tobacco, but it is presumed that it can achieve up to 35.000 tonnes. Tobacco's share in the total exports of Macedonia was 3.7% (Annual Statistics of RM for 2011). At first sight, this seems to be an insignificant segment in the foreign trade relations of the country, but its real value can be calcuated when we take into consideration that the total exports in 2015 was 20 663 tonnes of Oriental tobacco. The importance of tobacco for the Macedonian

economy is even more obvious when compared to the total exports of agricultural products in 2015, in which it participated by over 22%. All activities and processes related to production of this crop are regulated by law (The Tobacco and Tobacco Products Act). According to the provisions of this Act, tobacco producers are allowed to use only certified seed material and the only authorised institution for production of such material is the Scientific Tobacco Institute in Prilep.

The aim of our investigation was to study the importance of tobacco production in Macedonia for economic and social reasons.

CORESTA CONGRESS 2016

SMOKE SCIENCE and PRODUCT TECHNOLOGY

ORAL PRESENTATIONS

ST 01

BTFI - A descriptive overview concerning the papers and areas covered over the last 55 years

HELLER W.D.; BOENKE B.; MÜLLER B.; THIELEN A.; SCHERER G.

DZV Deutscher Zigarettenverband e.V., Unter den Linden 42, D-10117 Berlin, Germany

The following issues will be discussed:

1) Descriptions of the fields of publications and the enhancing and development of these fields are given. In context to this description "landmark" papers are highlighted: Seehofer, Dontenwill, Paschke, etc.
2) Which group of papers are important over a shorter time period, and which over a longer time period?
3) Development of the reviewing process over the years.
4) Changing of the editorial structure and the way the Journal is published.
5) Expectations: Which basic factors are necessary to secure a successful future for a scientific journal in the area of tobacco?

ST 02

BTFI - Devices for generation, exposure and collection of main stream smoke – from history to the future

MÜLLER L.; BOENKE B.; KLUS H.

Beitraege zur Tabakforschung Int'l GmbH, Unter den Linden 42, D-10117 Berlin, Germany

To gain insight into the physical properties, the chemical composition and the toxicological effects of smoke from different tobacco products, special smoking machines, suitable traps and exposure devices were designed for collecting and investigating whole smoke or specific smoke components. A large number of smoking and collection devices have been used over the last more than hundred years. This paper describes and discusses the development of devices for cigarette mainstream smoke generation, collection and exposure of microorganism, cell systems as well as animals for evaluation of the toxic properties of tobacco smoke over the course of time.

ST 03

BTFI - Toxicological sciences in 'Beitraege zur Tabakforschung International' (BTFI) over the last 55 years

SCHERER G.(1); HELLER W.D.(2)

(1) ABF GmbH, Goethestrasse 20, D-80336 München, Germany
(2) DZV Deutscher Zigarettenverband e.V., Unter den Linden 42, D-10117 Berlin, Germany

From the start of the Journal in 1961, articles with a toxicological relationship were published in BTFI. Most of those papers deal, of course, with toxicants present in tobacco and tobacco smoke. However, also articles which investigated toxic effects of tobacco smoke in *in vitro* and *in vivo* systems appeared in the Journal. Studies with human tobacco users in terms of their behaviour, smoke exposure as well as risks for diseases found their way to publication in BTFI in the last more than five decades. The time profile of the publication of the various toxicological aspects in BTFI well reflects the changes which took place in the smoking and health field and within the tobacco industry in the last more than 50 years.

ST 04

Reduction of harmful or potentially harmful constituents (HPHCs) following partial or complete substitution of cigarettes with electronic cigarettes in adult smokers

D'RUIZ C.D.(1); O'CONNELL G.(2); GRAFF D.W.(3)

(1) Fontem Ventures, B.V., Greensboro, NC, U.S.A.
(2) Fontem Ventures, B.V., Scientific and Regulatory Affairs, Amsterdam, The Netherlands
(3) Celerion, Lincoln, NE 68502, U.S.A.

Changes in fifteen urine, blood, and exhaled breath biomarkers of exposure (BoE) to harmful and potentially harmful constituents (HPHCs), representing major classes of compounds reported by the FDA to be significant contributors to smoking-associated disease risks, were measured in 105 randomized and clinical-confined subjects following a five-day forced-switch study from usual brand conventional combustible cigarettes to: (i) exclusive use of commercial e-cigarettes; (ii) dual-use of commercial e-cigarettes and the subject's usual conventional cigarette brand; or (iii) discontinued use of all tobacco or nicotine products. Levels of urinary biomarkers in subjects that completely substituted their usual cigarette brand with e-cigarettes were significantly lower (29%-95%) after five days. Percent reductions in eight of the nine urinary BoEs measured were indistinguishable to smokers who had quit smoking, with the exception of nicotine equivalents, which were reduced by 25%-40%. Dual users who halved their self-reported daily cigarette consumption of conventional cigarettes with e-cigarettes exhibited reductions of 7%-38% in eight of nine urinary biomarkers, with a statistically insignificant 1%-20% increase in nicotine equivalents. The observed reductions in the dual use group were broadly proportional to the reduced numbers of cigarettes smoked. After five days, blood nicotine biomarker levels were lower in subjects in the cessation (75%-96%) and exclusive groups (11%-83%); dual users did not experience any significant reductions. All subjects experienced significant decreases in exhaled CO, with decreases in cessation and exclusive groups ranging from 88%-89% and in the dual group from 27%-32%. Exhaled NO was observed to increase in cessation and exclusive groups (46%-63% respectively), whereas the dual user group experienced minimal changes. This study indicates that smokers who completely or partially substitute conventional tobacco cigarettes with e-cigarettes over five days experienced reductions in BoEs associated with exposure to a number of select HPHCs. While exclusive e-cigarette users displayed similar reductions to smokers who quit smoking over the same period of time.

ST 05

Impact of smoking cessation on the metabolic profile of former smokers

GOETTEL M.; MUELLER D.; PLUYM N.; SCHERER G.; SCHERER M.

ABF Analytisch-Biologisches Forschungslabor GmbH, Goethestrasse 20, 80336 Munich, Germany

Smoking is a major cause for several diseases but still the underlying pathophysiological mechanisms are not completely understood. Compounds which indicate the smoking induced perturbations in biochemical pathways, so-called biomarkers of effect, are needed. As presented at previous CORESTA Congresses and Meetings, we successfully developed a GC-TOF-MS method for untargeted metabolomic profiling. Analysis of bio-fluids from smokers (S) and non-smokers (NS) revealed several altered endogenous pathways including fatty acid, amino acid and energy metabolism. A second, diet-controlled clinical study was conducted with 60 healthy smokers willing to quit smoking. The subjects were followed over a time span of three months of smoking abstinence with the aim to determine the impact of smoking cessation on their metabolome. Biological samples (plasma, urine, saliva) were collected at baseline (when still smoking), after 1 week, 1 month, and 3 months and analysed by untargeted metabolomics profiling for altered endogenous compounds (so called target hits) after cessation in all compliant subjects (N = 39).

Metabolic fingerprinting revealed several altered metabolic pathways after cessation e.g. amino acid (in particular tryptophan) metabolism or analytes associated with energy metabolism. As expected, the number of target hits increased over time after smoking cessation. The presentation exemplifies the observed changes for the fatty acid (FA) profile as their metabolism was observed to be significantly affected after three months of smoke abstinence, comparable to the observed differences between S and NS in our first study. Apparently, there is a strong impact of smoking on FA metabolism. For further investigation, a GC-TOF-MS method for the simultaneous quantification of 44 saturated and unsaturated FAs over a broad range of chain lengths from C4 to C32 was developed and validated according to FDA guidelines.

The outcome of the targeted FA analysis with the aim to reveal suitable biomarkers of effect relevant to tobacco smoke exposure will be discussed in this presentation.

ST 06

Evaluation of a biomarker of oxidative stress: 8-iso-prostaglandin $F_{2\alpha}$ / prostaglandin $F_{2\alpha}$ ratio

NEWLAND K.(1); MILLER P.(1); FARMEN R.(1); TRAN C.T.(2); HAZIZA C.(2); PICAVET P.(2)

(1) Celerion, Inc., 621 Rose Street, Lincoln, NE 68502, U.S.A.
(2) Philip Morris Products S.A. (part of Philip Morris International group of companies), Quai Jean Renaud 5, 2000 Neuchâtel, Switzerland

It is understood that exposure to free radicals related to tobacco use negatively impacts smoker health. Studies have been performed measuring fatty acid oxidation products (F_2-Isoprostanes) in human urine as biomarkers of harm from free radical exposure. A challenge noted with this approach is that the measured biomarkers are formed through enzymatic pathways and chemical lipid peroxidation initiated by free radical exposure. A novel approach to differentiate the biomarker components specific to the enzymatic and radical peroxidation pathways was developed by van't Erve et al. (Free Radical Biology and Medicine 2015). The analysis of a ratio of stereo-isomers, 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in human plasma was suggested as a possible method improvement in the determination of oxidative stress. Initial published concentration results from non-smoker subjects ranged from

approximately 45 to 110 pg/mL for both analytes. The mean ratio non-smoker plasma samples of 8-iso-$PGF_{2\alpha}$ and $PGF_{2\alpha}$ was reported as 0.88 ± 0.26.

Working to further investigate this biomarker of oxidative stress, an assay was developed to measure the stereo-isomers in human plasma samples. To aid in reproducibility of the measurement, the samples were stabilized with indomethacin (inhibition of cyclooxygenase-1 and -2) and butylated hydroxytoluene (BHT) (inhibition of chemical peroxidation). A selective analytical approach was developed with reversed phase gradient chromatography on a UPLC system prior to detection by MS/MS on a SCIEX Triple Quad® 6500. Samples were prepared for injection via mixed-mode solid phase extraction.

Samples collected with the added inhibitors were significantly lower in measured concentrations of both analytes with a range of 6 to 15 pg/mL. Mean ratios of 8-iso-$PGF_{2\alpha}$ and $PGF_{2\alpha}$ observed in smoker and non-smoker samples were between 0.77-1.14 and 0.95-1.12 pg/mL respectively. Further evaluation of the ratios was performed with samples incubated in an attempt to reproduce the published work.

ST 07

A distributed computational fluid dynamics (CFD) model for estimation of room air levels of selected aerosol chemicals from emission of e-vapour products (EVP)

ROSTAMI A.A.(1); AGYEMANG S.(2); PITHAWALLA Y.B.(1)

(1) Altria Client Services LLC, Research, Development and Regulatory Affairs, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.
(2) TriMech Solutions LLC, 4461 Cox Road, Glen Allen, VA 23060, U.S.A.

At a 2015 “Electronic Cigarettes and the Public Health” workshop, among other topics, the FDA expressed interest in gathering scientific information on: (1) how far can exhaled e-cigarette aerosols travel in a confined environment to impact non-users? and (2) how do exhaled aerosol properties impact potential secondhand exposure? To conduct a systematic risk assessment of impact on non-users, one would need to estimate the potential range of second-hand exposures, resulting from different use scenarios. It is difficult and cost prohibitive to estimate the potential exposure ranges using controlled clinical studies, especially considering the multitude of possible variations in e-liquid compositions, individual usage behaviour and indoor space characteristics (size, ventilation, etc.). We have developed a distributed model, using CFD and thermodynamics principles that predict aerosol dispersion in indoor spaces. The model includes evaporation and condensation of selected chemicals from the dispersed aerosol. The model can dynamically estimate the spatial and temporal variations of the room concentration of selected aerosol chemicals. Results from the model are in good agreement with published experimental data. Modelling results indicate that, in close proximity of the source, concentrations of selected aerosol chemicals strongly depend on the distance and orientation of the sampling point with respect to the source. The direction of recirculation air within the room also has a strong effect on the concentration at the sampling point. The model may be used for estimating (a) the exposure level of non-users to selected chemicals in an indoor space where EVPs are used and (b) estimate the level of particulate matter and chemicals from EVP use in a variety of enclosed spaces (e.g. cars, homes and office settings).

ST 08

Analysis of N-nitrosodimethylamine in smokeless tobacco by use of UHPLC-MS/MS

ERICSSON D.; PATRING J.; KALVO D.; LINDHOLM J.

Swedish Match North Europe AB, P.O. Box 170 37, SE-104 62 Stockholm, Sweden

N-nitrosodimethylamine (NDMA) is a member of the compound group N-nitrosamines and is a carcinogenic substance belonging to IARC Group 2A. In addition, NDMA is on the FDA "Established List for Harmful and Potentially Harmful Constituents in Tobacco Products and Tobacco Smoke". Therefore a sensitive and reliable NDMA analysis method is important for many tobacco industry laboratories.

In this presentation, an UHPLC-MS/MS (Ultra High Performance Liquid Chromatography-Tandem Mass Spectrometry) NDMA analysis method which has been operating reliably since 2008 will be presented.

NDMA is extracted for 40 min from the smokeless tobacco sample with ethyl acetate after the addition of deuterium-labelled internal standard (NDMA-d6). The extraction is followed by centrifugation and finally transfer of the particle-free ethyl acetate extracts to vials for UHPLC-MS/MS analysis. Although NDMA is injected in ethyl acetate (organic solvent) onto a reversed-phase column, the NDMA is well-retained on the column and elutes in a sharp, symmetric chromatographic peak. The working range of the method is 0.6-150 ng/g (standard sol. 0.1-50 ng/ml)

The method is accredited according to ISO17025 and the validation has been performed for snus, moist snuff and chewing tobacco with respect to specificity, precision, accuracy, limit of quantification and detection (LOQ and LOD), linearity, recovery, matrix effects and robustness.

To conclude; this is a sensitive and reliable method with a simple sample extraction procedure which has been analysing over 10000 sample extracts over the last seven years.

ST 09

Analysis of coumarin in tobacco and smokeless tobacco by use of UHPLC-MS/MS

PATRING J.; KALVO D.; ERICSSON D.; LINDHOLM J.

Swedish Match North Europe AB, P.O. Box 170 37, SE-104 62 Stockholm, Sweden

The demand for chemical analysis is increasing; the number of routine assays is steadily increasing as well as requests for new assays required by the regulators. Coumarin is one of the constituents of regulatory concern on FDA's established list for Harmful and Potentially Harmful Constituents (HPHC) in tobacco smoke or Smokeless Tobacco Products (STPs). There is currently no recommended method for coumarin for these matrices, neither by CORESTA or any other organisation.

The aim of this study was to develop a method for the quantitative analysis of coumarin in tobacco and smokeless tobacco products using UHPLC-MS/MS (Ultra High Performance Liquid Chromatography-Tandem Mass Spectrometry).

In this method, coumarin is extracted from the sample matrix (1 g of sample) with a methanol/water mixture after the addition of deuterium-labelled internal standard (coumarin-d4). Following extraction, the sample is diluted with water and finally filtered directly into a LC-compatible vial by using syringeless, Mini-UniPrep filters. The sample extract is then analysed using a fast UHPLC-MS/MS method with a cycle time of less than 3 minutes.

The method has been validated for tobacco and several smokeless products for specificity, precision, accuracy, limit of quantification and detection (LOQ and LOD), linearity (0.015-9 µg/g in sample), recovery, matrix effects and robustness.

Altogether, a robust, sensitive and easy-to-use method has been developed that has the capacity to meet the demands of tobacco industry laboratories. This method has several advantages including a short analysis time, simple clean-up step and the ability to analyse up to 150 samples per day.

ST 10

Design of experiments on heat-not-burn tobacco substrates to characterise factors affecting aerosol composition by low temperature pyrolysis GC/MS

DUROT N.(1); RAVERDY-LAMBERT D.(1); HERVE R.(2)

(1) SWM Intl, c/o LTR Industries, Usine Le Mans, Allonnes Cedex 72702, France
(2) SWM Intl, c/o PDM industries, Kerisole, Quimperlé, France

Heat-not-burn (HnB) tobacco products comprising an aerosol tobacco substrate are typically heated at a temperature below 350 °C, which is sufficient to release nicotine and some other tobacco volatile components, but not high enough to generate a significant level of pyrolysis and combustion products often involved in cigarette smoke toxicity.

Low temperature pyrolysis is used to instantly heat tobacco substrates and characterise aerosol emission through mass spectrometry after gas chromatography separation.

The objective is to define the contributions of the following factors on aerosol composition through an experimental design: heating temperature, heating duration and tobacco substrates. Tobacco leaf and reconstituted tobacco (RTL) are compared for different tobacco types.

The release of glycerol is higher below 200 °C and decreases at higher temperature whatever the tobacco substrate. The nicotine release increases with temperature from 150 to 250 °C, and depends also on the tobacco substrate.

HnB reconstituted tobaccos show a higher aerosol amount than corresponding natural tobaccos with glycerol, particularly in nicotine (+35 to 75%), glycerol (+20 to 80%), and volatile aromatic components such as terpenoids, ketones, aldehydes, fatty acids, alcohols.

For example at 250 °C for 2 min, a RTL vs a natural tobacco aerosol is 45% higher in nicotine and 1.5 to 6 times higher in volatile aroma for flue-cured, respectively, and 70% higher in nicotine and 1.2 to 3 times higher in volatile aroma for Burley.

Variation of heating duration from 1 to 5 min shows low variation in aerosol composition up to 250 °C.

Finally, the heating temperature and the tobacco matrix itself are more important for the aerosol composition than the duration of the heating.

In conclusion, this study provides interesting indications on the potential of heated tobacco substrates for aerosol generation even if the approach is not quantitative and very simplified versus aerosol flows in a HnB product design.

ST 11

Authenticity, quality, and identity of natural products by means of NMR-spectroscopy

SCHWARZINGER S.(1); RÖSCH P.(1); BLUMENSTOCK M.(2); WESKE D.(2)

(1) ALNuMed GmbH, Am Aubach 5, 95448 Bayreuth, Germany
(2) British American Tobacco (BAT) Germany, Product Centre Europe, Weiherstraße 26, 95448 Bayreuth, Germany

Quality of products, including food and plant materials, has traditionally been evaluated by analysis of a large number of parameters determined by targeted analysis with multiple methods. Such conventional analysis typically involves several methods and is time consuming. With nuclear magnetic resonance (NMR) spectroscopy a method is available that allows rapid targeted analysis of a large number of parameters in a simple measurement lasting just a few minutes. It is already industrially applied for, e.g. quality testing of foods such as honey, fruit juices, and wine, where up to 54 parameters are simultaneously determined in a quantitative manner. Such a large number of parameters resembles a quantitative product fingerprint and can harbour information about the authenticity and quality of the product. NMR fingerprints can be used to test, e.g. where a honey has its geographic origin, which variety it presents, and whether or not the product has been adulterated. Similarly, multi-parameter analysis can also be applied to compare samples, for instance, a market sample and the delivered batch. The principles of NMR based simultaneous testing of authenticity and quality will be explained. We will demonstrate that NMR can be applied for quantitative multi-parameter screening as well as for chemometric screening of tobacco blend constituents and tobacco ingredients. Furthermore, highly complex NMR spectra in conjunction with chemometric measures offer the potential for the determination of a product blend composition, i.e. the portions of different types of tobacco leaf. Finally, we will present first results showing how NMR developments can be transferred to optical spectroscopy to facilitate initial analytical screening at the point of need.

ST 12

Non-targeted high resolution screening of tobacco for potential contaminants (pesticides and chemical toxins) using UPLC/QTOF-MS

MERUVA N.(1); McCALL E.(2); CLELAND G.(1); BURGESS J.(1)

(1) Waters Corporation, 34 Maple Street, Milford, MA 01757, U.S.A.
(2) Waters Corporation, Altrincham Rd, Wilmslow SK9 4AX, U.K.

In this study a UPLC coupled to high resolution mass spectrometry (QTOF-MS) was used to increase the scope of analysis for multi-residue pesticides and other chemical contaminants in tobacco. Analytical methods for high throughput contaminant screening require non-targeted acquisition under generic conditions followed by targeted processing against a scientific library typically containing retention time and accurate mass data for both precursor and fragment ions for hundreds of chemical contaminants. The key to implementation of UPLC-HRMS for routine screening of tobacco to accurately detect residues at the required regulated concentrations was evaluated.

Reference and commercially available tobacco samples with incurred residues and varying complexity and moisture content were included in the study. The sample extraction was achieved using a generic QuEChERS method. Representative tobacco samples were pre- and post-spiked with a mixture of LC amenable pesticides at 0.01 and 0.05 mg Kg^{-1}. The experiments were performed using UPLC/QTOF-MS, run in electrospray positive (ESI+) mode and the data was acquired using MS^E, a data independent acquisition mode which simultaneously collects both accurate mass precursor and fragment ion data.

After automated data processing, data review workflows were used to quickly review HRMS data in a consistent and accurate manner and to reduce false positives and eliminate false negatives for routine tobacco screening. The contaminant identifications were made on the basis of retention time, accurate mass precursor and fragment ions and isotopic pattern score matching with the entries in the scientific library. Analytical strategies to increase compound coverage and confidence in identification of potential contaminants from high throughput screening of tobacco will be discussed.

ST 13

Determination of harmful components in mainstream cigarette smoke by FT-NIR spectrometry equipped with Cambridge filter pads

REN Jianxin(1); WANG Zhiguo(1); LIU Wei(1); DU Wen(1); ZHONG Kejun(1); LIU Huimin(2); HU Qingyuan(3)

(1) Hunan Industrial Co., Ltd. of CNTC, Technonlgy Center, Changsha, Hunan 410007, P.R. China
(2) Zhengzhou Tobacco Research Institute of CNTC, Zhengzhou, Henan 450001, P.R. China
(3) China National Tobacco Quality Supervision & Test Center, Zhengzhou, Henan 450001, P.R. China

The yields of harmful components in cigarette smoke are basic parameters to evaluate the harm of cigarettes. However, the existing detection methods for most harmful components in mainstream cigarette smoke are complicated and time consuming. Therefore a simple, fast and efficient method that can simultaneously determine the yields of several harmful components in mainstream cigarette smoke was developed. The method presented is based on FT-NIR spectrometry equipped with Cambridge filter pads, which collect the particulate matter of cigarette smoke. Partial least square (PLS) method was adopted to establish prediction models between the NIR spectra of the filter pads and the yields of smoke components, including tar, nicotine, carbon monoxide, crotonaldehyde, phenol, HCN, NH_3, B[a]P and NNK. The yields of the nine components could be obtained within one minute after cigarette smoking through one run. The average prediction relative errors (APRE) of the established models were about 5% for components of milligram yield (tar, nicotine, CO), 10% for components of microgramme yield (crotonaldehyde, phenol, HCN, NH_3) and below 15% for components of nanogram yield (B[a]P and NNK). Compared with conventional methods, this technique is much safer, more economically efficient, and environmentally friendly due to detecting the NIR spectra of the pads directly. In addition, it may be possible for the method to be further developed and applied to the quantitative determination of the other harmful components in mainstream cigarette smoke for ensuring the consistency of cigarette quality.

ST 14

Determination of transfer rates of smoke constituents into mainstream smoke and the correlation to physical parameters

HUSSEIN L.; KAMM H.; WESKE D.; BLUMENSTOCK M.

British American Tobacco, Product Centre Europe, Weiherstraße 26, 95448 Bayreuth, Germany

The mainstream smoke of a combustible cigarette contains several thousand smoke constituents. Examining the levels of certain compounds of interest, which are transferred into mainstream smoke, e.g. substances applied as flavourings, and correlating these with the observed transfer rates (defined as into mainstream smoke transferred amount/amount on

product * 100%) and the physical-chemical properties of these compounds, could provide a deeper understanding of the transfer process.

We developed a method to determine such transfer rates for several compounds. These compounds vary in their physical-chemical properties such as boiling point (120-300 °C), molecular weight (120-190 g/mol) and vapour pressure (0.02-1700 Pa at 20 °C). The developed method is validated regarding selectivity, linearity, accuracy, and repeatability, and possible matrix effects were also taken into account. Smoking was conducted under the Health Canada Intense regime. As a trapping system, a combination of Cambridge filter pads and impingers filled with appropriate solvent ensured adequate collection of the target substances during smoking (breakthrough is below limit of detection). After work-up, gas chromatography-mass spectrometry was used for analytical measurements using internal standards. If commercially available, the deuterated equivalent of the target substance was used as an internal standard. Otherwise, a deuterated compound with comparable chemical and physical properties was used.

The determined transfer rates for the examined compounds varied from 17%-57%. The results demonstrate that while a general correlation between physical parameters and the transfer rates can be observed (lower boiling point/higher vapour pressure result in higher transfer rates), these parameters alone are not sufficient to predict the determined transfer rates of compounds into mainstream smoke. For example, two compounds with comparable boiling point, vapour pressure and same concentration on the product showed significantly different transfer rates (30% vs. 40%; based on 6 replicates per sample). Generally, the results indicate that besides physical parameters, the overall composition of the tobacco product (e.g. tobacco, physical design) has an influence on the transfer rates of single compounds.

ST 15

Estimation with values below the limit of quantification

MORTON M.J.; LION K.

Altria Client Services LLC, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.

For analytes that are present at trace levels, it is common to have a mix of results above and below an analytical reporting threshold, such as the analytical limit of quantification (LOQ). A determination must be made as to how to treat values below the reporting threshold when estimating, for example, the mean and standard deviation of the analyte value.

Two common approaches that are employed by some laboratories are either dropping the values below the reporting threshold or treating them as zeros. Either approach biases the summary results. Dropping results below the threshold biases the summarized value on the high side and treating those values as zero biases the results on the low side.

Several statistical approaches are possible to report trace level analytes, and two of those were examined: (1) using the instrumental value even if below the reporting threshold and (2) treating the values as censored and using statistical methods for censored data (censored data are data values that are only partially known, for example, in this instance, the values are treated as only known to be within the interval 0 to LOQ).

These approaches are illustrated with data from a tobacco-specific nitrosamine (TSNA) tobacco survey.

Using the instrumental value or censored data methods can reduce the biases associated with the two common approaches listed above. The simpler approach is generally to use the instrumental value even if below the reporting threshold. Most censored data methods are much more complicated and may not be applicable in smaller data sets.

ST 16

Tease tipping: to protect and to attract

LINDNER M.; GUTWALD M.

TANNPAPIER GmbH, Johann Roithner-Strasse 131, A-4050 Traun, Austria

The generation of open windows on the tipping paper represents a smart way for the extraordinary realisation of ventilated filter cigarettes. During a sophisticated die-cutting process, accurate laser application or mechanical punching technology on the tipping paper surface provokes the formation of macroscopic perforation holes on the millimetre scale with unlimited possibilities with respect to dimensions and geometries. The stabilities of hole parameters, air permeabilities and cigarette design properties like filter ventilation and pressure drop by using open windows are comparable to the standard pre-perforation techniques of electrostatic and laser perforation. Cigarettes made with tease tipping also reveal the same efficiency in ventilation rates and smoke yields reduction as conventionally perforated cigarettes due to the controlled, undisturbed air flow through the customised ventilation zone. The first goal of the present study was to demonstrate tease tipping as an alternative method to dilute cigarette smoke by conducting a physical and chemical analysis of specifically designed cigarette samples. The second part of this study refers to visual benefits of window shape variations. Die-cut windows on the tipping paper provide a perfect view of special cigarette filter features such as segment, chamber, charcoal or coloured plug wrap. In conclusion: tease tipping comprises the outstanding option to combine technical cigarette functionalities, compliance with tobacco regulations and appealing design features.

ST 17

Paper filters – the influence of different paper composition and paper properties on filtration efficiency and pressure drop

GLEINSER M.; BACHMANN S.; VOLGGER D.

Papierfabrik Wattens GmbH & Co. KG, Ludwig-Lassl-Straße 15, 6112 Wattens, Austria

Smoke yields, smoke composition and the taste of a cigarette can be modified by changing different parts of the cigarette, especially the different filter design. Cellulose acetate is currently the most common material for cigarette filters, but also other materials, such as paper, are available with different filtration properties.

This study investigates the impact of the fibre type and paper properties, especially air permeability and thickness, on pressure drop and filtration efficiency. In a first step papers with different fibre types and paper properties were produced. Then filters were manufactured from the papers and cigarettes were produced using these filters. The filtration efficiency for tar or nicotine in % was determined by measuring nicotine or the retained particular phase of tar on the Cambridge filter pad divided by the total nicotine or particular phase for NFDPM (Cambridge filter pad and cigarette filter).

It was found that different fibre types can increase the pressure drop of a filter by 50 mmWG even at a constant filter rod weight. Additionally, the filtration efficiencies could be reduced by approx. 5% using different fibre types. When evaluating different paper properties and keeping the fibre type constant, the influence of air permeability and thickness of the paper was investigated. The results show that at the same filter rod weight, air permeability and thickness could vary the pressure drop in a range from 200 - 500 mmWG. As a consequence, by varying the paper properties it is possible to adjust the pressure drop, and consequently the filtration efficiency, for tar and nicotine from 30 up to 60%.

In summary it can be concluded that the fibre type and paper properties such as thickness and air permeability have an influence on pressure drop and filtration efficiency and thereby allow to fine-tune the design of paper filters.

ST 18

Effect of polylactic acid (PLA) filter on mainstream cigarette smoke and deliveries of phenol

JIANG Wen; WU Jingqiang; SHEN Jingxuan; LIU Weijuan; XIAO Weiyi; XU Lanlan; SUN Jun; ZHANG Yong

Yunnan Reascend Tobacco Technology (Group) Co., Ltd., Kunming, Yunnan 650106, P.R. China

Most cigarette filters are manufactured from cellulose acetate (CA) or polypropylene (PP). These materials generally degrade slowly in the natural environment. Polylactic acid (PLA) is a type of thermoplastic which is produced from plants such as corn, sweet potatoes and sugar cane. PLA can degrade completely into water and carbon dioxide in a natural environment. The routine components in mainstream smoke (MS) from cigarettes with PLA filters are almost the same as those from cigarettes with CA filters. However, the adsorptive properties of PLA for selected constituents such as phenol are expected to be different, relative to CA or PP. The objective of this study was to compare the chemical components of MS from cigarettes with PLA and CA filters, and to investigate the influence of additives to PLA filters on the deliveries of phenol. Cigarettes made with either CA or PLA filters were smoked on HBRM/cs20 rotary smoking machines by ISO puffing regimen. Different types and levels of additives were incorporated into the PLA filters in order to check the deliveries of phenol in MS. The results showed that the puff number and nicotine deliveries remained unchanged, and TPM, water, tar and CO were almost the same. The harmful substance content of cigarette smoke was basically similar except for an increase in phenol with PLA (27.33 µg/cig) from cigarettes with CA (17.23 µg/cig) filters without additives. Triethyl citrate (TEC), tributyl citrate (TBC) and acetyl tributyl citrate (ATBC) added to PLA filters decreased deliveries of phenol, with the greatest reduction in phenol observed with TEC. The extent of phenol reduction increased with increasing level of TEC (mass fractions were 2.0%, 2.6%, 3.2%), and the deliveries of phenol was reduced to 19.66 µg/cig, 18.68 µg/cig and 17.59 µg/cig respectively. It was found that addition of TEC to PLA filters effectively reduced phenol levels in MS.

ST 19

Smoke analysis of fine-cut tobacco (Part 3) – Physical characterisation of fine-cut smoking articles and their variability

TEILLET B.(1); DESTRUHAUT S.(1); CHAVES L.(2)

(1) SEITA-Imperial Tobacco, 48 rue Danton, 45404 Fleury-les-Aubrais, France
(2) Reemtsma Cigarettenfabriken GmbH (an Imperial Brands PLC Company), Albert-Einstein-Ring 7, D-22761 Hamburg, Germany

There is current regulatory interest in emissions from fine-cut tobacco. Various approaches exist for measuring emissions from fine-cut tobacco, such as ISO Standard 15592, and some countries have established their own specific methods (e.g. Canada and The Netherlands).

At the CORESTA Congress in 2014 (ST05), we reported that the TNCO yields obtained from four different fine-cut smoking articles (FCSA) made following the ISO standard were correlated. Consequently, we developed a predictive model based on the data obtained from a single smoking article. At the following CORESTA SSPT meeting in 2015 (ST86), we reported that the preparation of FCSAs with highly expanded tobacco led to a pressure drop outside of the

acceptable tolerances of the smoking machine, mainly due to the need for a higher volume of this tobacco at a given weight.

Further to our previous findings, additional investigations on physical characterisation of FCSAs (weights, pressure drops, tobacco density and firmness of the smoking articles) are required.

FCSA protocols are based on either a targeted tobacco weight or a targeted pressure drop, using a tube with or without a filter and incorporating expanded and non-expanded tobacco blends. Variability is then assessed from the distribution range of the physical parameters.

Results demonstrate that FCSAs are more variable than factory-made cigarettes as their preparation involves a manual process that is also more sensitive to the environmental conditions, i.e. temperature and humidity. This needs to be considered during the development of potential future regulations.

ST 20

The influence of storage conditions and packaging on acetic acid generation in cellulose acetate fibers

TATUM C.E.; WILSON S.A.; CASPER R.G.; PEARCE M.J.

Eastman Chemical Company, P.O. Box 511, Kingsport, TN 37662, U.S.A.

The cellulose acetate polymer used in cigarette filters is made by acetylating wood pulp with acetic anhydride. One advantage of this modified natural polymer is that it readily degrades by multiple mechanisms. While the ultimate products are carbon dioxide and water, the interim products are acetic acid and cellulose from the deacetylation caused by microbes or chemical hydrolysis.

Recent experimental work has confirmed that Estron acetate tow has a shelf life of two years when stored in the original unopened package, in an enclosed area protected from moisture, high humidity, and extreme temperatures. The shelf life was confirmed by evaluating the odor threshold for acetic acid in cellulose acetate fibers and by measuring the rate of acetic acid generation. The acetic acid was measured in tow samples which were stored at 26 °C and 51 °C in both cardboard bales and new vacuum packaged bales. The results show a 10 fold increase in the amount of acetic acid generated at 51 °C as compared to 26 °C. No difference in the acetic acid generation rate was observed in regards to the packaging; thus, tow stored in both cardboard bales and vacuum-packaged bales has a two-year shelf life.

ST 21

Characterization of puff topography during 8-hours of *ad libitum* use of MarkTen® e-vapor products

EDMISTON J.; LIANG Q.; VANSICKEL A.; LIU J.; ZHAO Y.; SARKAR M.

(1) Altria Client Services LLC, Research, Development and Regulatory Affairs, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.
(2) Eurofins Lancaster Laboratories, c/o Altria Client Services LLC, Research, Development and Regulatory Affairs, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.

Introduction: The purpose of this study was to characterize the puff topography variables following use of MarkTen® electronic cigarettes by adult conventional cigarette smokers [CS], adult cigarette and e-vapor dual users [DU] and adult exclusive e-vapor users [EV].

Method: Generally healthy CS (n=30, both classic and menthol users), DU (n=30) and EV (n=30) were enrolled in the study (53% male). Each participant used their preferred flavor with a

previously validated SODIM Smoking Puff Analyzer-Mobile (SPA-M) device for 8 hours in a confined clinical setting. Puff topography was recorded with the SPA-M and analyzed using SODIM SodAfc software. Each device consisted of a battery (3.7 volts) and cartridge containing propylene glycol, glycerol, flavors and 2.5% by weight USP grade tobacco derived nicotine (approximately the size of a King Size traditional cigarette).

Results: Overall (n=90), the mean values over the 8 hours were: puff count 141 (SD: 96, First Quartile (Q1): 85, Third Quartile (Q3): 172) puff volume 58.77 ml (SD: 23.63, Q1: 41.96, Q3: 69.98), puff duration 3.13 sec. (SD 1.44, Q1: 2.18, Q3: 3.78), flow rate 20.5 ml/sec (SD: 5.75, Q1: 17.49, Q3: 23.68) and inter puff interval (IPI) 75.24 sec. (SD: 39.88, Q1: 49.13, Q3: 89.68). The rank order for the mean topography parameters for each of the subpopulations were as follows: total puff counts, mean puff volume and puff duration were CS < DU < EV; mean flow rates and IPI were CS > DU > EV. The variability (CV%) was relatively large with inter-subject variability ranging from 26-46% and intra-subject variability ranging from 32-51% for puff count, puff volume and puff duration. The variability was much larger for inter puff interval (60-182%).

Conclusions: These results suggest that, on average, EV used MarkTen® e-vapor devices differently than CS or DU, with EV taking longer slower puffs with larger volumes. The topography variables were highly variable.

ST 22

"Addictiveness" – measures and methods

SHERWOOD N.

Neil Sherwood Consulting, 22 Route de Marnex, CH-1291 Commugny, Switzerland

Regulatory demands for information on the “addictiveness” of consumer nicotine or tobacco products and their component ingredients are now common. However there has been a notable lack of guidance regarding the core scientific constructs which are assumed to underlie “addictiveness”, what metrics exist or may need to be developed and what type of additional data, if any, may need to be generated. Based upon a review of the scientific literature this presentation will explore the theoretical basis of “addictiveness” and consider what information Regulators may wish to examine in reaching a determination of “addictiveness”. Firstly the historical background of “addictiveness” will be considered and how this relates to “addiction” and “abuse liability”. The presentation will then consider the lessons which may be learnt from the pharmaceutical industry (particularly in the development of pharmaceutical nicotine preparations) where the assessment of “abuse liability” is common if compounds are believed to exert a psychopharmacological effect. Historical “addictiveness” data relating to tobacco, nicotine and other consumer products are then discussed and recent relevant research explained. Finally a list of candidate measures which might be used in a determination of “addictiveness” will be presented, together with a palette of related methods.

ST 23

Pharmacokinetics of nicotine following single controlled use of a new type of tobacco: heated tobacco product

GILES L.(1); YUKI D.(2); SHERWOOD N.(3); GRAFF D.W.(4); NEWLAND K.(4)

(1) JT International S.A., 201 Galgorm Road, Lisnafillan, Ballymena, Co. Antrim, BT42 1HS, U.K.
(2) Japan Tobacco Inc., 6-2, Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan
(3) Neil Sherwood Consulting, 22 Route de Marnex, CH-1291 Commugny, Switzerland
(4) Celerion, 621 Rose Street, Lincoln, NE 68502, U.S.A.

Nicotine is one of the most characteristic compounds contained in any type of tobacco product including cigarettes. Previous studies have reported that different types of tobacco products display differences in nicotine pharmacokinetics. NHTP is a new type of heated tobacco product and there are currently no published studies examining the nicotine pharmacokinetics of this product. The objective of this clinical study was to investigate the pharmacokinetics of nicotine following use of NHTP as compared to a combustible cigarette (CC1, 1 mg tar). This open-label, randomised, two-period, two-sequence crossover study was conducted in healthy adult smokers in the U.K., and assessed the pharmacokinetics of nicotine after a single controlled use of NHTP or a CC1. During the 4-day study confinement period, blood samples were drawn from subjects for measurement of plasma nicotine concentrations and nicotine intake was estimated from mouth level exposure (MLE). The resulting C_{max} and AUC_{last} values showed 5 to 7 times lower nicotine uptake with NHTP use as compared to CC1 use. Estimated MLE following use of NHTP was half that obtained following the use of CC1 and the t_{max} value was longer for NHTP as compared to CC1. The results from this study showed differences in the nicotine pharmacokinetic profiles between NHTP and CC1. Such differences might be explained by differences in product use behaviour, as demonstrated by the differences observed in MLE between NHTP and CC1. Furthermore, it may be postulated that the differences in nicotine pharmacokinetic profiles observed in the present study may be due to differences in the nicotine absorption site and/or the rate of nicotine absorption from NHTP or CC1.

ST 24

A CFD model for the smouldering combustion of the cellulosic substrate during the ignition propensity test

GUINDOS P.(1); PATEL A.(1,2); KOLB T.(1)

(1) Fraunhofer WKI, 54E Bienroder Weg, 38104 Braunschweig, Germany
(2) TU Braunschweig, Pockelsstr. 14, 38106 Braunschweig, Germany

An experimental and numerical investigation was conducted to characterise the influence of the cellulosic substrate used to assess the ignition propensity (IP) of cigarettes. The objective was to better understand the poor repeatability of the test and eventually propose alternative substrates. It is believed that such a low test reproducibility is mainly due to the variability of the cigarette itself but it is unclear as to what extent the substrate may also influence the variability of the test results. An insight to this question was obtained by first characterising the thermodynamic behaviour of the substrate experimentally and then constructing a numerical model that quantified the relative influence of the distinct parameters. A suite of experimental methods that included TGA, DSC, infrared measurements and laser triangulation among others was used to measure the thermophysical properties of the substrate. These properties were then used to build a CFD model that simulated the smouldering combustion experienced by the substrate during the IP test. After validating the model against contactless temperature measurements, a parametric study consisting of 363 IP simulations was performed, which served to quantify the relative importance of each influencing parameter. The results indicated that the heat capacity, pyrolysis activation energy, and air gap thickness are the most influencing aspects of the substrate. The latter parameter was found to significantly vary within each test and its influence

was comparable to that of some major properties of the cigarette such as the cigarette's temperature and burning rate. It is therefore postulated that the variability of the substrate itself plays an important role on the poor repeatability of the test and it may comprise its reliability. A software called SIMULIP-Software was developed to facilitate the calculation of effect of the substrate in the IP testing and could be used for future developments.

ST 25

Has the EU reduced cigarette ignition propensity standard led to fewer fires and fire deaths?

SONDIK E.; SCHAENMAN P. (presented by GHOSH D.)

TriData LLC, 2111 Wilson Blvd, Arlington, Virginia, U.S.A.

Careless smoking is one of the highest causes of fire deaths in most western nations. To reduce fire deaths, the EU mandated that all cigarettes sold from November 17, 2011, must pass an ISO test standard intended to reduce their ignition propensity.

We examined whether the standard has made its intended difference and joins other effective fire prevention methods including public fire education and smoke alarm systems. Our analysis uses a three factor model: the historical trend in cigarette caused fires and fire deaths, cigarette sales volume and a variable representing the introduction of the standard. We hypothesized that the standard's impact would be a sharp reduction in deaths or fires in the immediate years following implementation. We used data on cigarette-caused fires and fire deaths from five EU nations. Our model accounts for declining cigarette sales volume.

We found that introduction of the standard is consistent with modest decreases in fire deaths in the year after implementation for two nations (Great Britain and Estonia), a possible larger decrease in Finland, and no impact in Poland or the Czech Republic. No impact was as great as the 75% reduction initially forecast by the US National Fire Prevention Association, nor their later revision to 30%. We conclude that there is no strong indication that implementation of the Reduced Cigarette Ignition Propensity standard in these five EU nations achieved substantial reductions in smoking-related fires or fire deaths.

Our approach fits general trends well but the highly variable rates of fires and fire deaths in smaller countries limit our conclusions. We also found that there is a need within the EU to agree on definitions of fire data, and report this data to Eurostat. Further, government agencies should evaluate the effectiveness of new product standards and not leave the evaluation to manufacturers.

ST 26

Impact of self-extinguishment during smoking on consumer exposure – Part I: Modelling

COLARD S.; VERRON T.

SEITA-Imperial Tobacco, 48 rue Danton, 45404 Fleury-les-Aubrais, France

The implementation of lower ignition propensity (LIP) regulations, e.g. in the U.S.A., Canada or Europe, has led cigarette manufacturers to develop products with a certain probability of self-extinguishment under standard laboratory testing conditions (ISO 12863).

Usually, LIP products have narrow paper bands along the tobacco rods with low porosity to restrict coal combustion. An increase of the risk of free-air self-extinguishment (FASE) is then unavoidable with LIP compared to non-LIP product designs. The relighting of the cigarette by the

smoker after extinguishment increases the quantity of tobacco smoked by puffing, and consequently the exposure to smoke compounds.

In order to better understand the impact of self-extinguishment, a mathematical model reproducing each temporal phases of smoking was developed. The following parameters were considered: tobacco burning rates during and between puffs, tobacco burning rates between and on LIP bands, puffing conditions (puff and inter-puff interval durations, puff volume), filter and paper ventilations, LIP band positions and probability of extinguishment on each band.

A comprehensive description of the mathematical model and the main outputs will be given. Among these outputs, the total quantity of tobacco burned during the puffs for a range of smoking conditions and product designs can be derived. The model has then been used to assess the impact of cigarette self-extinguishment on consumer exposure (see Part II: Simulations).

ST 27

Impact of self-extinguishment during smoking on consumer exposure – Part II: Simulations

VERRON T.; COLARD S.

SEITA-Imperial Tobacco, 48 rue Danton, 45404 Fleury-les-Aubrais, France

Cigarette mainstream smoke yields are broadly proportional to the quantity of tobacco burned during puffs. A mathematical model was developed previously (see Part I: Modelling) to calculate this quantity of tobacco burned for lower ignition propensity (LIP) cigarettes presenting a certain probability of self-extinguishment during smoking.

The model was used to assess the impact of the probability level on exposure of a virtual population of cigarette consumers. Various smoking conditions were considered with puff durations ranging from 1 to 3 seconds, puff interval from 30 to 90 seconds and puff volume from 20 to 70 millilitres. The distance between bands was kept constant but the first band was positioned randomly as is usually the case with manufactured products.

For a given cigarette design, simulations showed a wide range of tobacco burned during puff for a population of smokers. Interestingly, the distribution of tobacco burned during puff moves to upper levels when the risk of self-extinguishment increases and the impact is inversely proportional to the smoking intensity. This means that consumers having non-intense smoking behaviour seem to be more impacted by self-extinguishment than consumers having intense smoking behaviour.

ST 28

Puffing profile effects on carbonyl formation in e-cigarette aerosols

MILLER J.H.; FLORA J.W.; WILKINSON J.; WILKINSON C.; SINK K.M.; GARDNER W.P.; KARLES G.

Altria Client Services LLC, 600 East Leigh Street, Richmond, VA 23219, U.S.A.

It has been previously reported that aerosols from e-cigarettes can contain carbonyls such as formaldehyde, acetaldehyde, acrolein, and crotonaldehyde due to elevated temperatures of the heating coils. There have been several publications about thermal degradation products formed from the heating of carrier components (e.g. propylene glycol and glycerin) in e-cigarettes. Most of this previous work only investigated single aerosol collection regimes and it did not evaluate the impact of different aerosol collection conditions on the formation of carbonyls. The effect of puff duration and puff volume on e-cigarette aerosol collection has also been previously

investigated. It was observed that puff duration, not puff volume, played a key role in the amount of aerosol mass and the collected nicotine concentration. Aerosol mass and nicotine collected were observed to increase linearly with puff durations from 2 to 5 seconds for single device design. The purpose of this work was to evaluate the effect of puff volume, puff duration, and puff interval on carbonyl formation in aerosols produced by prototype and commercially available e-cigarettes. This information is important to identify appropriate aerosol generating regimes to be used for analytical testing.

ST 29

Do temperature regulated e-cigarettes prevent the formation of thermal decomposition products under dry wick conditions?

GILLMAN I.G.; HUMPHRIES K.E.

Enthalpy Analytical Inc., 800-1 Capitola Dr., Suite 1, Durham, NC 27713, U.S.A.

The study objective was to determine if temperature regulated e-cigarette (TR-EC) devices could reduce the formation of thermal decomposition products under simulated “dry wick” conditions. Three different TR-EC devices were evaluated under two conditions: 1) Aerosol samples were collected under normal conditions with a full tank of e-liquid. 2) Aerosol samples were collected under simulated “dry wick” conditions in which the e-liquid was removed from the tank prior to collection. For comparison, samples were also collected from non-TR-EC devices under the same two conditions. Aerosol samples were collected with all TR devices set to 230 °C using an analytical smoking machine set to deliver a 55 mL puff with a four second duration. Aerosol samples were analysed for the production of formaldehyde, acetaldehyde, and acrolein under each condition. These results were used to estimate daily exposure to formaldehyde, acetaldehyde, and acrolein from EC aerosols and were compared to estimated exposure from consumption of cigarettes and to occupational and work place limits. All devices yielded low levels of aldehydes under normal, full tank, conditions. However, the two non-TR-EC devices produced higher levels of aldehydes under simulated “dry wick” conditions. The ability of the TR-EC devices to limit the formation of thermal decomposition products under “dry wick” conditions varied greatly between the devices. A comparison between the TR and non-TR devices will be presented along with information on how coil construction may affect the ability of TR devices to limit the formation of thermal decomposition products under “dry wick” conditions.

ST 30

Effects of common alcohols on the particle size distribution of atomised aerosol in e-cigarette vapour

WANG Shitai; JIN Yong; LI Ke; ZHAO Guoling; PENG Xinhui; FAN Hongmei; TAN Haifeng; LIU Jinyun; YU Saibo

Hunan Industrial Co., Ltd. of CNTC, Technonlgy Center, Changsha, Hunan 410007, P.R. China

In order to investigate the effects of the constituents of e-liquids on the particle size distribution of atomised aerosol in e-cigarette vapour, the particle size distributions in e-cigarette vapour were determined by using an electrical low pressure impactor (ELPI) under the atomisation conditions known from e-cigarettes. Various alcohols were subject of our study: propanol, butanol, ethylene glycol, propylene glycol, 1,2-butanediol, glycerol, 1,2,4-butanetriol, polyethylene glycol 200 (PEG200), and polyethylene glycol 400 (PEG400).

The results showed that: 1) The type of alcohol influenced the particle size distribution of atomised aerosol. The aerosol particle size distributions (number concentration: NC) of alcohol, propanol, butanol, ethylene glycol, 1,2-propanediol, 1,2-butanediol could be characterised by a

curve with single peak; the peak ranged from 0.02 µm to 0.05 µm. The aforementioned alcohols showed the following order with respect to their maximum peak value of the concentration of particles: alcohol ≤ propanol < butanol, ethylene glycol < 1,2-propanediol < 1,2-butanediol. 2) The aerosol particle size distributions (NC) of glycerol and 1,2,4-butanetriol resulted in a curve with a double peak, and the peaks appeared in 0.02-0.05 µm and 0.1-1 µm, respectively. The peak value of particle number concentration of 1,2,4-butanetriol was higher than that of glycerol and was within the range of 0.1-1 µm. 3) The aerosol particle size distributions (NC) of PEG200 and PEG400 also resulted in a curve with a double peak, with the two peaks appearing in ranges 0.05-0.1 µm and 0.1-1 µm. 4) With the increase of glycerol and the decrease of propylene glycol in the e-liquid, the number of large particles in atomised aerosol increased whilst the number of small particles and the particle number concentration in aerosol decreased. The results suggest that the molecular structure and physical properties of alcohols in e-liquids are the main factors influencing the aerosol particle size distribution of e-cigarette vapour.

ST 31

Effect of sucrose on selected toxicant yields in e-cigarettes

MARGHAM J.; McADAM K.

British American Tobacco, R&D Centre, Regents Park Road, Millbrook, Southampton SO15 8TL, U.K.

Background: A plethora of e-cigarette flavours are available to consumers, some of which focus on the sweet palate, including fruit and dessert-like flavours. To achieve these characteristics, manufacturers mainly use mixtures of substances to create an aroma. However, some vapers have a preference for using e-liquids based on natural food-grade ingredients which can contain sugars. A concern with these compounds is their known thermal decomposition properties that can lead to the formation of carbonyls and benzene when heated to temperatures where decomposition can occur. It is possible that the same decomposition may occur in e-cigarettes. In this study, we have investigated the effect of sucrose on these selected analytes to understand whether this ingredient has an effect on the emissions of an e-cigarette.

Methods: Six e-liquids, including a control, were manufactured with varying levels of sucrose concentration. A commercially available modular e-cigarette device was used for testing the e-liquids. Samples were tested both at a 10 w and 20 w setting for 50 puff blocks (80 mL puff volume, 3 second duration, 30 second puff interval, square wave profile). The 20 w setting was used to represent the upper end of the useable range and 10 w represented a controlled power option.

Results: Twenty analytes were measured in aerosol for both power settings. At 10 w, six analytes were shown to increase in quantifiable yield in a linear relationship with sucrose. At 20 w, the quantifiable yields of analytes were higher in comparison to the 10 w setting. Seven analytes were shown to increase linearly in yield with respect to sucrose concentration, in comparison to the control. Significant charring of the wicks was observed with the higher sucrose concentration liquids.

Conclusions: The use of sucrose increases the levels of carbonyls in e-cigarette emissions and could have a negative effect on device performance. Manufacturers should avoid the use of sugar in e-liquids to limit vapers' exposure to these toxicants.

ST 32

Responsible practice in e-vapour products (EVP) stewardship: batch release and post-marketing surveillance

WALELE T.

Imperial Tobacco Limited, 121 Winterstoke Road, Bristol BS3 2LL, U.K.

Awareness and the use of e-vapour products (EVPs) has increased rapidly in recent years. For all its popularity however, there has been a measureable decline in consumer confidence surrounding its use. The number of people in the U.K., for example, who believe EVPs are more harmful than or equally as harmful as cigarettes has risen since 2014, with the number rising from 15.1% in 2014 to 22.1% in 2015. Furthermore, misleading claims and sensationalist headlines have influenced public perceptions of the category. For responsible manufacturers, it is imperative that appropriate stewardship is undertaken to provide reassurance to both consumers and regulatory authorities.

Imperial (Fontem Ventures) has developed a comprehensive product stewardship programme to evaluate and assess its e-vapour products. Experience and knowledge of tobacco products has been harnessed along with expertise from the fields of pre-clinical and clinical assessment. At the 2015 CORESTA Smoke-Techno Meeting, we presented testing approaches and strategies to evaluate EVPs prior to launch; referred to as pre-market product stewardship. In this presentation we will focus on what we believe a responsible manufacturer should do at batch release as part of manufacturing and product quality and post-market product stewardship assessment strategies.

Manufacturing and product quality is designed to ensure that product manufacture takes place under strict controls with high levels of incoming component quality control, standard operating procedures, on the line supervision, batch traceability and routine testing of devices prior to release.

Post-market product stewardship assessment strategies include but are not limited to: consumer behaviour assessment; monitoring potential safety and health effects of products either by conducting clinical trials or collating information via registries; biomarker assessments of smokers switching to EVPs; investigating claims through data interpretation and modelling to ascertain whether EVP could be a gateway to smoking; and the potential impact of EVP use on a bystander.

ST 33

PAHs in mainstream smoke of selected commercial products

PETTIT M.(1); SONGIN K.(1); CARRADUS M.(1); FIEBELKORN S.(2); TICHA J.(2); WRIGHT C.(2)

(1) Marchwood Scientific Services, 371 Millbrook Road West, Southampton SO15 0HW, U.K.
(2) British American Tobacco, R&D Centre, Regents Park Road, Millbrook, Southampton SO15 8TL, U.K.

Mainstream cigarette smoke (MS) contains a number of toxicologically significant chemicals that are of regulatory interest such as polycyclic aromatic hydrocarbons (PAHs). Sixteen PAHs have been included in the US FDA Harmful and Potentially Harmful Constituents (HPHCs) list. All of these 16 PAHs have been previously reviewed by the International Agency for Research on Cancer (IARC), with the majority of them (14) being classified as probably or possibly carcinogenic to humans.

Of the 16 FDA PAHs, robust Margin of Exposure assessments have been generated for only two (benzo[a]pyrene and naphthalene), principally due to a lack of inhalation toxicological data and quantitative smoke chemistry. Availability of such data would allow not only an exposure

risk/threshold assessment but also guide the establishment of expected performance data (including reporting limits) for regulatory reporting methods.

The quantitative measurement of PAHs in cigarette smoke is notoriously challenging due to the complexity of the matrix and their (ultra) low concentration levels. Fit for purpose (sensitive, robust and rugged) analytical methods are required to generate appropriate data.

In the present study, all 16 FDA PAHs were measured in the mainstream cigarette smoke, generated under Health Canada Intense smoking conditions, from nine commercial cigarette products with different blends and tar yields and from University of Kentucky 3R4F Reference Cigarettes. The analytical method comprised the addition of isotope labelled (13C, 2D) internal standards, Accelerated Solvent Extraction (ASE) to ensure exhaustive extraction of the samples, dual stage Solid Phase Extraction (SPE) clean-up and analysis using High Resolution Gas Chromatography coupled to High Resolution Mass Spectrometry. The Limits of Quantification (LOQs) for the PAHs were generally <0.01 ng/cigarette. The generated data will be presented and their use for assessment of potential risk to the consumer will be discussed.

ST 34

Computational modeling of e-vapor aerosol dynamics and deposition in the respiratory tract

ROSTAMI A.A.(1); CASTRO N.(1); PITHAWALLA Y.B.(1); OLDHAM M.J.(1); ZHANG J.(2); LI W.(1); FENG Y.(3)

(1) Altria Client Services LLC, Research, Development and Regulatory Affairs, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.
(2) Eurofins Lancaster Laboratories, c/o Altria Client Services LLC, Research, Development and Regulatory Affairs, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.
(3) School of Chemical Engineering, Oklahoma State University, Stillwater, OK 74078, U.S.A.

It is important for risk assessment of e-vapor products to understand: (1) how much of each aerosol constituent is deposited in the buccal cavity, upper and lower respiratory tracts? and (2) in what form (vapor or liquid) each aerosol constituent is deposited? It is extremely difficult to answer these questions *in vivo* or in human clinical studies, especially considering all possible variations in e-vapor products, e-liquid compositions, and individual usage behavior. A three-dimensional computational fluid dynamics model, using particle tracking approach, has been developed to estimate the vapor and particle deposition rates in the buccal cavity and upper respiratory tract (URT). Vapor-particle interactions, thermodynamic effects, airway humidity, particle growth, and all key deposition mechanisms are accounted for in the computational model. Parallel to the computational method, a physical model of the buccal cavity and URT was used to measure particle growth and vapor and particle deposition. The internal surface of the physical model was kept saturated with water to simulate wet respiratory tract walls. The computational model prediction of particle growth and nicotine deposition compared well with the data measured from the physical model. Sensitivity analyses were performed using the computational model, to estimate the effects of various parameters on the deposition fraction. Results of the sensitivity analysis showed that mouth hold time has a strong effect on the deposition fraction in the buccal cavity. Results from computer simulation models can inform risk assessments and strengthen the scientific understanding of respiratory tract deposition patterns expected from inhaled e-vapor aerosols.

ST 35

Development of a method for trace level analysis in the aerosol from a novel tobacco vaporiser

MASUGI E.(1); SASAMOTO K.(2); OCHIAI N.(2); DAVID F.(3); SANDRA P.(3)

(1) Japan Tobacco Inc., Product Quality Research Center, 6-2 Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan
(2) GERSTEL K.K., 1-3-1 Nakane, Meguro-ku, Tokyo 152-0031, Japan
(3) Research Institute for Chromatography, President Kennedypark 26, Kortrijk 8500, Belgium

In some markets, a variety of tobacco vaporisers is commercially available. One of the characteristics of tobacco vaporisers is that the generated aerosol contains a lower number of combustion-derived chemicals due to the particular heating system which avoids burning of the tobacco. One novel tobacco vaporiser, available on the Japanese market quite recently, generates the aerosol by heating e-liquid mainly composed of propylene glycol and glycerol. The aerosol is then passing through a capsule filled with tobacco in order to deliver nicotine and tobacco aroma. To characterise the trace level compounds in aerosols from tobacco vaporisers, highly resolving and sensitive analytical methods are required.

In this presentation, a method for the separation and identification of the trace level compounds in tobacco vaporisers will be presented.

A thermal desorption (TD) system combined with one-dimensional gas chromatography coupled to a quadrupole mass spectrometer (GC-QMS) was initially used. The aerosol generated by the vaporiser was collected onto a TD tube and injected by desorption into the GC-QMS. However, in order to fully understand the characteristics of trace levels of compounds in the aerosol from the tobacco vaporiser, higher analytical resolution and sensitivity was required. In order to increase the sensitivity, the sample injection volume and the number of puff counts were increased whilst removing propylene glycol and glycerol from the aerosol by applying a Cambridge filter. Moreover, much better performance in terms of resolution and sensitivity was obtained by using two-dimensional gas chromatography coupled to the time-of-flight mass spectrometry (GC×GC-Q-TOF-MS).

More than 100 compounds were detected by TD-GC×GC-Q-TOF-MS in the aerosol from the tobacco vaporiser, which is five times higher than the results obtained by one-dimensional GC-QMS.

ST 36

An evaluation of electronic cigarette formulations and aerosols for HPHCs typically derived from combustion

WAGNER K.A.; AVERY K.C.; BALLENTINE R.M.; BROWN A.P.; FLORA J.W.; JIN X.; MELVIN M.S.

Altria Client Services LLC, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.

In May 2016, the U.S. Food and Drug Administration (FDA) published draft guidance entitled Premarket Tobacco Product Application for Electronic Nicotine Delivery Systems. In this document, FDA recommends reporting the quantities of designated harmful and potentially harmful constituents (HPHCs) in e-cigarettes and formulations (e-liquids). This list contains both tobacco derived constituents such as nicotine and tobacco-specific nitrosamines (TSNAs) and combustion related constituents such as benzo[a]pyrene (B[a]P) and benzene. E-cigarette formulations and aerosols are known to contain trace levels of tobacco derived constituents; however, combustion related HPHCs are not likely to be found due to the relatively low operating temperatures of an e-cigarette relative to a tobacco cigarette. The objective of this work was to utilize highly sensitive and selective methods to determine if three classes of combustion related

HPHCs were detectable in e-cigarette formulations and aerosols. These compounds include three aromatic amines, five volatile organic compounds, and B[a]P. Results from a set of internally prepared reference products and commercially available e-cigarettes and formulations will be presented. The observations from our analysis demonstrate that these compounds are not present at measurable levels in e-cigarette formulations or aerosols. FDA may want to consider results from this and other studies as it makes decisions regarding inclusion of such compounds in a potential HPHC list for e-cigarettes.

ST 37

Determination of 2,3-butanedione, 2,3-pentanedione, and acetoin in electronic cigarette formulations and aerosols by gas chromatography-mass spectrometry

MELVIN M.S.; AVERY K.C.; FLORA J.W.; WAGNER K.A.

Altria Client Services LLC, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.

The presence of the flavor compounds, 2,3-butanedione (diacetyl), 2,3-pentanedione (acetyl propionyl), and acetoin in electronic cigarette (e-cigarette) formulations and aerosols has been reported in the scientific literature. Several methods of analysis for these compounds have been reported in the literature that rely upon sample derivatization with either 2,4-dinitrophenyl hydrazine (DNPH) or o-pentafluorobenzyl hydroxylamine (PFBHA). The purpose of this work was to develop a rapid, sensitive, and reproducible method for the quantitation of these compounds that does not rely upon complex derivatization chemistry. Samples were prepared using a simple bi-phasic extraction prior to analysis by GC-MS. The method demonstrated acceptable linearity for all compounds over a concentration range of 0.01 µg/mL to 3.88 µg/mL with coefficients of determination greater than 0.995. The accuracy of the method was demonstrated through recovery studies of fortified formulations and aerosols resulting in recoveries between 80% and 120% for all analytes. The sensitivity of the method was also evaluated and the method limit of quantitation was determined to be 0.33 µg/g of formulation and 0.67 µg/g of aerosol or 4 ng/puff. The results of the validation indicated that the developed method was fit for purpose for the analysis of the analytes in e-cigarette formulations and aerosols.

ST 38

Nicotine related impurities in e-cigarette cartridges and refill formulations

FLORA J.W.; WILKINSON C.; SINK K.M.; McKINNEY D.L.; MILLER J.H.

Altria Client Services LLC, 600 East Leigh Street, Richmond, VA 23219, U.S.A.

The nicotine used in e-cigarette formulations is extracted from tobacco, and the purity of the nicotine can vary depending upon manufacturer and grade. The U.S. and European Pharmacopeias make recommendations for the purity of nicotine intended for pharmaceutical products; however, there is no official purity recommendation for the nicotine used in e-cigarettes. To date, there are only a few published reports on nicotine-related impurities in e-cigarette formulations. The objective of this work was to develop a sensitive, selective, and robust analytical method (LC-MS) for the quantitation of nicotine-related impurities (LC-MS) in e-vapor products and to evaluate these impurities in a variety of commercial e-cigarette cartridges and refill solutions (e-liquids). The nicotine-related impurities listed in the European Pharmacopeia guidelines were quantitatively investigated in 10 commercial e-cigarette cartridges and 10 refill solutions purchased from retail. For all products investigated, myosmine, anabasine, ß-nicotyrine, cotinine and nornicotine were well below 0.3% of the labelled nicotine concentrations. Anatabine levels exceeded 0.3% for 3 products and nicotine-N-oxide levels exceeded 0.3% in six products. In several cases, some nicotine related impurities increased with product age (as indicated by

product sell-by-dates). The transfer efficiency of the nicotine-related impurities to the aerosol was also evaluated. Most of the nicotine-related impurities were observed to transfer to the aerosol. Nicotine-N-oxide showed low transfer efficiency and demonstrated significant thermal degradation. This selective and sensitive method is suitable to provide quantitative data for risk assessment analysis and for use in e-vapor product and refill solution stability studies as one of the stability indicating measures.

ST 39

The importance of method validation in the determination of 2,3-butanedione in e-liquids

MARTIN A.(1); GILLMAN I.G.(2)

(1) Enthalpy Analytical Inc., 1470 East Parham Rd, Richmond, VA 23228, U.S.A.
(2) Enthalpy Analytical Inc., 800-1 Capitola Dr., Suite 1, Durham, NC 27713, U.S.A.

There are many thousands of flavors potentially used in e-liquids. One particular flavoring of concern is 2,3-butanedione (diacetyl), the inhalation of which is associated with “popcorn lung”. In our experience, the determination of diacetyl and other associated diketones in e-liquid or e-aerosol can be complicated due to the number of other aldehyde and ketone compounds used in the production of e-liquid flavors. The complexity of these samples can easily lead to false positives and it is essential to use validated methods to verify compound identification in this matrix.

The purpose of this study was to evaluate if OSHA Method 1012, using gas chromatography with a non-selective detector, was fit for use for the determination of diacetyl in e-liquids and also to compare some published results with data obtained using our in-house validated method which uses gas chromatography/mass spectrometry (GC-MS). In this study, we analyzed a variety of samples for diketones using the same GC column and derivatization reagent given in OSHA method 1012 but, instead of GC-ECD, we analyzed the samples by GC-MS. Unlike ECD, MS provides confirmation of compound molecular mass and also provides information on the molecular structure. Using these techniques, we found that results obtained were significantly lower than have been reported elsewhere.

While the OSHA method has been validated for determination of acetoin and diacetyl in workplace air, we believe it has not been adequately demonstrated to be fit for use with e-cigarette aerosol. The derivatization reagent, PFBHA, reacts with the wide range of aldehydes, organic acids, and ketones found in flavorings, which can lead to false positives. Based upon our results, we conclude that OSHA method 1012 is not fit for use with the e-liquid matrix due to the complexity of the samples and, instead, only validated, proven fit for purpose methods should be used.

ST 40

Development of on-line coupled LC–GC/MS for analysing flavour constituents in e-liquids

GAO Yihan; ZHENG Saijing; LIU Baizhan

Shanghai Tobacco Group Co., Ltd., Technology R&D Center, Shanghai 200082, P.R. China

A novel fully automated on-line coupled liquid chromatography–gas chromatography/mass spectrometry (LC-GC/MS) method was established to analyse flavour constituents in e-liquids. A pressure switching LC-GC interface was developed by using double Y-connectors. Under gas back-flush mode, intake of solvent vapour into mass spectrometry was completely avoided. In

addition, the high boiling point components could be removed from the capillary column at the conditioning stage, and the separation column could be changed easily dispensing with MS venting. The interface described above took advantage of solvent trapping effects to reduce the loss of volatile components. The established on-line LC-GC/MS was applied to the analysis of flavour constituents in commercial e-liquids. By using a silica LC column and employing pentane/methanol elution, glycerol, propylene glycol and nicotine were removed while the flavour constituents were transferred into the GC/MS. Compared with solvent extraction-GC/MS methods, the proposed isopropanol extraction-LC-GC/MS method avoided interferences by glycerol, propylene glycol and nicotine, while the recovery and detection of polar flavours in e-liquids were not affected. The developed method is simple, accurate and sensitive with good reproducibility and reduced solvent consumption, and is suitable for the analysis of batches of various e-liquids.

ST 41

Analysis of the 'butter-type' flavour compounds diacetyl, acetoin and 2,3-pentanedione in e-cigarette liquids

RIEDEL K.; SCHERER G.; SCHERER M.; PLUYM N.

ABF Analytisch-Biologisches Forschungslabor GmbH, Goethestrasse 20, 80336 Munich, Germany

E-cigarettes have become a popular alternative to combustible cigarettes (CCs) in recent years. E-cigarette liquids (e-liquids) are marketed with different flavourings, from tobacco and common fruit flavours to "exotic" kinds like "magic stardust". This implies the use of a broad range of aroma chemicals, and one flavour may consist of several compounds. Although the majority of flavours used are "generally recognised as safe" (GRAS) food additives, little is known about their potential harm after inhalation. Diacetyl (DA), acetoin (AC), and 2,3-pentanedione (PD) are approved for ingestion and commonly used as food additives to give a butter-like aroma, but also frequently present in e-liquids. However, they have shown adverse health effects when inhaled and therefore, the National Institute on Occupational Safety and Hazards (NIOSH) has proposed exposure limits for DA and PD (NIOSH limits).

This prompted us to develop a method for the simultaneous quantification of DA, AC, and PD based on gas chromatography coupled to mass spectrometry with negative chemical ionisation (NCI-GC-MS) after derivatisation and liquid-liquid extraction.

A lower limit of quantification (LLOQ) of 0.013 µg/g liquid was obtained for all three analytes. Initially, a small set of 26 e-liquids was investigated yielding concentrations above LLOQ for 96% (DA), 73% (AC), and 35% (PD) of the analysed e-liquids proving the suitability of the method for the intended purpose. Assuming a daily e-liquid intake of 3 ml, levels above the NIOSH exposure limit for DA (65 µg/d) were observed for two e-liquids. In contrast, all e-liquids were within the NIOSH limit for PD of 137 µg/d.

The study will be extended to a larger set of e-liquids and to aerosol. Current findings and an interpretation related to potential respiratory effects will be discussed in this presentation.

ST 42

Exhaled aerosol properties in a room following use of electronic cigarettes and conventional cigarettes

CAHOURS X.(1); MARTUZEVICIUS D.(2); PRASAUSKAS T.(2); SETYAN A.(3,4); O'CONNELL G.(5); JULIEN R.(1); WANG J.(3,4); COLARD S.(1)

(1) SEITA-Imperial Tobacco, 48 rue Danton, 45404 Fleury-les-Aubrais, France
(2) Department of Environmental Technology, Kaunas University of Technology, 50254 Kaunas, Lithuania
(3) Laboratory for Advanced Analytical Technologies, Empa (Swiss Federal Laboratories for Materials Science and Technology), 8600 Dübendorf, Switzerland
(4) Institute of Environmental Engineering, ETH Zürich, 8093 Zürich, Switzerland
(5) Fontem Ventures B.V., HN Amsterdam 1083, The Netherlands

Use of electronic cigarettes (e-cigarettes) is rapidly increasing among smokers as an alternative to conventional tobacco cigarettes (CC). As e-cigarettes do not contain tobacco and do not require combustion, the mechanism of aerosol generation within an e-cigarette and CC is fundamentally different. There is little data available on the properties of exhaled e-cigarette aerosols in the scientific literature and as a result there is a growing discussion amongst the public health community as to whether the "particles" exhaled following use of such products has potential implications for indoor air quality. This study aimed to investigate the aerosol properties within a room during use of e-cigarettes and smoking of CC.

A room-simulating chamber with controllable ventilation rates was used with a bystander simulated using a heated mannequin. Human volunteers vaped an e-cigarette or smoked a CC according to a set puffing regime (i.e. 1 puff every 30 seconds; total 5 puffs). Aerosol particle samples were analysed using a Fast Mobility Particle Sizer (FMPS) spectrometer, Electrical Low Pressure Impactor (ELPI), and Scanning Mobility Particle Sizer (SMPS) at the bystander's position. The influence of several parameters was tested during this study: product type, room ventilation rate, and the distance between the volunteer and the bystander mannequin.

Our results suggest that particles exhaled following use of e-cigarettes are liquid droplets mainly composed of water. These particles evaporated very fast and disappeared within 10-15 seconds after the puff and were independent of the ventilation rate. By contrast, combustion particles emitted during smoking of CC were much more stable than those exhaled during e-cigarette use (30-45 minutes), and were dependent upon the ventilation rate. This study shows the clear and substantial differences between exhaled e-cigarette liquid droplets and CC smoke particles and should have a positive implication for continued use of e-cigarettes in indoor areas.

ST 43

Analysis of nicotine and nicotine related substances in electronic cigarette refill solutions and aerosols by liquid chromatography-tandem mass spectrometry

LIU X.; JOZA P.; RICKERT W.S.

Labstat International ULC, 262 Manitou Dive, Kitchener, Ontario, N2C 1L3, Canada

The objective of this study was to develop and validate an analytical method for determining nicotine, and nicotine related compounds (i.e. nicotine-N-oxide, cotinine, nornicotine, anatabine, myosmine, anabasine, and ß-nicotyrine) in e-cigarette aerosols and e-liquids.

Aerosol collection was achieved using a Cambridge collection pad. The sample preparation consisted of adding deuterated internal standards to the collection pad and extracting with 100 mM ammonium acetate solution using a wrist action shaker. The filtrate was then analyzed by LC-MS/MS using a Gemini NX C18 column (3 um, 150×3 mm) with a mobile phase gradient system consisting of acetonitrile and 10% acetonitrile in 10 mM ammonium bicarbonate (pH=8.0)

and electrospray ionization (ESI) in the positive mode. The e-liquid was analyzed using the same instrumental parameters, but simplifying the sample preparation procedure by adding deuterated internal standards to the 100 mg sample and the sample was then extracted with 100 mM ammonium acetate solution, sonicated, and filtrated.

In this study, the method's accuracy, robustness, and reliability were enhanced by using deuterated analogues of each analyte as internal standard and by applying two ion-transition pairs for each analyte for the confirmation and quantification. Validation experiments demonstrated good sensitivity, specificity and reproducibility. All target analyte curves exhibited good linearity from 50 to 5000 ng/ml ($r^2 > 0.995$). The average recoveries for e-liquids varied from 85.2% (nicotine-N-oxide) to 110% (ß-nicotyrine) with all CV < 5.0%. Similarly, the average recoveries for e-cig aerosols varied from 87.8% (for nicotine-N-oxide) to 111% (for myosmine) with all CV < 8.8%. The LOD and LOQ for e-liquids for all target analytes ranged from 0.234 and 0.781 µg/g (cotinine) to 1.66 and 5.48 µg/g (nicotine-N-oxide). For e-cig aerosols these ranged from 0.094 and 0.312 µg/collection (cotinine) to 0.872 and 2.87 µg/collection (nicotine-N-oxide).

ST 44

Development and validation of an ICP-MS method for simultaneous determination of metals in electronic cigarette aerosols

OHASHI S.(1,2)

(1) Japan Tobacco Inc., Product Quality Research Center, 6-2, Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan
(2) JTI-Ökolab GmbH, Koppstrasse 116, A-1160 Vienna, Austria

Due to the rapid gain of market share of electronic cigarettes (ECs), concerns about their safety and quality have been raised over the last few years. In Europe, safety and quality standards for ECs have been introduced regionally. For example, the British Standards Institution (BSI) specified the metals to be monitored in e-liquids and aerosols of ECs[1]. The method for the determination of these metals was validated and reported[2]. In addition, the United States Food and Drug Administration (FDA) reported that safety concerns exist regarding EC user exposure to harmful and potentially harmful constituents (HPHCs). To date, a study for a simultaneous determination method for the metals described in the HPHC list in addition to the metals specified by BSI has not been reported. The purpose of this study was to develop and validate a method for the simultaneous determination of 13 metals (Be, Al, Cr, Fe, Co, Ni, Cu, As, Se, Ag, Cd, Sn and Pb) in EC emissions.

EC aerosol was generated according to CORESTA Recommended Method No. 81 and collected by electrostatic precipitation. Based on Standard Methods 3125, developed by American Public Health Association, the condensate was dissolved in nitric acid and an aliquot of this solution was analysed by inductively coupled plasma mass spectrometry (ICP-MS) equipped with a collision/reaction cell. Requirements for method validation such as linearity, accuracy and precision were evaluated. For example, for each metal the linearity of the calibration curve was verified to yield a correlation coefficient larger than 0.999 for the range from 0.02 to 10 µg/50 puffs. Also matrix effects were evaluated in the presence of glycerol or propylene glycol which ranged from 100 to 300 mg/50 puffs.

[1] British Standards Institution, PAS 54115, 2015
[2] OTTE S., NOWAK S., INTORP M.; CORESTA SSPT 2015, ST 10

ST 45

Soft-ionisation mass spectrometric on-line analysis of e-cigarette and heat-not-burn tobacco product vapours and pyrolysis products

EHLERT S.(1,2); HEIDE J.(2); BENTE v FROWEIN M.(1); WALTE A.(1); ZIMMERMANN R.(2)

(1) Photonion GmbH, Hagenower Str. 73, 19061 Schwerin, Germany
(2) University of Rostock, Dept. of Analytical Chemistry, Dr.-Lorenz-Weg 1, 18059 Rostock, Germany

Within the last few years, e-cigarettes and other new smoking/vaping products have became more and more commonly used. Glycerol (VG) and propylene glycol (PG) are two important ingredients used in e-cigarettes and heat-not-burn (HnB) products either as principal components of e-liquids or as additives in HnB products. A wide range of pyrolysis products as well as thermal fragments of tobacco and tobacco ingredients could be determined with several analytical methods such as GC-MS or NMR. An on-line puff-by-puff analysis of thermal degradation products has the challenge to enable an analyte separation without pre-separation although the starting point of the thermal decomposition is a complex mixture and will provide multiple reaction pathways. Besides a fast sampling, a soft ionisation and an online data acquisition, mathematical methods, such as non-negative-matrix factorisation (NNMF), help to distinguish between different processes taking place during smoking/vaporisation and the respective analysis.

The measurements in this study were mainly performed using a modified Borgwaldt KC LM1 smoking machine coupled to a laser-photo-ionisation time-of-flight mass spectrometer. The modification of the smoking machine refers to 'cold-spot'-free sampling without affecting flow velocities and volumes. Our study was conducted with commercially available vaping as well as smoking products such as e-cigarettes, e-liquids and heat-not-burn products.

Pyrolysis products and thermal fragments such as carbonyls and other relevant classes of substances were investigated on a puff-by-puff basis. NNMF was used as a statistical tool to refer to the source of the detected analyte and to the specific reaction pathway. Especially for HnB products and flavoured e-liquids, analytes/substances besides VG and PG as well as their respective pyrolysis products were determined.

In summary, soft-photo-ionisation mass spectrometry was found to be as powerful for the investigation of emissions of new smoking/vaping products, such as e-cigarettes and HnB products, as it was already demonstrated for conventional combustible products.

ST 46

Determination of e-cigarette aerosol pH using ISFET pH electrode and nicotine absorption by saliva using a glassmouth and saliva pH change

LAUTERBACH J.

Lauterbach & Associates, LLC, 211 Old Club Court, Macon, GA 31210-4708, U.S.A.

E-liquid pH-values continue to be of regulatory interest. Some have used pH-values of aqueous dilutions of e-liquids to estimate pH-values of aerosols generated from those e-liquids, and they have used those pH-values along with the Henderson-Hasselbalch equation to estimate unprotonated nicotine in the aerosols in a manner similar to those that have been used with pH-values observed for mainstream tobacco smoke (MSS). Such approaches suffer from problems obtaining accurate pH-values for dilute aqueous solutions (sometimes suspensions) of e-liquids, the assumption that compositions of e-cigarette aerosols are the same as the e-liquids used to generate them, and compositional differences between e-cigarette aerosols and MSS aerosols. Consequently, two different approaches were developed to determine the pH-values of e-cigarette aerosols. One involved modification of the Health Canada smoke pH method (T-113)

using ISFET or flat/conical electrodes replacing the modified glass electrode. That approach worked (data will be presented), but it does not permit differentiation of multiple-stream versus single-stream devices; and observed pH-values were dependent on aerosol concentration in the T-113 smoke trap. These problems were overcome with use of a glassmouth that has ports for pH-electrodes and a depression for saliva or other absorbent. Systems are based on a IQ Model 150 pH meter and a Hach PH77-SS ISFET pH electrode or a Hanna HI-1053B conical pH electrode; puffing conditions followed CRM No. 81. A multiple-stream device (2.5% nicotine) gave a maximum (25 puffs) pH of 7.7 versus 8.2 for a single-stream device (2.4% nicotine). In another experiment, saliva (5 mL, pH 7.7) was used. Single-stream devices (2.4% nicotine, but with different reported e-liquid pH-values, 4.78±0.09 for Red and 9.42±0.04 for Green) gave approximately the same increase in saliva pH (0.46 versus 0.52 pH units). Other examples showing the utility of pH determinations with the glassmouth will be presented.

ST 47

An alternative strategy for the determination of metals in the aerosol from electronic cigarettes

JOZA P.; DONISA C.; RICKERT W.S.

Labstat International ULC, 262 Manitou Dive, Kitchener, Ontario, N2C 1L3, Canada

Methods for the determination of metals in mainstream cigarette smoke, in general, have limited flexibility and are susceptible to sporadic high levels of background contamination when applied to the collection of aerosol from electronic devices. To address these limitations, a procedure using pad collection principles and a standard linear smoking machine was developed for the analysis of 21 metals (Ag, Al, As, Au, Be, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, Se, Sn, Sr, Ti, W, Zn, Zr) in aerosol from electronic cigarettes.

Multiple collection strategies were investigated with aerosol collection onto a quartz fiber filter providing the lowest, and most consistent, levels of background for most of the metals investigated. The capacity of the collection pad was determined to be approximately 400 mg accumulated aerosol mass. The sample preparation procedure was finalized to a simple extraction with 35 mL of 10% (v/v) ultrahigh purity nitric acid solution. The extract was analysed directly using ICP-MS.

The limits of quantification (LOQ) range from 2 ng/collection for Ag, to 59 ng/collection for Zn. The potential impact on quantification of the metals from the aerosol matrix was investigated by comparing slopes from calibrations built with standard solutions containing 10% methanol (for impact of carbon load), and various ratios of propylene glycol (PG) to glycerol (GLY), with calibrations of standards prepared in 10% HNO_3. For most metals, matrix matched calibration slopes ranged of 80 to 100% of the slope from standards prepared in acid. However, metals such as As and Se, showed significant differences with slopes ranging from 39-56% and 30-43% respectively, dependent on the specific matrix. In cases where quantifiable levels of these metals are identified, a standard additions analysis using aliquots of the original extract would be required for accurate quantification.

For many metals, the background levels found in the collection pad were below the LOQ. However, quantifiable levels of Al, As, Cr, Fe, Mo, Ni, Pb, Sr, W, Zn and Zr were found. Collection of air blanks by drawing room air through the collection pads under the same puffing regime as test samples suggests metals from the environment are insignificant in comparison to the levels measured. The collection pads showed some lot-to-lot, as well as package-to-package variability within the same lot. However, pads within a single package showed very consistent background levels. Strategies for handling test sample results and background will be further discussed. This procedure can reduce the variability in the background which could mask the deliveries of metals in aerosol from electronic cigarettes.

ST 48

The proposed use of human cells to cover the 'R' end point for EUTPD2 data requirements from a 21st century toxicology perspective

SIMMS L.(1); PALMER J.(2)

(1) Imperial Tobacco Limited, 121 Winterstoke Road, Bristol BS3 2LL, U.K.
(2) Stemina, 504 South Rosa Road, Suite 150, Madison, WI 53719, U.S.A.

In 2007 the National Academies of Sciences released "Toxicity Testing in the 21st Century: A Vision and a Strategy", a new toxicological paradigm, moving away from the idea of high dose animal tests as the 'gold standard' and focusing on the use of human cell lines.

The European Tobacco Product Directive (2014/40/EU), requires data for priority additives regarding Carcinogenic, Mutagenic or Reproductive (CMR) properties either in the neat form, or increasing the CMR properties of the combusted product to a significant or measurable degree.

Traditional reproductive toxicity testing utilises a significant proportion of the animals used for toxicity testing, with a limited ability to predict reproductive toxicity in humans. Previous reproductive studies with rats exposed to either 1R4F or 2R4F cigarette smoke, exhibited no adverse effects on various reproductive endpoints in a multi-generational study. The only treatment related effects were decreased body weight gain in both dams and pups, associated with delayed ossification of occipital bones and sternebrae.

A human developmental toxicology screening assay is considered more appropriate to look for the possible effects of additives on humans than animal related assays, due to known species differences.

Stemina have developed a metabolomics based assay using human Induced Pluripotent Stem Cells (IPSCs) looking at the disruption in the ratio of two key amino acids (ornithine and cysteine), as a biomarker of development toxicity. The assay has a high concordance with both human developmental toxicants and non-developmental toxicants (82% overall, 0.71 sensitivity and specificity 1.0). This assay has been used by the United States Environmental Protection Agency (EPA) as part of ToxCast library for >1000 chemicals in support of Tox21. This is the first time a cigarette smoke condensate has been used in the assay, however, it appears to be able to measure the relative developmental toxicity potential of tobacco condensates.

ST 49

Changes in e-cigarette aerosol during transport for toxicology studies

TINDALL I.; JONES C.; COLE A.

Cerulean, Rockingham Drive, Milton Keynes, U.K.

Emerging regulation of e-cigarettes may require that cell cultures and the like are exposed to whole e-cigarette aerosols. Part of the mechanics of this process involves first the generation of the aerosol and then the transport to the exposure system, the desire being that the aerosol used on the cell culture to be substantially similar to that generated at the mouth end of the e-cigarette.

An ideal puffing system was constructed and its performance explored using a number of different "cig-a-like" products A to G.

Firstly the mass lost in the system was examined and then the change in aerosol droplet size distribution through the puffing process. Brand to brand differences were observed as were differences associated with the experimental set up and puffing parameters.

Mass losses through the equipment were surprisingly low, between 3% and 7% by weight of the total being retained in the system. A "priming" process was observed where losses diminished with puffing.

Aerosol droplet size distribution varies on the basis of brand and puffing conditions at the mouth end of the e-cigarette and this has an observed impact on droplet size distribution at the exhaust of the system. When the median droplet diameter exceeded a defined size there was a noticeable loss of larger droplets through the system. Where the median distribution was below this limit the distribution of droplets at the exhaust was largely maintained unless the exhaust path was lengthened where upon an increase in larger droplets was observed presumably due to inelastic droplet/droplet collisions within the exhaust path.

The significance of these findings is discussed in the context of equipment design for use in non-clinical studies.

ST 50

Between vapour and smoke? Prooxidant activity of electronic cigarette emissions

TROFIMOV A.V.; MENSHOV V.A.

Emanuel Institute of Biochemical Physics, Russian Academy of Sciences, ul. Kosygina 4, Moscow 119334, Russian Federation

Electronic cigarettes (e-cigs) are considered among the most promising tools designed for the reduction of a potential harm derived from smoking. Nevertheless, debates exist over the scientific evidence for claims that e-cigs have no health-related ramifications. In this context, the purity of constituents of e-liquids, which are the solutions that are heated up and converted to an aerosol and inhaled by e-cig users, are in the spotlight. But more important are possible chemical changes of the e-liquid components in the e-cig atomiser and the formation of species potentially active towards biomolecules. The objective of this study was to examine such a possibility. In the present pilot work, we investigated the generation of prooxidants in emissions derived from e-cigs available on the local market (Russian Federation) through harnessing the redox-sensitive chemiluminescence probes. Delivering the nicotine vapour while using e-cigs proceeds without combustion products responsible for most of the damaging impacts in a human organism; however, heating the e-liquids may account for the generation of reactive oxygen species (ROS) and other prooxidants, which may trigger certain oxidative developments *in vivo*. Our study has revealed that heating the aerated mixtures of propylene glycol, glycerin and nicotine in e-cigs atomisers with coils from various metals indeed causes activation of molecules with the subsequent formation of reaction products, which exhibit prooxidant activity as manifested by the chemiluminescence derived from model oxidation processes. The plausible mechanisms of the formation of such reactants, as well as the possibility of the oxidative stress developments in e-cigs users are discussed. The results of this work should be of interest for improving the design of e-cigs and optimising their functioning.

ST 51

Preliminary investigation on interaction mechanism between nicotine and pepsin

HAN Jingmei(1); LIAO Xiaoxiang(1); YUAN Dalin(1); LEI Ping(1); ZHENG Xudong(1); FAN Ying(1); SHANG Shanzhai(1); LI Zhiqiang(1); TANG Jianguo(1); LI Hui(2); CHEN Yongkuan(1)

(1) Non-traditional Tobacco Products Institute, Technology Center, China Tobacco Yunnan Industrial Co., Ltd., Kunming 650231, P.R. China
(2) Sichuan University, School of Chemical Engineering, Chengdu 610064, P.R. China

In order to investigate the absorption characteristics of the human stomach to nicotine (NIC) originating from tobacco products for oral use, different concentration levels of nicotine in a citric acid and sodium citrate buffer solution (pH = 2.0) were used to stimulate gastric conditions. After

interaction of pepsin with the different levels of nicotine, the spectrum changes of the pepsin before and after addition of different levels of nicotine were observed by various spectroscopic methods (i.e. fluorescence spectrum, ultraviolet spectrum, infrared spectrum, circular dichroism spectrum) and molecular docking simulation technology. Consequently, the action mechanism between nicotine and pepsin was preliminarily studied. The results of spectroscopy and molecular docking simulation indicated that: 1) The quenching mechanism of NIC on pepsin was static fluorescence quenching; 2) NIC and pepsin interacted spontaneously via hydrogen bonding and van der Waals forces; 3) There existed a single high-affinity binding site between NIC and pepsin; 4) The interaction between NIC and pepsin not only increased the polarity of the microenvironment of amino-acid residues in pepsin, but also changed the C=O, C-H and N-H of polypeptide chain of pepsin, which further resulted in the variation of conformation and spatial structure of pepsin; at the same time NIC obviously promoted the activity of pepsin when it was added into pepsin at different concentrations. The results from the study provide references for the development, quality control and health risk evaluation of tobacco products for oral use.

CORESTA CONGRESS 2016

SMOKE SCIENCE and PRODUCT TECHNOLOGY

POSTERS

STPOST 01

Determination of benzo[a]pyrene in electronic cigarette formulations and aerosols by gas chromatography–mass spectrometry

BALLENTINE R.M.; AVERY K.C.; FLORA J.W.; WAGNER K.A.

Altria Client Services LLC, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.

In May 2016, the U.S. Food and Drug Administration (FDA) published draft guidance entitled Premarket Tobacco Product Application for Electronic Nicotine Delivery Systems. In this document, FDA recommends reporting the quantities of designated Harmful and Potentially Harmful Constituents (HPHCs) in e-cigarettes and formulations (e-liquids). B[a]P is listed on the FDA Premarket Tobacco Product Application (PMTA) HPHC list. This work describes a sensitive and selective method for determining B[a]P in both formulations and aerosols. Formulation samples were prepared using a one-step biphasic extraction procedure. Aerosol samples were collected on Cambridge filter pads (using the puffing regimen of 55 cc puff, 5 second duration) which were subsequently extracted with toluene. Prepared samples were analyzed by gas chromatography-mass spectrometry (GC-MS) in selected ion monitoring mode. The method demonstrated acceptable linearity over a concentration range of 0.5 ng/mL to 50 ng/mL with a coefficient of determination of 0.995 or higher. The accuracy of the method was demonstrated through recovery studies of fortified formulations and aerosols resulting in recoveries between 80% and 120%. The method limit of quantitation was determined to be 2.5 ng/g of formulation and 50 ng/g of aerosol or 0.2 ng/puff.

STPOST 02

Determination of volatile organic compounds (VOCs) in electronic cigarette formulations and aerosols by gas chromatography-mass spectrometry

BROWN A.P.; AVERY K.C.; FLORA J.W.; WAGNER K.A.

Altria Client Services LLC, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.

In May 2016, the U.S. Food and Drug Administration (FDA) published draft guidance entitled Premarket Tobacco Product Application for Electronic Nicotine Delivery Systems. In this document, FDA recommends reporting the quantities of designated Harmful and Potentially Harmful Constituents (HPHCs) in e-cigarettes and formulations (e-liquids). This work describes a sensitive and selective method for determining the five volatile organic compounds (VOCs) listed on the FDA Premarket Tobacco Product Application (PMTA) HPHC list in both formulations and aerosols. The VOCs include 1,3-butadiene, isoprene, acrylonitrile, benzene and toluene. Preparations of formulations require a simple dilution prior to analysis. Aerosol samples are collected using a Cambridge filter pad (using the puffing regimen of 55 cc puff, 5 second duration) followed by a single fritted impinger containing 20 mL of cryogenically cooled methanol.

Samples are analyzed by GC-MS in selected ion monitoring mode (SIM). The method demonstrated acceptable linearity for all compounds over a concentration range of 0.05 to 20 µg/mL with coefficients of determination ≥ 0.995. The accuracy of the method was demonstrated through recovery studies of fortified formulations and aerosols resulting in recoveries between 80% and 120% for all analytes. The method limit of quantitation was determined to be 1.7-16.7 µg/g in formulation and 0.04-0.4 µg/puff in aerosol for the five VOCs.

STPOST 03

E-cigarette aerosol collection: 44 mm Cambridge filter pad capacity

MILLER J.H.; WILKINSON J.; SPICER-GLAVE S.; FLORA J.W.; GARDNER W.P.

Altria Client Services LLC, 600 East Leigh Street, Richmond, VA 23219, U.S.A.

CORESTA has developed recommended (or standardized) methodologies to measure most of harmful and potentially harmful constituents (HPHCs) in cigarette smoke. CORESTA Recommended Methods (CRMs) specify the collection of cigarette smoke under standardized conditions referred to as ISO and/or Canadian Intense (CI) conditions where smoke particulate matter is trapped on 44 mm Cambridge filter pads (CFP). Many of the methodologies being developed to collect and measure constituents in e-cigarette aerosols are based on CRMs developed for cigarette smoke. However, there are fundamental differences between the processes of generating tobacco cigarette smoke compared to e-cigarette aerosol. For example, cigarettes are burned and the puff counts collected under standardized puffing regimes range from ~5 to ~14 puffs per cigarette depending upon design and puffing conditions. E-cigarettes provide far more puffs than conventional cigarettes and it is common to collect up to or more than 100 to 150 puffs per device with 5 and 3 second puff durations, respectively. The purpose of this work was to evaluate the collection capacity of 44 mm CFP relative to e-cigarette aerosol mass (AM), nicotine, and menthol. Results showed that the CFP could collect 650 mgs of AM without measurable breakthrough. When using a 5 second puff (the maximum puff duration for conventional cigarette smoking machines), a 55 cc puff volume, and a 30 second puff interval, a linear increase was observed for AM, nicotine, and menthol. This was observed when measuring 20 puff increments, using new devices per incremental collection, and collecting up to 650 mgs of AM. Similar results were observed for different puff durations (e.g. 3 seconds).

STPOST 04

Impact of different vaping machines on metal contaminations of e-cigarette aerosols

OTTE S.; NOWAK S.; INTORP M.

Reemtsma Cigarettenfabriken GmbH (an Imperial Brands PLC Company), Albert-Einstein-Ring 7, D-22761 Hamburg, Germany

The presence of trace metals in e-liquids or aerosol of electronic cigarettes (e-cigarette) has been previously reported[1]. It has also been demonstrated that contamination with tin, aluminum, copper, iron and nickel may occur from certain parts of a standard smoking machine[2]. Therefore, the risk of a possible transfer of trace metal from e-liquids or components of e-cigarette devices into aerosol needs to be carefully investigated.

In this study, both rotary and linear smoking machines fitted with impingers and electrostatic precipitation trapping systems were used for sample collection. For the quantification of trace metal concentration levels in aerosols, the fully validated method as presented during the CORESTA Smoke/Techno Joint Study Groups Meeting 2015 was applied. The measurements

encompassed seven metals including aluminium, nickel, iron, chromium, copper, tin and silver and were performed with an Inductively Coupled Plasma - Optical Emission Spectrometer (ICP-OES).

The aerosols were generated from clearomiser samples by applying vaping conditions according to the CORESTA Recommended Method (CRM) No. 81.

The quantification was carried out using Yttrium as an internal standard. For the e-aerosol emissions, the limits of quantification (LOQ) ranged from 0.003 µg/10 puffs (copper, iron) to 0.04 µg/10 puffs (tin).

In this study, metal contaminations could be detected in aerosol and blank samples on a similar level when the investigated smoking machines were used. The results obtained by the rotary and linear type devices and the different trapping systems will be compared and discussed along with suggestions to reduce possible contamination sources.

[1] M. Williams, A. Villarreal, K. Bozhilov, S. Lin, P. Talbot; PLOS ONE; Volume 8; Issue 3, 2013
[2] Otte S., Nowak S., Intorp M.; Method Development and Validation: Quantification of Metals in Liquids and Aerosol of e-cigarettes; CORESTA Congress Presentation ST 10; 2015

STPOST 05

Simultaneous determination of nicotine and related impurities in e-liquids using UPLC-UV-MS

MERUVA N.(1); BENVENUTI M.(1); McCALL E.(2); CLELAND G.(1); BURGESS J.(1)

(1) Waters Corporation, 34 Maple Street, Milford, MA 01757, U.S.A.
(2) Waters Corporation, Altrincham Rd, Wilmslow SK9 4AX, U.K.

A dilute and shoot method for simultaneous determination of nicotine and related impurities in e-liquid formulations by UPLC-UV-MS has been developed for routine QC testing of e-cigarette formulations. Due to the differences in the concentration of nicotine (mg level) and related impurities (µg level) in e-liquids, two separate analyses are typically performed for nicotine using GC-FID or LC-UV and for impurities using GC-MS or LC-MS/MS.

E-liquid manufacturing and nicotine purity standards are currently being established worldwide. As nicotine used in e-liquids is extracted from tobacco, the extracted nicotine contains varying amounts of related alkaloid impurities. The concentration of nicotine and related impurities in e-liquids and e-cigarettes is known to change during the product shelf-life as nicotine degrades or gets oxidized to nicotine-n-oxide. The American E-liquid Manufacturing Standards Association (AEMSA) recommends using USP or certified nicotine with purity greater than or equal to 99%, with nicotine-n-oxide less than or equal to 1% and total contaminants less than or equal to 1%.

Simultaneous determination of nicotine and related impurities in e-liquids was achieved using a seven minute UPLC method and two detectors (UV and MS). A wide linear dynamic range for nicotine (2.5-500 µg/mL) using PDA detector and for related impurities (0.005-0.5 µg/mL) using MS detector was used. Six different commercially available e-liquids and e-cigarette cartridges were analyzed. E-liquid samples were diluted 100-fold before analysis, eliminating the need for multiple methods.

The measured nicotine levels in e-liquids were observed to be within 15% of labeled claims. The levels of impurities in e-liquids ranged from not detected to 2.79% of nicotine concentration. Single quadrupole MS detection can be incorporated into existing LC workflows to provide orthogonal detection to UV detectors for simultaneous analysis of nicotine and related impurities on a single instrument platform in a single injection.

STPOST 06

Determination of tobacco-specific nitrosamines in electronic cigarette liquids and aerosols by UPLC/MS/MS

JIN X.; AVERY K.C.; FLORA J.W.; WAGNER K.A.

Altria Client Services LLC, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.

Electronic cigarette (e-cigarette) formulations typically contain tobacco derived nicotine and therefore, may contain other tobacco related components, such as tobacco specific nitrosamines (TSNAs). Previous research using analytical methodologies developed for cigarette filler and smoke has shown that TSNAs can be detected in e-cigarette formulations and aerosols at trace levels. The levels found in e-cigarettes are far lower than those found in tobacco and smoke and are often below the limit of quantitation when using methods designed for tobacco products. The purpose of this work was to develop a sensitive and rapid method specifically designed to measure low levels of TSNAs in e-cigarettes using Ultra-Performance Liquid Chromatography (UPLC) with tandem mass spectrometry detection (MS/MS). Linearity was demonstrated with coefficients of determination of greater than 0.995 for the calibration ranges of 0.1 ng/mL-20 ng/mL for NNK and 0.25 ng/mL to 20 ng/mL for NNN. The recoveries for NNK and NNN were 90%-120% for fortified formulations and aerosols. The method limits of quantitation were determined to be 2 and 5 ng/g of formulation for NNK and NNN, respectively. The limits of quantitation for aerosol were determined to be 0.04 ng/puff and 0.1 ng/puff for NNK and NNN, respectively.

STPOST 07

Is a method to determine polycyclic aromatic hydrocarbons in cigarette smoke applicable to aerosols generated from heated tobacco products?

GADOIS-POMMEREUL S.; BRU G.; PERROT R.; TROUDE V.

SEITA-Imperial Tobacco, 48 rue Danton, 45404 Fleury-les-Aubrais, France

The market offers various new generation products, including heated tobacco products. In order to measure constituents in the aerosol of such products, methods developed for cigarette smoke were investigated for their applicability.

This study is focused on the determination of polycyclic aromatic hydrocarbons (PAHs) in aerosols generated from heated tobacco products.

We have developed and validated a PAH method in cigarette mainstream smoke using ISO and intense smoking regimes by applying GC-MS/MS[1]. The purpose of this study was to investigate how to adapt a method originally developed and validated for the measurement of PAHs in mainstream cigarette smoke condensate for the measurement of aerosols from heated tobacco.

Potential matrix effects and sources of contamination were a focus of this study as PAH levels are likely to be significantly lower compared to yields obtained from cigarette smoke.

An experiment in which aerosols collected on Cambridge filter pads (CFP) were spiked with PAHs was carried out in order to clarify these questions.

Furthermore, when the accuracy and precision of the adjusted method were compared with the original validation data obtained from cigarette smoke it was found that the tolerance intervals were comparable to those determined for cigarette smoke condensates. However, as the PAH emissions of such products are expected to be significantly lower than those in smoke condensates, particular attention was given to analytical criteria such as blank levels, signal/noise ratio (S/N) and specificity. These analytical criteria will be highlighted for each of these different types of products.

[1] V. Troude et al, HPHCs-validation of method: limitations in the “accuracy profile”, 68th TSRC, 2014

STPOST 08

Analysis of 12 polycyclic aromatic hydrocarbons compounds in aerosol

CORREIA D.; ROTA D.; SAUSER Y.; LE BOUHELLEC S.

Philip Morris International, Research & Development (R&D) Innovation Cube, Quai Jeanrenaud 5, CH-2000 Neuchâtel, Switzerland

Polycyclic aromatic hydrocarbons (PAHs) comprise a large class of chemical compounds known to be carcinogenic. These compounds are present in cigarette smoke, normally at low concentrations (ng/cig or lower).

The purpose of this study was to improve and re-validate a method for the determination of PAH compounds in cigarettes smoke and in aerosol from potential Reduced-Risk Products[1] (RRPs).

This method comprises the determination of 12 PAHs (pyrene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[j]fluoranthene, benzo[a]pyrene, indeno[123-cd]pyrene, dibenz[a,h]anthracene, dibenz[a,l]pyrene, dibenz[a,e]pyrene, dibenz[a,i]pyrene, dibenz[a,h]pyrene) using gas-chromatography with mass spectrometer detection using electron ionization mode (GC-EI-MS) and selected ion monitoring (SIM).

Kentucky reference cigarette 3R4F mainstream smoke and potential RRP (*iQOS*) mainstream aerosol were generated under Health Canada Intense smoking regime in linear smoking machines and particulate matter collected in glass fiber filter pads (CFP). Due to the high complexity of the matrix, an optimided two step solid-phase extraction (SPE) clean-up procedure is applied to whole extract for removal of interferences and trace-enrichment of PAHs. The clean-up procedure was optimised and transferred to an automated system, increasing the throughput of the method.

As a result of the modifications to the previous methodology, linearity problems were corrected, peak shape and resolution for the different target analytes were significantly improved, including for the high molecular weight isomers present at levels of concentration below 1 ng/cig. The improved baseline and chromatographic peak profile allowed a better resolution, easier identification and integration of the compounds of interest.

The method of analysis of 12 PAHs was shown to be selective, precise, linear and accurate. Lower limits of quantification (LOQ) were achieved with validated methodology. Percent reduction of PAHs in *iQOS* to Kentucky 3R4F was between 92% and 98%.

[1] Reduced Risk Products (RRPs) is the term used to refer to products with the potential to reduce individual risk and population harm in comparison to smoking cigarettes.

STPOST 09

Analysis of mercury in mainstream smoke or aerosol

HOFER I.; YERLY M.; MARCHESE A.; LE BOUHELLEC S.

Philip Morris International, Research & Development (R&D) Innovation Cube, Quai Jeanrenaud 5, CH-2000 Neuchâtel, Switzerland

Mercury has been identified as a harmful, or potentially harmful, constituent of tobacco smoke. For this reason, methods for quantifying the mercury content in the mainstream smoke of cigarettes, or in the aerosol of potential Reduced Risk Products[1] (RRPs), are of interest.

The purpose of the developed method is to quantify mercury in the mainstream smoke of cigarettes and in the aerosol of potential RRPs by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

The mercury content in the particulate and gas vapour phases of mainstream smoke from Kentucky reference cigarette 3R4F and the aerosol of potential RRPs (*iQOS*) was evaluated. The

mainstream smoke was generated under ISO smoking regimen. The particulate phase was collected using an electrostatic precipitation trap and in parallel the vapour phase was trapped using two impingers. After collection, both phases were analysed by ICP-MS and compared. The mainstream smoke and the aerosol were trapped in an acid solution and analysed by ICP-MS.

Only mercury in the gas vapour phase could be quantified whereas the signal of mercury in the particulate phase was found close to those of blank solutions and was not measurable. The nominal content obtained under Health Canada (HC) and ISO smoking regimens was respectively 4.5 ng/cig and 2.0 ng/cig for 3R4F and 1.1 ng/item and 0.45 ng/item for RRPs.

The method has been validated for the mercury quantification in the gas vapour phase of mainstream smoke from Kentucky reference cigarette 3R4F and the aerosol of potential RRPs (*iQOS*) collected under HC and ISO smoking regimens, analysed by ICP-MS. Validation results demonstrated the selectivity, precision, linearity and accuracy of the method (from 25 pg/mL to 1000 pg/mL).

[1] Reduced Risk Products (RRPs) is the term used to refer to products with the potential to reduce individual risk and population harm in comparison to smoking cigarettes.

STPOST 10

Analysis of the polyphenols of tobacco using pressurized liquid extraction (PLE) and ultra performance liquid chromatography with electrospray ionization – tandem mass spectrometric detection (UPLC-ESI-MS/MS)

SHIFFLETT J.R.; WATSON L.; McNALLY D.J.; BEZABEH D.Z.

United States Department of the Treasury, Alcohol and Tobacco Tax and Trade Bureau (TTB), 6000 Ammendale Road, Beltsville, MD 20705, U.S.A.

Tobacco curing is a drying process that is used to transform recently harvested tobacco leaves into material suitable for incorporation into consumer products such as cigars and cigarettes. Different types of tobacco (bright, Burley, Oriental, etc.) are cured by different methods (air-curing, flue-curing, sun-curing) to achieve distinct organoleptic properties in the dried leaf or the smoke produced from burning the dried leaf. Additionally, the curing method affects the chemical profile of the dried leaf and significant variations can be observed between leaves processed under different curing conditions. Polyphenols are chemicals found in tobacco that are affected by the method used to cure the leaf. The purpose of this work was to develop an analytical method to investigate the levels of six polyphenols found in tobacco leaves and tobacco products: 3-O-Caffeoylquinic acid (Chlorogenic acid), 4-O-Caffeoylquinic acid (Cryptochlorogenic acid), 5-O-Caffeoylquinic acid (Neochlorogenic acid), Kaempferol 3-O-rutinoside (Nicotiflorin), Quercetin 3-O-rutinoside (Rutin), 6-Methoxy-7-hydroxycoumarin (Scopoletin). Extraction conditions for sample preparation using PLE and instrument conditions for analysis by UPLC-MS/MS were optimized and validated. Results from the analysis of 30 cured tobacco leaves and various tobacco products are presented and discussed in the context of each curing method represented. Total polyphenol levels for flue-cured, Oriental, and air-cured leaves were determined to be in the ranges of 18-41 mg/g, 5-27 mg/g, and 0.5-3 mg/g respectively. Additionally, cigarette polyphenol levels were found in the range of 4-16 mg/g and cigar polyphenol levels were less than 1.5 mg/g. The trends observed in the results reflected the type of curing process used or the composition of cured tobacco blends traditionally used in each type of product.

STPOST 11

Quantitative discrimination of the flavour type of flue-cured tobacco and correlation between flavour type and planting area

LI Chao(1); MIAO Mingming(1); QIN Yunhua(1); LI Exian(2); WU Yiqin(1); XU Yong(1); YANG Guangyu(1); ZHANG Chengming(1)

(1) Cigarette Product Quality Inspection Institute, Technology Center, China Tobacco Yunnan Industrial Co., Ltd., No. 181, Hongjin Road, Kunming 650231, P.R. China
(2) Institute of Biotechnology and Genetic Resources, Yunnan Academy of Agricultural Sciences, Kunming 650223, P.R. China

To study the relationship between chemical composition and flavour type of flue-cured tobacco, 500 tobacco samples were collected from 29 areas in 14 Chinese provinces in 2012. A classical sampling method was applied. Following relevant methods described in industrial standards and the published literature, we determined 117 chemical indicators which significantly affect the quality of flue-cured tobacco. The obtained data were processed by using multiple factor analysis for dimension reduction. A Bayes quantitative discriminant model was established and verified according to the scores of factors. A correspondence analysis between flavour type and planting area was also conducted. The results showed that 24 common factors, extracted from the original indicators, could explain 83.7% of the total variance. The quantitative discriminant model accurately predicted the flavour type of tobacco leaves on the basis of megastigmatrienone, furan, alkali and other substances. The accuracies of internal cross validation and external validation were ≥ 89.5% and ≥ 97.0%, respectively. The results of the correspondence analysis between flavour type - discriminated by the established model - and planting areas of the tobacco were generally in line with the results of sensory evaluation. Among the leaves from 14 provinces, those from Yunnan represented best the typical fresh flavour style, followed by those from Fujian and Sichuan; for leaves of medium flavour style, the planting areas were ranked as follows: Chongqing > Guizhou > Heilongjiang > Hubei; the robust flavour style was best in Henan, followed by Anhui, Guangdong, Jiangxi, Shaanxi, Shandong, Hunan. The discriminant model proved to be suitable for a fast and objective quality evaluation of tobacco leaves.

STPOST 12

Determination of tobacco-specific N-nitrosamines (TSNAs) in mainstream cigarette smoke by heart-cutting two dimensional liquid chromatography–tandem mass spectrometry (2DLC-MS/MS)

CHEN Min; WANG Leijun; QI Dawei; LIU Baizhan; WU Da

Shanghai Tobacco Group Co., Ltd., Technology R&D Center, Shanghai 200082, P.R. China

Accurate results are often hard to obtain in the determination of tobacco-specific N-nitrosamines (TSNAs) in mainstream smoke of Virginia type cigarette due to the low deliveries of TSNAs and the complicated matrix disturbance; therefore, a novel heart-cutting two dimensional liquid chromatography–tandem mass spectrometry (2DLC-MS/MS) method was developed. The method offers simple sample preparation involving only extraction and filtration, with almost no matrix interference and low quantitative limits compared with traditional methods. A strong cation exchange (SCX) column is utilised for first dimensional separation, which effectively removes the acidic and neutral components in smoke. To reserve TSNAs on the trap column, a compensate pump is applied for on-line dilution and pH adjustment during the period of TSNAs fraction transfer and enrichment. Next, a C18 column is employed for 2nd dimensional separation and coupled to tandem mass spectrometry under multiple reaction monitoring (MRM) mode. The 2DLC-MS/MS method with isotope deuterated internal standards is applied to the determination of TSNAs levels in mainstream cigarette smoke, the limits of detection of NNN, NAT, NAB and NNK were 0.023, 0.028, 0.019 and 0.028 ng/cig, and their spiked recoveries ranged from 93.6%

to 108.6% with RSDs within 5.4%. The developed method is simple, automatic, sensitive, accurate and reliable; it is suitable for determining the deliveries of TSNAs in mainstream smoke of cigarette, especially Virginia type cigarettes.

STPOST 15

Indoor air chemistry: an exploratory study on e-cigarettes shows no negative impact on indoor air quality

GOUJON GINGLINGER C.; MITOVA M.; MOTTIER N.; ROUGET E.; THARIN M.; MAEDER S.

Philip Morris Products S.A. (part of Philip Morris International group of companies), PMI R&D, Quai Jeanrenaud 5, CH-2000 Neuchâtel, Switzerland

The impact on indoor air quality when using e-cigarettes is expected to be very different to cigarette use and has been subject of numerous research papers. The published literature is often of somewhat limited value in evaluating the impact of e-cigarettes on indoor air quality since the experiments were either conducted with smoking machines, based on theoretical calculations (i.e. modelling experiments) or performed in rooms with limited control. Philip Morris International has built an environmentally controlled, furnished room and developed analytical methods to measure air pollutants under diverse simulated indoor environments focusing on: (i) ISO measurement standards for Environmental Tobacco Smoke and, (ii) selected carbonyls and volatile organic compounds. The room is fully controlled and adjustable in terms of air renewal and the analytical methods have been developed, validated and accredited under ISO 17025. An exploratory study on Indoor Air Quality for e-cigarettes was performed focused on relevant analytes in the context of e-cigarettes, i.e. particulate matter, nicotine and selected carbonyls. Three different e-cigarette products were tested, representing a range of e-liquid compositions and product designs. Multiple replicates with panelists, under one simulated condition (residential with air renewal set at 1.2 per hour, according to CEN-EN 15251:2007) were performed, including “background” sessions (no products used), against which vaping results are compared. During vaping sessions, panelists used the assigned product once every 40 minutes for 10 minutes duration over the course of 5 hours. Results show that all analytes measured when e-cigarettes are used were not different from background levels, with the exception of nicotine, glycerin and propylene glycol. Nicotine levels ranging from 0.3 µg/m^3 to 6.5 µg/m^3 were observed and were directly correlated with the amount of e-liquid consumed during the different sessions. Based on these results, we conclude that using e-cigarettes indoor does not negatively impact air quality.

STPOST 16

The stability of diacetyl and acetyl propionyl and the conversion of acetoin to diacetyl in e-liquids

VAS C.; MARGHAM J.; WATERS G.; SANCHEZ M.; COSTIGAN S.; McADAM K.

British American Tobacco, R&D Centre, Regents Park Road, Millbrook, Southampton SO15 8TL, U.K.

Background: Diacetyl (DA) and acetyl propionyl (AP) have been reported to be used in many e-liquid flavours. Inhalation of DA in occupational settings has been shown to lead to the onset of a decline in respiratory function and a condition known as *bronchiolitis obliterans*. Limited evidence suggests that AP may have the potential to act in the same way upon inhalation. Acetoin (AC) has been used as an alternative to DA/AP in e-cigarette liquids, but while the toxicological data on it shows little of concern, its chemical similarity to DA means that its

conversion to DA could occur. This study was conducted to understand the stability of these ingredients, and whether AC is a precursor to DA.

Method: Part One: e-liquids were spiked with DA and AP and analysed by GC-MS 18 days after spiking. Part Two: e-liquids were spiked with AC; samples were analysed by GC-MS on the day of spiking, and additional time points for up to eight weeks. All measurements were completed by Enthalpy Analytical Inc.

Results and Conclusions: Part One: Substantial reductions in the concentration of both DA and AP in the e-liquid were observed after 18 days. Part Two: For the eight week time course study, conversion from AC to DA was not measurable in nicotine-free e-liquids. However, nicotine-containing e-liquids showed clear conversion of AC to DA over this time period.

Conclusion: DA and AP have limited stability in e-liquid formulations. This work has shown that AC is a precursor to DA in nicotine containing e-liquids. Action should be taken by e-liquid manufactures and flavouring suppliers to eliminate the use of DA, AP and AC as flavour ingredients.

STPOST 17

Determination of leakage in e-vaping products

GOKSEL H.; JACQUES T.

Philip Morris Products S.A. (part of Philip Morris International group of companies), PMI R&D, Quai Jeanrenaud 5, CH-2000 Neuchâtel, Switzerland

An important quality aspect of e-vaping products is the absence of liquid leakage from the cartridge. Therefore it is highly important to verify cartridge integrity with a reliable method.

Various external conditions could influence product leakage and should be considered from the design phase, to the manufacturing and use of the product.

Currently AFNOR XP D90-300-1 is the best known standard for leakage testing of e-vaping consumables. As per this standard, absence of leakage of an e-vaping product is verified by placing the consumable on an absorbent paper, upside-down vertically and horizontally for a minimum of 6 hours per position. The verification must be conducted at a controlled temperature of 20 °C ± 5 °C.

The e-vaping consumable leakage testing method proposed by PMI R&D addresses product leakage robustness in a sequence of dynamic conditions: temperature, movement and pressure are considered as variables that can influence leakage.

- Temperature testing is done at 22 °C and 42 °C
- Shaking testing from 0 to 250 rpm and back
- Pressure from atmospheric pressure to 800 mBar absolute pressure (pressure exposure in planes).

With this method, the e-vaping cartridge weight is recorded individually and inserted into a glass tube within one single testing instrument (Buchi Syncore) with multiple glass tube holders. In a pre-defined time and sequence the above conditions are applied. Afterwards the cartridges are removed from the glass tubes, externally dried with a clean towel and re-weighed. The leakage is quantified by the difference in weight before and after the applied conditions.

STPOST 18

Assessment of the so-called “gateway effect” based on electronic vapour product classifications

VERRON T.(1); CAHOURS X.(1); CERSON L.(2); COLARD S.(1); O’CONNELL G.(3)

(1) SEITA-Imperial Tobacco, 48 rue Danton, 45404 Fleury-les-Aubrais, France
(2) Imperial Tobacco Limited, 121 Winterstoke Road, Bristol BS3 2LL, U.K.
(3) Fontem Ventures B.V., HN Amsterdam 1083, The Netherlands

Since electronic cigarettes (e-cigs) became popular as alternatives to conventional cigarettes, with subsequent market growth amongst smokers, there is currently a debate as to whether e-cigs may be a "gateway" to conventional cigarette smoking or not. Such fears are related to the possibility that prior use of e-cigs could conceivably result in conventional cigarette smoking initiation amongst never smokers. Although the common definition of a “gateway effect” is based on the concern that current use of a potential low-risk product could facilitate the use of higher-risk products in the future, there is actually no agreed method for assessing a "gateway effect" for e-cigs. Consequently, this creates a lack of clarity and confusion among researchers, politicians, media, vapers and smokers, which often leads to misleading study interpretations and conclusions being drawn.

To this end, we have described a framework based on product classification to assess any so-called “gateway effect”: ‘alternative product’, ‘transition product’, ‘substitution product’ or ‘gateway product’.

Each of these four categories corresponds to a different probability of a consumer switching from a potential low-risk product to a high-risk product, and vice versa, based on the motives for using them. Using an approach such as dynamic population modelling, it will be possible to classify e-cigs in one of these four product categories and thereby to assess whether e-cigs are a ‘gateway’ or a ‘roadblock’ to conventional cigarette smoking. Here we describe this innovative approach.

STPOST 19

Exposure atmosphere characterization using tank vaping products

GUPTA A.; HAYDEN B.; BEHRINGER S.; PETRELLE L.; PSURNY E.

Battelle Memorial Institute, 505 King Avenue, Columbus, OH 43201, U.S.A.

Recent market trends indicate that tank vaping products are gaining increasing popularity. Characterization of the health effects of individual products may include conduct of nonclinical investigations. This study demonstrates an approach for generating and characterizing the output from commercially available tank products as part of preparation for the conduct of *in vivo* inhalation studies.

In the present work, we evaluated some of the variables associated with tank products and their influence on aerosol atmosphere delivered to a nose only inhalation exposure carousel. The two products selected had coil resistances of ~1.8 and ~0.6 ohms. The evaluation included: device power settings; output consistency from a full tank to an empty tank; new coil versus an aged coil, orientation of the device; and impact of puff durations.

A manifold to mount the device at pre-determined angles and an automated button-activation mechanism were developed. The exposure atmosphere was characterized using: a real time aerosol monitor; determination of propylene glycol, glycerol and nicotine; cascade impactors for aerosol particle size. Transfer efficiency of aerosol from the device to breathing zone of the animals was determined.

For both devices, aerosol output per puff was higher as compared to conventional e-cigarettes. Aerosol concentration at the nose-port was temporally stable after a startup period. Aerosol output and particle size were comparable among new and aged coils. Aerosol output increased with increasing power. Ratios of chemical constituents were comparable among numerous power setting, orientations and puff durations that were tested.

STPOST 20

Alternative additives for cigarette filters

BENSE T.; RUFENER C.; TAROCO E.; RODRIGUEZ G.; BONILLA T.; UMPIÉRREZ E.

Compañia Industrial de Tabacos Montepaz S.A., San Ramon 716, CP 11800 Montevideo, Uruguay

For many years, activated carbons have been extensively used for water treatment and gas adsorption because their large surface area. More recently, new generations of carbon structures built at nanoscale with relatively large surface areas and exhibiting novel electronic and chemical properties open new horizons for achieving enhanced adsorption and new and sophisticated applications.

In this work we tried to find whether these novel carbon structures were capable of selectively absorbing polyaromatic hydrocarbons (PAHs) from cigarette smoke using a device similar to a cigarette filter. We selected different allotropic forms of carbon as adsorbents, that is to say, same composition but different structure: graphene, graphene oxide, different grades of functionalised graphene, fullerene, single and multiwall nanotubes and combination of them.

Different filter structures were designed to support the different additives. Firstly, cavities with the additives under cellulose acetate plugs, and then cellulose acetate filter with very low-pressure drop that were immersed in an aqueous suspension of the adsorbent. Different types and quantities of surfactants were tested to improve dispersion of the additives in water. Plain cellulose acetate filters submerged in water with no additives were used as reference. The filter plugs with the different additives and the reference filters were placed in specially designed acrylic nozzles and attached to the same cigarette tobacco column. Cigarettes were smoked following the ISO regime with a puff time modification, Cambridge filters were extracted with solvent and then analysed by GC-MS-MS and retention efficiency against the reference cigarettes was tested for eleven PAHs including benzo[a]pyrene.

We found that graphene could selectively retain PAHs, and depending on the particle size, selectively retain the lower or higher molecular weight compounds. Functionalisation of graphene could decrease the retention rate of PAHs, and multiwall nanotubes of small size seemed to more effectively retain high weight PAHs.

STPOST 21

Puff-by-puff tar, nicotine and carbonyl profiles under different smoking regimens of commercial cigarettes with various carbon filter technologies

ROBERTSON R.M.; NORFLEET K..; CANTERBURY W.B.

Celanese, 3520 Virginia Drive, Narrows, VA 24124, U.S.A.

Puff-by-puff smoke analysis has shown that the CelFX® carbon filter technology has similar nicotine and tar smoke delivery profiles but with significantly reduced carbonyls, particularly in the first 6 puffs, compared to a cellulose acetate filter. Prior work used a 3R4F Kentucky reference cigarette tested with ISO methodology but with vent holes blocked.

This work will investigate the differences in puff-by-puff results between various commercial cigarettes containing different filter configurations including the novel CelFX® carbon filter, carbon-on-tow filters and cellulose acetate filters. Additionally, the impact of ISO versus Canadian Intense on puff-by-puff basis will also be analyzed. Prior work used hand assembled cigarettes with no ventilation showed a reduction in gas-phase efficiency in later puffs. This work will seek to assess that impact in ventilated designs, as well as to show the results under more intense smoking regimes. The delivery profiles of tar, nicotine, and carbonyls reductions will be summarized.

STPOST 22

Impact of naturally-porous tipping paper on the delivery of harmful substances and the sensory quality

LIANG Jie; ZHAI Jilan

Mudanjiang Hengfeng Paper Co., Ltd, Mudanjiang 157013, P.R. China

Filter ventilation technology is one of the widespread strategies to reduce the delivery of tar and other harmful substances. Due to its easy-operability and pronounced advantages, this technology is widely used in cigarette production. At present, it can be achieved by using the filter with perforated tipping paper. In this study, a naturally porous tipping paper was used instead of perforated tipping papers during cigarette production. Tipping papers with different porosities were evaluated as regards the delivery of harmful components in the mainstream smoke, the ventilation rate, the variation coefficient of ventilation, and the sensory quality. It was found that, for naturally porous tipping paper, the delivery of nicotine, tar, crotonaldehyde, carbon monoxide, etc., was much less than perforated tipping paper at the same porosity. The average value of ventilated variation coefficient was 5.84%, which is lower in comparison with the perforated tipping paper (6.67%). In the case of porosity of less than 160 CU, the cigarette aroma was more plentiful, smooth and elegant, and the aftertaste was more comfortable. These results showed that naturally-porous tipping paper did reduce the delivery of tar and other harmful substances. In addition, since its mainstream smoke was diluted more uniformly, the stability and sensory evaluation of the samples was much better.

STPOST 23

Influences of sugars in cigarette paper additives on the pyrolysis products of cigarette papers

LIU Lin; WU Jingqiang; SHEN Jingxuan; XIAO Weiyi; XU Lanlan; HUANG Haiqun; SHEN Yan; LIU Zhe

Yunnan Reascend Tobacco Technology (Group) Co., Ltd., Kunming, Yunnan 650106, P.R. China

Cigarette paper additives have been shown to play an important role during the cigarette burning process. For example, different additives may impact the cigarette burning state, adjust ash appearance characteristics, improve cigarette quality, and potentially reduce the tar and harmful components. The objective of this study was to examine the influences of sugars in cigarette paper additives on the pyrolysis products of cigarette papers. Six natural plant extracts rich in sugars were added to the cigarette papers as flavouring additives. The water-soluble sugars of flavouring additives were analysed with high performance liquid chromatography (HPLC) and the pyrolysis products of flavouring additives and cigarette papers were analysed with pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS). The contents of fructose and glucose in additives were the highest. Fructose content was 15.78%-32.88% and glucose content was 16.74%-22.33%, so we selected fructose and glucose as the main reference indexes. In pyrolysis

products of six additives, ten different kinds of chemical constituents were detected when the temperature was 200-900 °C, which included acids, alkenes, aldehydes, ketones, alcohols, esters, furans, phenols, polycyclic aromatic hydrocarbons (PAHS) and benzenes. 5-hydroxymethyl-furfural, furfural and 5-methyl-2-furfural were the compounds with relatively high levels in pyrolysis products, which were 7.20%-16.98%, 7.55%-19.58% and 2.57%-4.49% respectively in flavouring additives, 6.98%-49.82%, 4.21%-5.99%, 0.80%-1.32% respectively in flavoured cigarette papers. The changes of fructose and glucose contents in flavouring additives and 5-hydroxylmethyl-furfural content of the pyrolysis products of additives and cigarette papers were consistent. The fructose and glucose contents had no significant effect on the contents of furfural and 5-methyl-2-furfural. Therefore, it was concluded that, more fructose and glucose in cigarette paper additives should increase the 5-hydroxymethyl-furfural content of pyrolysis products of cigarette papers, which could increase the aroma and nuances of cigarette smoke.

STPOST 24

The effects of cigarette papers on the ash stability, ash colour, hot cone fall out propensity and emissions of slim cigarettes

WEN Siru; CHEN Yuxiang; BAI Liangliang

Mudanjiang Hengfeng Paper Co., Ltd, Mudanjiang 157013, P.R. China

In recent years, slim cigarettes are more commonly used by consumers, however, only a low number of references can be found with respect to the type of cigarette papers (CPs) applied on that type of product. This paper provides an overview on how main CP properties, such as CP basis weight, CP porosity, CP burn additive content, type of CP burn additive (potassium citrate and sodium citrate) and CP ash content impact the ash stability, ash colour, hot cone fallout propensity (HCFP) and the emissions of a burning slim cigarette. In summary, the effects of cigarette paper (CP) properties on the ash stability and ash colour of slim cigarettes are studied systematically in this paper.

The influence of CPs on the ash stability and the ash colour of slim cigarettes were investigated using image analysis. As one of the results, it was found that increased ash content or increased sodium citrate content (at the same citrate level) of the applied CPs improved the ash stability and the ash colour of the burning slim cigarette.

Hot cone fall out is a commonly known phenomena for slim cigarettes. We investigated the influence of the main CP properties on the hot cone fall out propensity (HCFP) of slim cigarettes by using a HCFP analyser.

CP is known to have a significant impact on the emissions, such as tar and CO, of a burning cigarette. In our study it was shown that an increased CP ash content, an increased CP porosity, an increased CP burn additive content or an increased potassium citrate content (at the same citrate level) of CP led to a significant reduction of tar and CO in the emissions of the investigated slim cigarettes. On the contrary, an increased CP basis weight also led to a reduction of tar, whilst the CO significantly increased.

STPOST 25

Inner hollow tube diameter influence on cigarette smoke

MOSTOVOJUS V.; LUSCIKAITE L.; TUCINSKAS G.

Nemuno Banga LLC, Kestucio Str.1, Lentvaris, Lithuania

There has been a number of significant changes in the tobacco industry over the last few years driven by various new legislations. Over the next decade regulations on packaging will almost undoubtedly evolve into outright plain packaging in mature markets and then move on to emerging ones. This will and already is driving innovation in the filter industry to offer consumers more choice, unique taste experiences and visual differentiation lost through plain packaging, with special filters playing a key part in the creation of combustible tobacco products with more favourable smoke constituent profiles. Hollow filter has recently been growing in popularity on a large scale and we therefore would like to focus on this product.

In this study we analysed the influence of hollow tube inner diameter on cigarette smoke parameters. We made four different hollow filter cigarettes with inner tube diameter 2.0 mm, 3.0 mm, 4.0 mm and 5.0 mm, and then compared tar, nicotine, CO, level of triacetin and temperature of smoke in the middle of the hollow tube against a standard monoacetate and dual filter products. Cigarettes with different test filters were made in a standardised way and all confounding factors removed.

Cigarettes with the above described filters were made with 50% ventilation and without ventilation. The smoke was collected on Cambridge filter pads using the ISO and Canada Intense smoking regimes with some machine adjustments to measure smoke temperature. Analyses of smoke composites were made using Agilent 5973N GC-MS.

Our results showed that inner tube diameter and ventilation affects cigarette smoke temperature and triacetin levels. The amount of triacetin in non-ventilated cigarette smoke was higher than in ventilated due to the high concentration of triacetin in the hollow tube.

STPOST 26

A review of aerosol exposure systems relative to the analysis of cytotoxicity: a CORESTA *In vitro* Toxicity Testing Sub-Group perspective

THORNE D.(1); WIECZOREK R.(2); FUKUSHIMA T.(3); SHIN H.J.(4); LEVERETTE R.(5); BALLANTYNE M.(6); LI X.(7); BOMBICK B.(5)

(1) British American Tobacco, R&D Centre, Regents Park Road, Millbrook, Southampton SO15 8TL, U.K.
(2) Reemtsma Cigarettenfabriken GmbH (an Imperial Brands PLC Company), Albert-Einstein-Ring 7, 22761 Hamburg, Germany
(3) Japan Tobacco Inc., Scientific Product Assessment Centre, 6-2 Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan
(4) KT&G Corporation, 30 Gajeong-ro, Yuseong-gu, Daejeon 305-805, South Korea
(5) RAI Services Company, 401 North Main Street, Winston-Salem, NC 27102, U.S.A.
(6) Covance Laboratories Ltd, Otley Road, Harrogate HG3 1PY, U.K.
(7) Zhengzhou Tobacco Research Institute of CNTC, No. 2 Fengyang Street, High-Tech Zone, Zhengzhou 450002, P.R. China

Aerosol exposure systems offer researchers a variety of ways to customise the exposure set-up, modify experimental parameters and provide a novel and versatile platform for *in vitro* aerosol research. These systems produce an aerosol that more closely mimics the human smoking condition with associated aerosol interactions, an advantage over the potential limitation of using aerosol fractions alone. Exposure systems typically consist of two functional parts: the smoking machine / aerosol generator, and the exposure module/multiwell plate housing the cell system.

The possible combinations of exposure systems, modules and plate formats give rise to an *in vitro* aerosol research environment that is complex and diverse, resulting in unique combinations of variables that few laboratories share. Ultimately, this causes challenges in comparing data between set-ups using similar systems and an inability to compare data across some platforms, making tobacco aerosol research particularly difficult to contextualise across laboratories.

Over recent meetings, the CORESTA *In vitro* Toxicity Testing Sub-Group has discussed the developing field of aerosol exposure research. Given the diversity of techniques, exposure parameters and biological end-points being deployed, it was considered a high priority to establish a strategy to assess these systems and the responses obtained. Twelve global companies with expertise in *in vitro* aerosol research met to discuss this topic and identify potential areas of alignment. A detailed and comprehensive survey was conducted on over 40 parameters ranging from aerosol generation, dilution, biological methodology, data analysis and dosimetry approaches, across eight independent laboratories.

Survey results demonstrate the diversity and provide awareness of the exposure systems, parameters, methodological nuances, and data analysis. Results identify potential commonalities and important areas of consideration, which may be of substantial benefit to current smoke/aerosol researchers, scientists from intersecting fields of research, and new scientists and laboratories entering into this area of research.

STPOST 27

Interaction of nicotine, NAT and NAB with NNK and NNN induced by cytochrome P450 2A13 in aqueous solution

LIU Xingyu; ZHANG Jie; ZHANG Chen; ZHOU Jun

Beijing Cigarette Factory, Shanghai Tobacco Group Corporation, Ltd., CNTC, No. 99 Wan Sheng Nan Jie, Tongzhou District, Beijing 101121, P.R. China

NNK and NNN are considered to be the most carcinogenic of the four TSNAs. However, these TSNAs are carcinogenic to humans only after being metabolically activated by cytochrome P450 enzyme. There are other weak carcinogens, such as NAT and NAB, and non-carcinogenic compounds, such as nicotine, in tobacco. The structures of these compounds are similar to that of NNK and NNN. Therefore, whether NAT, NAB and nicotine could inhibit P450 enzyme-catalysed metabolism of NNK and NNN is worth investigating. NNK, NNN and their metabolites were analysed by HPLC coupled with triple quadruple mass spectrometry. The results of the *in vitro* study showed that the main products of P450 2A13-catalyzed NNK metabolism were OPB (4-Oxo-4-(3-pyridyl)-butanal), HPB (4-Hydroxy-1-(3-pyridyl)-1-butanone), OPBA (4-Oxo-4-(3-pyridyl)-butanoic acid), and those for NNN metabolism were HPB, hydroxy acid (4-Hydroxy-4-(3-pyridyl)-butyric acid), and OPB.

The kinetic parameters of the enzymatic reactions in aqueous solution were calculated with the Michaelis-Menten equation. A competitive inhibition effect on the metabolism of NNK and NNN was observed after adding nicotine, NAT or NAB. The values of inhibition constant Ki of the three inhibitors were calculated. For NNK metabolism, Ki values are as follows: a) 8.51 μM (nicotine), 0.21 μM (NAT) and 0.23 μM (NAB) for OPB, b) 25.01 μM (nicotine), 0.71 μM (NAT) and 0.87 μM (NAB) for HPB, c) 6.57 μM (nicotine), 0.36 μM (NAT) and 0.50 μM (NAB) for OPBA. For NNN metabolism, Ki values are as follows: a) 0.98 μM (nicotine), 1.37 μM (NAT) and 0.71 μM (NAB) for HPB; b) 1.35 μM (nicotine), 1.35 μM (NAT) and 1.01 μM (NAB) for hydroxy acid; c) 8.40 μM (nicotine), 3.40 μM (NAT) and 3.04 μM (NAB) for OPB. These results suggest that structurally similar compounds in tobacco, such as NAT, NAB, and nicotine, inhibit the metabolic activation of NNK and NNN, which might influence their carcinogenicity to a certain extent.

STPOST 28

Determination of benzo[a]pyrene in smokers' urine by online gel permeation chromatography–solid phase extraction–gas chromatography/mass spectrometry

SI Xiaoxi; ZHU Ruizhi; LIU Zhihua; ZHANG Fengmei; LIU Chunbo; SHEN Qinpeng; HE Pei; MIAO Mingming; YANG Guangyu

Key Laboratory of Tobacco Chemistry of Yunnan, R&D Center of China Tobacco Yunnan Industrial Co., Ltd., Kunming 650231, P.R. China

A new method for determining the benzo[a]pyrene (B[a]P) in samples of smokers' urine was developed with an online gel permeation chromatography-solid phase extraction-gas chromatography/mass spectrometry (GPC-SPE-GC/MS) hyphenated system. Urine samples from smokers were extracted with cyclohexane containing D12-B[a]P as an internal standard. The extract was separated by gel permeation chromatography and the B[a]P fraction was cut into a solid phase extraction cartridge and enriched. The enriched B[a]P was eluted with acetone and determined by gas chromatography-mass spectrometry (GC-MS). The method utilised the online connections of gel permeation chromatography, solid phase extraction and gas chromatography/mass spectrometry, eliminating the need for multi-step manual purification. By incorporating gel column in liquid chromatography, the volume of sample injection increased to 200 µL and sample loading capacity was 10 times that of a conventional micro silica gel column. The purified large volume fractions from liquid chromatography were injected into the gas chromatography directly via online solid phase extraction, enhancing sensitivity. For B[a]P concentrations in the range of 0.08-10 ng/mL, the method had good linearity with the correlation coefficient of 0.9992. The intra-day and inter-day relative standard deviations (RSDs) were 3.26% and 3.87%, the limit of detection (LOD) and limit of quantification (LOQ) were 0.022 and 0.093 ng/L, respectively; and the recoveries ranged from 95.6% to 97.2% at three spiked levels. The method is automated, simple, fast, sensitive, accurate, and suitable for determining B[a]P levels in smokers' urine.

STPOST 29

Establishment of a respiratory parameter measurement system for mice using an unrestrained whole-body plethysmograph

SUZUKI H.; MOTOHASHI Y.; OZAKI H.; MINE T.

Japan Tobacco Inc., R&D Group, Scientific Product Assessment Center, 6-2,Umegaoka, Aoba-ku, Yokohama, Kanagawa, 227-8512, Japan

Respiratory parameters, such as tidal volume (TV), breathing frequency (Bf) and minute volume (MV), are important factors in the evaluation of the effect of cigarette smoke (CS) on laboratory animals in *in vivo* inhalation studies. However, a measurement method for respiratory parameters during whole-body inhalation has not been established, especially for CS.

The objective of this study was to establish a respiratory parameter measurement system for mice using an unrestrained whole-body plethysmograph (WBP) and to investigate respiratory parameters in mice.

After CS was delivered from a nose-only exposure system to the WBP, CO and wet total particulate matter (WTPM) concentrations were measured both at a nose port of a nose-only exposure system and in WPB. In addition, kinetic changes in mice respiratory parameters were measured at different CS concentrations and with different exposure regimens (consecutive exposure and interval exposure).

Our results showed that CO and WTPM concentrations in WBP were comparable to those at the port of the nose-only exposure system, suggesting CS was successfully delivered to the WBP.

TV, Bf and MV respiratory parametres were depressed under both the consecutive and interval regimens during whole-body CS inhalation. Interestingly, temporal recovery was observed for Bf and MV during a break period in the interval exposure regimen.

In conclusion, we established a respiratory parameter measurement system for mice using unrestrained WBP. We also investigated kinetic changes in respiratory parameters and differences in respiratory behaviour between exposure regimens.

STPOST 30

Comparative study of cigarette smoke-induced effects among multiple strains of mice

MOTOHASHI Y.; SUZUKI H.; OZAKI H.; MINE T.

Japan Tobacco Inc., R&D Group, Scientific Product Assessment Center, 6-2, Umegaoka, Aoba-ku, Yokohama, Kanagawa, 227-8512, Japan

It is known that the effects of cigarette-smoke (CS) exposure on mice are strain-dependent, due to differences in genetic backgrounds. However, the susceptibility of the strains to CS-exposure is inconsistent across reports, probably because of differences in experimental conditions.

Here, we investigated the differences in the inflammatory and oxidative stress responses to CS-exposure among the strains which have been reported as 'the most CS-susceptible strains', using our well-controlled exposure system.

C57BL6/J, A/J, BALB/c, and AKR mice were exposed to CS in whole-body exposure chambers for five weeks, and inflammatory and oxidative stress responses in the lungs were assessed.

CS-exposure induced increases in the numbers of macrophages and neutrophils in bronchoalveolar lavage fluids (BALF) in all strains, whereas an increase in the number of lymphocytes was observed in the BALF of C57BL/6 and A/J mice. C57BL/6 and A/J mice showed up-regulation of some chemokines, interleukins and other inflammatory mediators in the lungs, while BALB/c and AKR mice showed down-regulation or no change in some interleukins and cytokines, such as IFN-γ and Fas ligand. In addition, BALB/c and AKR mice showed down-regulation of glutathione peroxidases, peroxiredoxins and other antioxidants in the lungs.

These data confirmed qualitative and quantitative variation in the inflammatory and oxidative stress responses to CS-exposure among strains. In our study, which was focused on inflammation and oxidative stress, C57BL/6 and A/J mice were more susceptible strains than BALB/c and AKR mice. It is important to consider the characteristics of each mouse strain to CS-exposure, such as inflammatory responses, when studying the molecular mechanisms induced by CS-exposure.

STPOST 31

Improvement of *in-situ* slide preparation procedure for semi-automated micronucleus counting system

ERAMI K.(1); SAWAMOTO Y.(1); YOSHINO K.(1); SOFUNI T.(2)

(1) Japan Tobacco Inc., Scientific Product Assessment Center, 6-2, Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan
(2) Genotoxic Consulting Services, 916-3 Koguki, Shiraoka, Saitama 349-0217, Japan

In-situ micronucleus (MN) test is an easier-to-use procedure than the traditional way such as a dish preparing approach. Nevertheless, the amount of resources needed for manual counting and the necessity of advanced preparations for reaching consensus of counting criteria between

counters stay unchanged in both procedures. Automated micronucleus counting systems are effective tools to address such difficulties of the MN test. It is expected that the combination of *in-situ* methods and automated counting systems provides a certain benefit for increasing laboratory throughputs.

Algorithms of automated systems for image analysis have two major functions such as cell outline recognitions and micronucleus findings. Adherent cells which are usually used for *in-situ* methods have infinite forms, which cause cell recognition algorithms more difficult to be developed. In addition, changing of cell morphology caused by chemical effects amplify the difficulties whilst developing required algorithms. In consequence, stabilising cell morphologies is a key for success whilst developing required algorithms.

In this study, we conducted several experiments for stabilising cell shapes to apply *in-situ* sample preparation required for automated counting.

Hypotonic treatment is one of the procedures for stabilising cell morphology. Although it is well known that the hypotonic solution changes cells to spherical shape in wet state without regards of culture condition, there are no reports for keeping the round shape on dried slides until micronucleus scoring step.

We found that cell fixation using a certain mixture of alcohol and acetic acid in a step by step manner is effective for keeping the round shape after hypotonic treatment without any dependency from cell culture conditions.

Finally, advanced staining methods resulted in a slide preparation procedure that delivered good recognition rates of cells and micronuclei for an automated system.

STPOST 32

Cellular transformation activity of different tobacco condensates in the Bhas 42 cell assay

STEVENSON M.(1); WIECZOREK R.(2); SIMMS L.(1); PANT K.(3)

(1) Imperial Tobacco Limited, 121 Winterstoke Road, Bristol BS3 2LL, U.K.
(2) Reemtsma Cigarettenfabriken GmbH (an Imperial Brands PLC Company), Albert-Einstein-Ring 7, D-22761 Hamburg, Germany
(3) BioReliance, Sigma-Aldrich Corp., 9630 Medical Center, Rockville, MD 20850, U.S.A.

Carcinogenesis has been described as a multi-stage process comprising of initiation, promotion and progression. Smoking is a cause of serious disease in smokers, including lung cancer. Determining the carcinogenic potential of a tobacco product ingredient is a key component of a stewardship assessment. The aim of the study was to assess the initiation and promotion potential of different tobacco condensates (factory manufactured cigarettes with and without additives) in the Bhas 42 Cellular Transformation assay. The ability of the assay to distinguish a dose response was also investigated.

Mainstream smoke from cigarettes was generated in accordance with ISO 3308. Total Particulate Matter (TPM) was collected on Cambridge filter pads (up to 600 mg per pad) and extracted with DMSO, achieving a TPM concentration of up to 50 mg/ml. The assay protocol consisted of two components, an initiator assay (Sasaki, et.al. 1988, 1990, Asada, et.al. 2005) and a promoter assay (Ohmori, et.al. 2004, 2005), in an attempt to detect tumour-initiating activity and tumour-promoting activity, respectively, of chemical carcinogens.

Out of the three different condensates only one sample displayed weak initiating activity. However for the promotor assay, statistically significant increases in the mean number of foci were observed for all condensates at concentrations ranging from 2.5-60 µg/ml. For each condensate, a clear dose response was observed in the promotor assay. The results from this study will be presented in detail and will highlight the potential of these assays for future tobacco product ingredient assessment.

STPOST 33

Accurate and reproducible dispensing of patterned picoliter quantities of tobacco extract onto apical surfaces of human 3D reconstructed airway tissues

BEHRSING H.(1); SADOWSKI N.(1); CONSTANT S.(2); HUANG S.(2); RAABE H.(1)

(1) Institute for In vitro Sciences, Inc., Gaithersburg, MD, U.S.A.
(2) Epithelix Sàrl, Geneva, Switzerland

There is an increasing need for researchers to understand the dynamic aspects of inhaled tobacco product exposure. Exposure-induced events can cause respiratory irritation, sensitisation, and other events that may lead to severe pulmonary disease. Available 3D human reconstructed airway tissues (RHuA) provide researchers with a more physiological platform that offers apical and basal compartments for flexibility in modelling relevant exposures. Commercially available instrumentation can generate smoke and aerosols from tobacco products (including e-cigarettes) and expose tissues at ALI, but the quantification of materials deposited at the exposure site remains a challenge. We have tested the Tecan D300 digital dispenser as a potential technical solution to deliver precise amounts of very small vehicle droplets to coat the apical surface of an available RHuA. The picoliter volume dispensing allows the direct dilution of vehicle to <0.1% levels based on estimated RHuA mucous layer volumes. During patterned TPM-dispensing onto apical surfaces of Epithelix MucilAir™ tissues, marker release (including cytokines) and the viability were compared in both the apical and basolateral compartments after 72 hr exposure. The dispensing precision and accuracy, as well as the effect of direct vehicle (DMSO) or DMSO solubilized TPM patterned dispensing onto apical surfaces were evaluated. No significant adverse effects up to 707 nL total dispensed volume was detected using LDH or WST-8 assays. However, the highest volume dispensed (707 nL) did adversely impact ciliary beat frequency. Hand-pipetting of larger volumes (20 µL) onto the apical surface of RHuA induced a greater baseline cytokine response than D300 dispensing. This novel technology demonstrated promising results as a method by which the agent to be tested (e.g. derived from tobacco product emissions) was exposed into an ALI-based culture format and onto the apical surface of RHuA tissue. The very low dispense volumes minimize effects on the rheology of RHuA apical surfaces.

STPOST 34

The mutagenic assessment of electronic-cigarettes and tobacco smoke using the Ames assay in strains TA98 and TA100

THORNE D.(1); CROOKS I.(1); HOLLINGS M.(2); SEYMOUR A.(2); MEREDITH C.(1); GAÇA M.(1)

(1) British American Tobacco, R&D Centre, Regents Park Road, Millbrook, Southampton SO15 8TL, U.K.
(2) Covance Laboratories Ltd, Otley Road, Harrogate HG3 1PY, U.K.

Salmonella typhimurium strains TA98 and TA100 were used to assess the mutagenic potential of a commercially available rechargeable, dual voltage, closed system modular electronic-cigarette (Vype® ePen, Nicoventures UK). Results were compared to a Kentucky reference (3R4F) cigarette. Two different test matrices were assessed. Aerosol generated from the e-cigarette was trapped on a Cambridge filter pad, eluted in DMSO and compared to cigarette smoke total particulate matter (TPM), generated in the same manner. Fresh e-cigarette and cigarette smoke aerosols were generated on the Vitrocell® VC 10 smoking robot and compared using a modified scaled-down 35 mm air agar interface (AAI) methodology. E-cigarette TPM (eTPM) was found to be negative in the 85 mm Ames assay in strains TA98 and TA100 conducted in accordance with OECD 471. Freshly generated e-cigarette aerosol was also found to be negative in both strains following a 3-hour AAI aerosol exposure. Positive control responses were observed in both strains, using benzo[a]pyrene and 2-aminoanthracene for TA98 and TA100 respectively. In

contrast, cigarette smoke TPM and whole aerosol from 3R4F reference cigarettes were found to be mutagenic in both tester strains, under comparable test conditions to that of e-cigarette exposure.

Currently, limited information exists on the mutagenic activity of captured e-cigarette particulates and whole aerosol AAI approaches. Regulatory standard product testing approaches as used in this study will become important when determining whether e-cigarette aerosols are less biologically active when compared to cigarette smoke, as suggested by the literature and data presented here.

STPOST 35

Comparison of the carcinogenic potential of tobacco smoke and electronic-cigarette aerosol using the Bhas cell transformation assay

BREHENY D.(1); OKE O.(1); PANT K.(2); BRUCE S.W.(2); LAFORCE M.K.(2); CELESTIN N.(2); GAÇA M.(1)

(1) British American Tobacco, R&D Centre, Regents Park Road, Millbrook, Southampton SO15 8TL, U.K.
(2) BioReliance, Sigma-Aldrich Corp., 9630 Medical Center, Rockville, MD 20850, U.S.A.

In vitro cell transformation assays (CTA) are used to assess the carcinogenic potential of chemicals and complex mixtures. They can detect non-genotoxic as well as genotoxic carcinogens. The Bhas 42 CTA has been developed with both initiation and promotion protocols to distinguish between these two classes of carcinogens. Cigarette smoke contains both genotoxic and non-genotoxic carcinogens. It has been shown to act as a tumour promoter *in vivo* and to be positive in the Bhas 42 promotion assay. In this study we have used the Bhas 42 promotion assay to assess the tumour promotion potential of an electronic cigarette (e-cigarette) compared to a conventional cigarette.

The activity of a commercially available e-cigarette (Vype® ePen) was compared to that of a reference cigarette (3R4F) in the Bhas 42 promotion protocol. A 24 mg/mL stock solution of 3R4F total particulate matter (TPM) was prepared using the Health Canada Intense (HCI) puffing regime (55 mL puff volume, 2 second puff duration, every 30 seconds; vents blocked on the 3R4F cigarettes). A modified version of this regime was used to prepare TPM stock solution from the Vype® ePen (eTPM). TPM was tested up to a maximum concentration of 120 µg/mL (n=3). 3R4F TPM produced a clear concentration-dependent response and induced significantly higher numbers of foci than control treatment at all concentrations (Dunnett's test; $p < 0.0001$). The overall activity of eTPM from the Vype ePen was found not to differ significantly from a DMSO control at any concentration tested.

This study shows that the Bhas 42 promotion assay can distinguish between TPM from a conventional cigarette and an e-cigarette and could be included as part of a weight of evidence package for comparative risk assessment of tobacco and nicotine based products.

STPOST 36

Ultra-sensitive method for the determination of nicotine in PK studies with new generation products

APPELT F.; PLUYM N.; SCHERER G.; SCHERER M.

ABF Analytisch-Biologisches Forschungslabor GmbH, Goethestrasse 20, 80336 Munich, Germany

The pharmacokinetics (PK) of nicotine uptake with new generation products such as electronic cigarettes (e-cigs), tobacco heating products (TBH) and others is an essential criterion for evaluating the performance of the product. When using new generation products, the nicotine uptake is frequently found to be much lower as compared to conventional cigarettes, sometimes even hardly exceeding the common background levels in human blood samples of non-users. Therefore, apart from having a highly sensitive analytical method for quantification of nicotine in serum or plasma, it is of paramount importance to reduce the ubiquitously occurring nicotine background levels as far as possible. This is required to be performed both for the blood sample collection (the clinic) on the one side, as well as for the analysis (the laboratory) on the other side.

In this presentation, we will provide an overview of provisions and their effectiveness to lower the nicotine background levels at both sides mentioned above so that a limit of quantification for nicotine of 0.1-0.2 ng/ml in serum or plasma can be achieved. Furthermore, we will present validation and application data of an UPLC-MS/MS method, which we have developed for the described purpose.

STPOST 37

UPLC-MS separation and analysis of carbonyl compounds in e-liquids aerosol samples

CARTER K.M.; MAINES H.; GILLMAN I.G.

Enthalpy Analytical Inc., 1470 East Parham Rd, Richmond, VA 23228, U.S.A.

The popularity and regulation of electronic nicotine delivery systems (ENDS) has drawn attention to the chemical composition of the e-liquids used in, and aerosols formed by these devices. The possible presence of carbonyl compounds like formaldehyde, acetaldehyde, diacetyl, acetylpropionyl, and acetoin are of concern due to their potential impact on human health when inhaled at sufficient concentrations. Flavoring added to the e-liquids may contain diacetyl, acetylpropionyl, and acetoin, which can be easily transferred to the aerosol. Propylene glycol and glycerin, the main carriers used in e-liquids, are heated during the formation of the aerosol and may undergo thermal decomposition leading to the formation of formaldehyde and acetaldehyde. Analytical detection of carbonyls is commonly achieved by analysis of their corresponding 2,4-dinitrophenylhydrazine (DNPH) derivatives using liquid chromatography. This method can be problematic in this complex matrix due to the possibility of interfering peaks and can result in incorrect reporting of data. The objective of this study was to develop a single rapid method for accurate detection and analysis of carbonyls that may be present in flavored e-liquid and aerosol samples. A method of determination of carbonyl compounds by ultra-performance liquid chromatography (UPLC) and electrospray ionization (ESI) tandem mass spectrometry (MS/MS) after derivatization with DNPH was developed and successfully applied to the evaluation of e-liquid and aerosol samples. This method allows for the detection and analysis of carbonyls including formaldehyde, acetaldehyde, acetone, diacetyl, acetylpropionyl, acrolein, acetoin, and crotonaldehyde in e-liquid and aerosol samples in less than ten minutes. The LC-MS/MS method will be compared to an existing UPLC method with an emphasis on its application to the analysis of complex, highly flavoured samples.

STPOST 38

The retention of nicotine from a new type of heated tobacco product in human respiratory tracts

SUZUKI T.; YUKI D.; KITAGAWA M.; SHIBATA T.

Japan Tobacco Inc., R&D Group, Scientific Product Assessment Center, 6-2, Umegaoka, Aoba-ku, Yokohama, Kanagawa, 227-8512, Japan

Nicotine is the major alkaloid in tobacco leaf and therefore present in tobacco products. Previous studies suggest that absorption sites of nicotine in human respiratory tracts affect nicotine pharmacokinetics. This study investigated the retention of nicotine in human respiratory tracts when using a new type of heated tobacco product (NHTP) in comparison with a conventional cigarette (CC1). Twenty-four male smokers used NHTP and smoked CC1 with two use/smoking patterns (normal inhalation: pattern A; mouth hold: pattern B). The total nicotine retention ratio, the ratio of the retained nicotine amount to the amount of nicotine taken by inhalation pattern A, was calculated from the amount of nicotine intake and the amount of exhaled nicotine where the intake was estimated as mouth level exposure (MLE). The MLE of NHTP and CC1 was estimated from the weight loss of NHTP and Part-Filter Method, respectively. The nicotine retention ratio for mouth was calculated in the same manner using the result of pattern B. The retention ratio for lower respiratory tract was estimated from the difference between the results of pattern A and pattern B. The mean (±S.D.) of total nicotine retention ratio was 93.1±8.9% and 94.9±11.4% for NHTP and CC1, respectively. The mean (±S.D.) of nicotine retention ratio for mouth was 57.9±15.7% and 36.4±11.8% for NHTP and CC1, respectively. Therefore, retention ratios for lower respiratory tract were estimated as 35.2% for NHTP and 58.5% for CC1, respectively. The result shows that retention profiles in human respiratory tracts were different between NHTP and CC1. It may be possible to understand the result taking into account the differences in chemical and/or physical properties of main stream smoke between NHTP and CC1. While, it may be presumed that nicotine pharmacokinetics profiles are different between NHTP and CC1.

STPOST 39

Estimation of smokers' mouth level exposure to cigarette smoke with filter analysis method

CAI Junlan; PENG Bin; WANG Bing; WANG Sheng; ZHAO Ge; YU Jingjing; GUO Junwei; ZHANG Xiaobing; XIE Fuwei

Zhengzhou Tobacco Research Institute of CNTC, No. 2 Fengyang Street, High-tech Industrial Development Zone, Zhengzhou, Henan 450001, P.R. China

Part-filter analysis method was used to estimate Chinese smokers' mouth level exposure (MLE) to nine chemical components in cigarette smoke. 1) A series of quantitative analysis methods for determining levels of nicotine, tar, solanesol and six harmful components (i.e. ammonia, crotonaldehyde, hydrogen cyanide, phenol, NNK and benzo[a]pyrene) in smoked cigarette filters was developed. The precision and recovery of the developed methods were satisfied for both whole-filter and part-filter. 2) According to the results of Chinese smokers' smoking behaviours investigation and relevant literature, six smoking regimes were designed to estimate Chinese smokers' MLE to cigarette smoke. Under the designed smoking regimes, the deliveries in mainstream cigarette smoke and retention by filter of the above nine target analytes were determined by taking cigarettes with different type filters (i.e. cellulose acetate filter, activated carbon filter, polypropylene fibre filter and paper filter) as samples. The linear regression equations of retention by whole-filter or part-filter versus the deliveries in mainstream cigarette smoke for the nine analytes were established, the correlation between linear regression equations for whole-filter method and part-filter method was investigated, and then part-filter analysis method was determined as the method used for estimating smokers' MLE to the nine

components. 3) A total of 123 Chinese male smokers were recruited and divided into three groups, each group smoked one of the three cigarettes with labelled ISO tar values of 6, 8 and 12 mg. The smokers' MLE to the nine components was estimated with the developed part-filter analysis method. The results showed that the smokers' mean MLE to smoke of cigarette with labelled ISO tar value of 6 mg was lower than that of 8 or 12 mg.

STPOST 40

Role of oxidative stress in the suppression of immune responses in peripheral blood mononuclear cells exposed to combustible tobacco product preparations

PRASAD G.L.(1); ARIMILLI S.(2); DAMRATOSKI B.E.(2)

(1) RAI Services Company, Scientific & Regulatory Affairs, 401 North Main Street, Winston-Salem, NC 27102, U.S.A.
(2) Wake Forest University Health Sciences, Department of Microbiology & Immunology, Winston-Salem, NC 27101, U.S.A.

Cigarette smoking is a major risk factor for several human diseases. Chronic inflammation, resulting from increased oxidative stress, has been suggested as a mechanism that contributes to the increased susceptibility of smokers to cancer and microbial infections. We have previously shown that whole smoke-conditioned medium (WS-CM) and total particulate matter (TPM) prepared from Kentucky 3R4F reference cigarettes potently suppressed agonist-stimulated cytokine secretion and target cell killing in peripheral blood mononuclear cells (PBMCs) under *ex vivo* conditions.

Here, we sought to investigate the role of oxidative stress from cigarette smoke exposure in the compromised immunity observed in smokers. Particularly, we investigated the mechanisms of WS-CM and TPM induced suppression of select cytokine secretions in Toll-like receptor (TLR) agonist-stimulated cells, and target cell killing by effector cells in PBMCs. Pre-treatment with N-acetyl cysteine (NAC), a precursor of reduced glutathione and an established antioxidant, protected against DNA damage and cytotoxicity (measured by γ-H2AX and 7-AAD staining, respectively) caused by exposure to WS-CM and TPM. Similarly, secretion of interferon-γ, tumor necrosis factor, interleukin (IL)-6 and IL-8 in response to TLR-4 stimulation was restored by NAC. Target cell killing, which is used as a functional measure of cytolytic cells in PBMCs, is suppressed by WS-CM. Pre-treatment with NAC restored the target cell killing in WS-CM treated PBMCs. This was accompanied by higher perforin levels in the effector cell populations. Collectively, these data suggest that reducing oxidative stress caused by cigarette smoke components restores select immune responses in this *ex vivo* model.

STPOST 41

Investigation on the chloranisoles in tobacco

WANG Jiale; LV Liangliang; ZENG Lingjie; QI Dawei; LIN Huaqing; WU Da

Shanghai Tobacco Group Co., Ltd., Technology R&D Center, Shanghai 200082, P.R. China

A novel analytical method for the determination of chloranisoles (2,4-dichloroanisole, 2,6-dichloroanisole, 2,4,6-trichloroanisole and 2,3,4,6-tetrachloroanisole) in tobacco was developed by using gas chromatography-tandem mass spectrometry (GC-MS/MS). In this method, Concurrent Solvent Recondensation Large Sample Volume Injection (CSR-LVI) was applied using a temperature-programmed inlet, and by applying a large injection volume of 25 µL, which greatly enhanced the analytical sensitivity of ultra-trace chloranisoles. Florisil SPE cleaning of tobacco sample extracts was performed for the sample pre-treatment, followed by a back-flush technique to expel the high boiling point components, reducing the contamination of the

chromatographic system. This method exhibited ultra-high sensitivity, excellent selectivity, recovery and repeatability, and was suitable for routine analysis of multitudinous tobacco samples. The method was applied to investigate changes in the contents of chloranisoles and in the musty odour of flue-cured tobacco during the mouldy tobacco cultivation process. The result showed that the contents of chloranisoles, especially of 2,4-dichloroanisole and 2,4,6-trichloroanisol, was correlated with the degree of musty odour, and a quantitative relationship between two chloranisoles and musty odour in tobacco could be established.

STPOST 42

A proposed approach for modeling HPHC yields

ROSTAMI A.A.; PITHAWALLA Y.B.; HANNEL T.; MORTON M.J.; WANG J.; GANNON T.; WAGNER K.A.

Altria Client Services LLC, Research, Development and Regulatory Affairs, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.

To ensure quality, consistency and supply security of its portfolio over time, a company may need to make changes that affect all or much of its portfolio of products. Among other requirements, prior to introducing a changed tobacco product into interstate commerce, FDA requires reporting of Harmful and Potentially Harmful Constituents (HPHC). In our proposed approach, instead of testing each product individually, we propose conducting a designed experiment of a subset of products that encompass the major design characteristics of the manufacturer's portfolio and use statistical modeling to determine the HPHC yield for the rest of the portfolio. Additionally, such a modeling approach could also potentially be used to generate supporting information for other premarket submissions such as Substantial Equivalence Reports.

To demonstrate feasibility, we used 30 representative products that cover the range of cigarette design and filler parameters of our entire portfolio. One set of 30 products was manufactured using current cigarette paper and another set using paper from an alternate supplier. The experiment was controlled to minimize product, manufacturing and analytical testing variations between the products with the two cigarette papers. Models were developed to correlate the HPHC yields of the changed product to yields of the control product. For model validation, 12 different products were randomly selected from the remaining products. The predicted yields from the model were compared with the measured yields. Model predictions were robust and differences between measured and predicted values were within the ISO repeatability limits, thereby demonstrating feasibility of our proposed approach.

STPOST 43

Potential impact of partial and total filter ventilation blocking on cigarette mainstream emissions during smoking

COLARD S.; VERRON T.; JULIEN R.; CAHOURS X.

SEITA-Imperial Tobacco, 48 rue Danton, 45404 Fleury-les-Aubrais, France

Cigarette mainstream smoke yields are broadly proportional to the quantity of tobacco burned during puffs. A mathematical model was developed to calculate this quantity during smoking, taking into account the tobacco burning rates during and between puffs, the burning rates between and on LIP bands, the puffing conditions (puff and inter-puff interval durations, puff volume), filter and paper ventilations, LIP band positions and the probability of extinguishment on each band.

The model was used to assess the impact of partial and total filter ventilation blocking on the exposure of a virtual population of cigarette consumers. A range of smoking conditions were considered with puff durations ranging from 1 to 3 seconds, puff interval from 30 to 90 seconds and puff volume from 20 to 70 millilitres.

For a given product design, calculations show a wide range of estimates for tobacco actively burnt resulting from the different puffing behaviours within a population of smokers. When the extent of blocked filter ventilation increases, the distribution of tobacco actively smoked moves to upper levels assuming the range of smoking behaviour is unchanged. This means that regular smokers of ventilated products could potentially be exposed to higher mainstream smoke yields if 100% of the ventilation holes were blocked.

These findings suggest that a filter ventilation ban should not be considered without a careful evaluation of the potential unintended consequences. In particular studies designed to understand the impact for regular consumers of filter ventilated product switching to non-ventilated products would be required.

STPOST 44

The development of a tobacco laboratory process control sample with elevated levels of selenium

WAGNER K.A.; DAVIS G.A.

Altria Client Services LLC, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.

In 2012, the U.S. Food and Drug Administration (FDA) established a list of harmful and potentially harmful constituents (HPHCs) for currently regulated tobacco products. This list included the following eight metals: beryllium, cadmium, chromium, cobalt, lead, mercury, nickel, and selenium. Selenium is an example of an HPHC that is present at low nanogram or non-detectable levels in tobacco, thus creating significant challenges regarding method development and the generation of reproducible results. Inductively coupled plasma–mass spectrometry (ICP-MS) is frequently employed due to high throughput and sensitivity; however, all of the major selenium isotopes suffer from polyatomic interferences, which can adversely impact accuracy with low level determinations. When conducting analytical assays such as elemental analysis, it is a common practice to include a standard reference material or process control sample as a measure of accuracy and/or to ensure the entire laboratory process is in control. It is important that the matrix of the reference material or process control sample is comparable to the sample matrix to ensure the two matrices behave similarly during sample preparation and analysis. The objective of this work was to produce tobacco with elevated levels of selenium for use as a process control sample. This was achieved by growing TN 90 LC tobacco plants in a greenhouse and adding sodium selenite to the soil during watering. After a growing period of approximately four months, the leaves were harvested, dried, ground and analyzed by ICP-MS. Selenium content in the test samples was determined to be 30.8 ppm demonstrating this process is a viable option for generating a process control sample for selenium.

STPOST 45

Status of benzo[a]pyrene in commonly consumed food products and in selected environments: a review

TYAGI K.; DINESH T.K.; SUDESNA P.; SATHISH KUMAR D.

ITC Limited, ITC Life Sciences & Technology Centre, #3 1st Main, Peenya Industrial Area, Phase 1, Bengaluru-560058, India

Benzo[a]pyrene (B[a]P) is an extensively studied compound among the polycyclic aromatic hydrocarbons (PAHs) in commonly consumed products and in the environment. B[a]P is formed due to incomplete combustion of organic matter and commonly consumed products can also be contaminated from environmental sources, food processing, domestic cooking, and transfer from soil and water. This poster summarises the levels of B[a]P, as studied in edible oils, commonly consumed food, smoked food, cooking fuels and tobacco products. There is no specified maximum limit for B[a]P for the majority of food products around the world except for a few such as edible oils, which have a maximum limit of 10 ppb in China and in Europe the maximum allowed limit is 2 ppb. The amount of B[a]P found in groundnut oil is the highest, i.e. 106 ppb, among edible oils and in common food products it ranges from not detected to 58.2 ppb. Smoked food contains significant amounts of B[a]P ranging from 0.1 to 54 ppb. Tea, which is one of the most highly consumed beverages, contains B[a]P from 0.6 to 21.9 ppb. B[a]P has also been reported in tobacco (0.11 to 19.3 ppb) and cigarette smoke (2.1 to 22 ng/cig). The concentration of B[a]P in the kitchen environment, where kerosene oil is used as fuel for cooking, is 52.8 ng/4h/day; however, where coal, wood and cattle dung are used as fuel for cooking, the amount of B[a]P available for a person to inhale is extremely high (3810 to 27951 ng/4h/day). This review of scientific findings indicates that almost all the food products, especially tobacco products, contain very low amounts of B[a]P; whereas in the kitchen environment, where coal, wood and cattle dung are used as fuels, much higher amounts of B[a]P can be found.