ABSTRACTS OF PRESENTATIONS MADE AT THE 2014 CORESTA CONGRESS IN QUEBEC, CANADA

SMOKE SCIENCE AND PRODUCT TECHNOLOGY

(in alphabetical order of first authors)
(Presenter's name is underlined when the main author listed first did not present the paper)

ARIMILLI S.(1); DAMRATOSKI B.E.(1); PRASAD G.L.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST38

Role of oxidative stress in the suppression of immune responses in peripheral blood mononuclear cells exposed to combustible tobacco product preparations

Chronic cigarette smoking is known to adversely impact innate and adaptive immune responses, resulting in immunosuppression, which has been linked to increased susceptibility of smokers to microbial infections and higher incidences of cancer relative to non-smokers. We have shown that Whole Smoke-Conditioned Medium (WS-CM) from Kentucky 3R4F cigarettes potently suppressed agonist-stimulated cytokine secretion and target cell killing in Peripheral Blood Mononuclear Cells (PBMCs) in *ex vivo* cultures, whereas nicotine effects were minimal. Here we investigated the mechanisms of WS-CM induced suppression of select cytokine secretion in Toll-Like Receptor (TLR) agonist-stimulated cells and the cell killing by effector cells in PBMC population.

We investigated the relationship between the oxidative stress from cigarette smoke exposure and the observed suppression of the immune responses in PBMCs utilizing the *ex vivo* model. The addition of N-acetyl cysteine (NAC), a precursor of reduced glutathione and an established antioxidant, during exposure to WS-CM, resulted in reversing the adverse effects of WS-CM. The DNA damage and cytotoxicity due to exposure of WS-CM were reversed. Similarly, a recovery in secretion of IFN-γ, TNF, IL-10, IL-6 and IL-8 in response to TLR-4 stimulation was observed. The inhibition in target cell killing, a functional measure of cytolytic cells in PBMCs, by WS-CM was also restored by NAC. This was accompanied by augmentation of perforin levels in the effector cell populations. Consistent with our previous set of studies, nicotine treatment minimally impacted cytokine secretion and target cell killing, and addition of NAC did not modulate those responses. Collectively, these data suggest that the oxidative stress caused by WS-CM in PBMCs resulted in the suppression of receptor-mediated immune responses.

1. *Department of Microbiology & Immunology, Wake Forest University School of Medicine, Winston-Salem, NC 27157, U.S.A.*
2. *R.J. Reynolds Tobacco Company, R&D Department, Winston-Salem, NC 27102, U.S.A.*

BASU S.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST32

Impact of carbon particle size and pressure drop of the CelFX™ Matrix Technology Section on carbonyl reduction at constant total cigarette pressure drop

The CelFX™ advanced filtration platform utilizes a binder and activated carbon to efficiently filter cigarette smoke. Previous studies have demonstrated the role of activated carbon in reduction of many vapor phase components including various carbonyls. CelFX™ based filters have a much better carbonyl removal efficiency compared to conventional carbon filters. In this study, we show the impact of both activated carbon particle size (30 × 70 to 12 × 40 mesh) and pressure drop of CelFX™ segment (1.3 to 4.2 mm of H_2O/mm of segment) on carbonyl removal efficiency at constant total filter pressure drop. The ISO 3308 smoking protocol was used but the ventilation holes were blocked.

Celanese, 3520 Virginia Drive, Narrows, VA 24124, U.S.A.

BEHRSING H.P.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST24

Toxic insult to rat precision cut lung slices increases tissue cytokine levels and activation of macrophages, and causes acute damage, while prolonged insult may lead to increased deposition of collagen – a marker of fibrosis

The use of *in vitro* or *ex vivo* models is intended to provide meaningful data that will identify or predict the adverse effects of tissue exposure. Precision-cut lung slices (PCLS) are used as a model that retains the heterogenous population of cells in the native architecture of the organ. The retention of native cells allows the study of the initial, dynamic events (such as inflammation) that occur following a toxic insult prior to overt tissue damage. The purpose of the reported studies was to identify initial inflammatory signals, acute toxicities, as well as markers associated with chronic toxicities of PCLS exposed to a toxic insult as a way to qualify the model for identifying such endpoints. Rat PCLS were exposed to several chemotherapeutics known to cause acute and/or chronic pulmonary damage. Time points for respective endpoints were chosen based on known response times of when relevant endpoints may change. Cytokines and acute toxicity were evaluated during initial days of exposure while activation of macrophages and collagen deposition were evaluated through four weeks of culture in other studies. Exposure of PCLS for 24 hours resulted in increased cytokine levels and 72 hour exposure caused overt toxicity, as assessed using tissue protein content and histologically using H&E and ED-1 staining. Long term exposure of PCLS to two agents known to cause fibrosis (bleomycin and carmustine) resulted in elevated numbers of macrophages and also increased collagen deposition. PCLS generate inflammatory cytokine signals and, if levels persist after insult removal, these signals may predict subsequent tissue damage. The expression of adverse markers of chronic exposures (collagen deposition) in PCLS may signify risk of fibrosis. Cytokine responses, macrophage activation, and fibrosis are hallmarks of tobacco related exposures. PCLS may elucidate acute and chronic adverse pulmonary responses when exposed to tobacco products.

Advanced In Vitro, LLC, Frederick, MD 21704, U.S.A.

BELUSHKIN M.(1); PIADÉ J-J.(1); CHAPMAN S.(2); FAZEKAS G.(2)

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Predictability of *in vitro* toxicological assessments of cigarettes: analysis of seven years of regulatory submissions to Canadian authorities

Canadian authorities have required the annual submission of a wealth of by-brand information about cigarettes – including analytical data for cut filler and mainstream smoke constituents, for more than a decade. In 2005, the reporting requirements were extended to include results of three *in vitro* toxicity tests. These toxicity tests are to be performed on each brand annually, regardless of whether modifications to the brand were made. In the present study[1], information covering the period 2006-2012 submitted by Rothmans, Benson & Hedges – the Canadian affiliate of Philip Morris International – was analysed to investigate the possibility of establishing quantitative models for the *in vitro* toxicological endpoint responses to cigarette smoke. For the first time this dataset has allowed the confirmation of previously published results concerning the influence of such factors as cigarette blend, diameter and filter type on *in vitro* toxicity at the level of a representative range of products on a market. Taking these cigarette design features into account and adding a limited amount of quantitative mainstream smoke composition information, it was shown that, within the boundaries of the considered cigarette design parameters, the *in vitro* toxicological response could be effectively predicted. *In vitro* tests of tobacco products are an invaluable initial comparative product assessment tool. The present results reveal the limited value of data from repeated tests on products within the specified design ranges.

[1] Belushkin M.; Piadé J-J.; Chapman S.; Fazekas G.; Investigating predictability of *in vitro* toxicological assessments of cigarettes: Analysis of 7 years of regulatory submissions to Canadian regulatory authorities. Regulatory Toxicology and Pharmacology 2014, **68** (2), 222-230.

1. *Philip Morris International, Research & Development, Philip Morris Products S.A., Rue des Usines 90, CH-2000 Neuchâtel, Switzerland*
2. *Rothmans, Benson & Hedges Inc., 174 Kennedy Road South, Brampton, Ontario L6W 3G6, Canada*

BENNING J.; LIU C.; McADAM K.G. et al.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST11

Arsenic speciation in selected tobacco products

Arsenic is classified as an IARC Group 1 carcinogen and is known to be present in tobacco and cigarette smoke. Arsenic's toxicity varies dependant on the species and the levels present. We have previously published work to investigate the arsenic species present in both cut tobacco and in mainstream cigarette smoke condensate; firstly using synchrotron-based X-ray spectrometry, and secondly using HPLC coupled with ICP-MS. In 3R4F reference cigarettes, the predominant species in the tobacco was found to be Arsenate (AsV), while in the smoke condensate both Arsenite (AsIII) and AsV were present and their ratio varied depending on the age of the smoke.

The objective of this study was to extend the arsenic speciation determination across a range of tobacco products using the HPLC-ICP-MS method. This included a range of smokeless tobacco products and tobaccos used in cigarettes with different tobacco types and grown in different geographical locations. The tobaccos selected gave varying pHs when extracted in water; this was to test the hypothesis that the pH of the tobacco affects the arsenic speciation. The tobacco samples were analysed using a sequential extraction followed by analysis using HPLC-ICP-MS. In addition, the total arsenic content was determined using microwave acid digestion followed by ICP-MS analysis.

The predominant extractable arsenic species across all the samples was found to be AsV, which is consistent with the finding for 3R4F cigarette tobacco. Additionally, small quantities of Arsenite and the organic forms Monomethylarsonic acid (MMA) and Dimethylarsinic acid (DMA) were also detected. For the cigarette tobaccos studied, the results suggested that as the tobacco pH increased, the percentage of the total As extracted decreased; however this would need to be confirmed by further work. The results also showed that the predominance of AsV was consistent across different tobacco products and different pHs.

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

BOMBICK B.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST77

***In vitro* toxicity testing of tobacco products: the CORESTA *In vitro* Toxicity Testing of Tobacco Smoke Task Force**

Results from *in vitro* toxicity tests have been reported for several decades. Methods for several *in vitro* toxicity tests have been formalised, accompanied by both national and international guidelines for toxicity test batteries. Cigarette smoke (cigarette smoke condensate/total particulate matter, "whole smoke") has been shown to elicit positive responses in many of these assays. The CORESTA *In vitro* Toxicity Testing of Tobacco Smoke Task Force was established in 2002, "to prepare a report covering the rationale and strategy for conducting *in vitro* toxicity testing of tobacco smoke" and "to identify key procedures based upon internationally recognised guidelines, adapted to accommodate the nature and unique properties of tobacco smoke". The Task Force's mandate subsequently modified as follows: "To conduct a proficiency testing programme to evaluate cigarette smoke using a common experimental protocol and the Task Force's recommended test battery". While Task Force proficiency trials have primarily focused on "cigarette smoke condensate" (smoke particulate extracted in DMSO), *in vitro* evaluation of "whole smoke" has become an increasingly active discussion topic. This presentation will provide a brief review of *in vitro* toxicity testing, a summary of Task Force accomplishments and some thoughts on *in vitro* toxicity testing in a regulatory context.

R.J. Reynolds Tobacco Company, R&D 611-12/250, P.O. Box 1487, Winston-Salem, NC 27102, U.S.A.

BROKL M.(2); FOÇANT J-F.(2); TICHA J.(1); BISHOP L.(1); WRIGHT C.(1)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST67

Application of multi-dimensional GC techniques to the analysis of cigarette smoke

Tobacco smoke is an extremely complex and dynamic aerosol consisting of liquid/solid droplets (particulate phase) suspended in a mixture of gases and semi-volatiles (gaseous phase). It is formed during overlapping processes of oxidation, pyrolysis, pyrosynthesis, distillation, sublimation, condensation, filtration and elution. Smoke is emitted either as the mainstream smoke from the cigarette filter or from the smouldering cigarette in the form of sidestream smoke. Mainstream smoke consists of over 6000 identified compounds and some reports claim the number of unidentified compounds might reach up to 100,000.

Methods have been developed for the separation and identification of mainstream smoke constituents that include headspace solid-phase microextraction (HS-SPME) and comprehensive two-dimensional gas chromatography (GC×GC) coupled to time-of-flight mass spectrometry (TOFMS). Such approaches allow evaluation of the profiles of volatile and semi-volatile compounds present in mainstream tobacco smoke. Data analysis methodology has been developed and used to evaluate the capability to distinguish quantitative or qualitative differences between samples by the use of statistical comparison and principal component analysis (PCA).

Examples will be presented of chemical profile differentiation, for example, those associated with modifications to cigarette filter materials and the potential use of this technique for characterisation of non-combustible products.

1. *British American Tobacco, Group R&D, Regents Park Road, Millbrook, Southampton SO15 8TL, U.K.*
2. *University of Liège, Mass Spectrometry Laboratory (CART), Allée du 6 août, B6c (R.1), B-4000 Liège, Belgium*

CABOT R.(1); JOHNSON T.J.(2); OLFERT J.S.(2); YURTERI C.Ü.(1); McAUGHEY J.(1)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST83

Steady-state and transient effective density of cigarette smoke

Effective particle density is an important aerosol characteristic and relates a particle's mobility diameter to its mass; an understanding of both properties is important to model impaction and settling losses, such as in human lung deposition. The nature of tobacco smoke formation with water present and where early coagulation processes dominate, suggest that droplets should be spherical and of uniform composition across the size distribution. In this study, mobility diameter was measured by electrical mobility (DMS-500, Cambustion, UK) and mass by using opposing electrical and centrifugal fields (CPMA, Cambustion, UK). Smoke sampling into a Tedlar® bag with sequential DMA-CPMA classification gave a measure of steady-state density. Direct puff sampling (Smoking Cycle Simulator, Cambustion, UK) with sequential CPMA-DMS classification yielded puff by puff transient density data. The steady-state average effective particle density for a University of Kentucky 3R4F cigarette was 1180±113 kg/m^3 and was independent of particle mobility-size (i.e. the particles were spherical with constant density). The steady state values were consistent with earlier published data of 1120±40 kg/m^3 (Lipowicz, 1988: 1R3F; Chen, 1990: 2R1F). Transient data were measured for 3R4F and a range of commercial cigarettes from 1-7 mg ISO pack tar. The average effective particle density (puffs 3 to 6) varied from 1090-1518 kg/m^3, with a majority falling within 1300-1394 kg/m^3. Density was typically greater for the commercial cigarettes than 3R4F. No effect on density was seen within a puff, with puff number, with cigarette format, or between ISO and Health Canada puff parameters, although the puff parameters in particular affected both particle diameter and concentration significantly. In conclusion, these studies offer a new method of real-time aerosol classification and have shown that smoke density is relatively consistent for any cigarette type and over the full smoke particle size distribution.

1. *British American Tobacco, Group R&D, Regents Park Road, Millbrook, Southampton SO15 8TL, U.K.*
2. *University of Alberta, Department of Mechanical Engineering, Edmonton T6G 2G8, Canada*

CABOT R.; YURTERI C.Ü.; McAUGHEY J.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST40

Droplet size measurement of e-cigarette aerosol

Electronic cigarettes (also known as e-cigarettes or Electronic Nicotine Delivery Systems – ENDS), are a new type of product rapidly gaining popularity with adult cigarette smokers. They typically produce a condensation aerosol by quickly evaporating a formulation containing nicotine and water with glycerol, propylene glycol (PG) or a mixture of each. This study sought to measure droplet size distributions by real-time analytical methods using commercially available equipment. Measurements were conducted by electrical mobility (EM: Model DMS-500 MkII, Cambustion, UK) and by laser diffraction (LD: Spraytec, Malvern, UK). The Smoking Cycle Simulator (SCS: Cambustion, UK) was used to generate appropriate puff profiles, and to minimise dilution and potential droplet evaporation. Core profiles included 50, 55, 70 and 80 mL puffs of 3 s duration every 30 seconds. Volume-weighted median droplet diameters (d_{50}) from a variety of e-cigarette devices were typically less than 500 nm by LD and less than 300 nm for EM, slightly larger than equivalent tobacco smoke measurements of approximately 210 nm. The electrical mobility data were larger than previous published data suggesting evaporation was in part suppressed. Precision data were dependent on the e-cigarette tested but coefficients of variation of less than 4-5% were observed for the better performing products. This degree of precision meets the acceptance criteria for droplet size distribution ($d_{50} \pm 20\%$ for $d_{50} < 1$ µm) for laser diffraction measurements for similar aerosol products. No equivalent standards are available for electrical mobility measurements. In conclusion, droplet size measurement for e-cigarettes can be readily achieved using commercially available sampling and measurement instrumentation, with suitable precision for product comparisons and to meet any potential regulatory requirements.

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

CAHOURS X.; VERRON T.; JULIEN R.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST50

Collaborative studies: definitions, concepts and outcomes

Whenever decisions are based on analytical results, it is important to assess the quality of the results, that is, the extent to which they can be relied on for the purpose at hand. In some sectors of analytical chemistry, it is now a formal requirement for laboratories to introduce quality assurance measures to ensure that they are capable of providing data of the required quality. Knowledge of the uncertainty of measurement results is essential to their interpretation. Without quantitative assessments of uncertainty, it is impossible to decide whether observed differences between results reflect more than experimental variability, whether test items comply with specifications, or whether laws based on limits have been broken. Without information on uncertainty, there is a real risk of either over- or under-interpretation of results.

The purpose of a collaborative study is to determine estimates of the attributes of a method, particularly the "precision" of the method that may be expected when the method is used in actual practice. The two terms to define the precision of a method under two circumstances of replication are repeatability and reproducibility.

The results of collaborative studies yield a set of performance figures (sR, sr, and, in some circumstances, a bias estimate) that form a 'specification' for the method performance. It should be stressed that a collaborative study is primarily a test of the method and not of the laboratory.

The aim of this presentation is to briefly describe some of the factors that need proper attention in the design of collaborative studies and some of the aspects of statistical treatment of the data by ISO 5725-2 (1994) that are left somewhat open to interpretation. The use of precision estimates will also be discussed.

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

CHAMBERS O.; JI H; GEARY J.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST30

Information on the cooperative agreement between the Center for Tobacco Products and the University of Kentucky to develop a "Cigarette Tobacco Reference Product Program"

The United States Food and Drug Administration's Center for Tobacco Products (CTP) recently announced a cooperative agreement with the University of Kentucky to develop a cigarette tobacco reference product program. The University of Kentucky has provided reference cigarettes as the standard for non-clinical investigational purposes by tobacco manufacturers, contract and government laboratories, and academic institutions since 1968. The new project will produce and characterize 50 million, high-quality reference cigarettes representative of an American blended cigarette that are manufactured in one manufacturing run with certain physical and chemical characteristics to allow for proficiency testing, instrument calibration, method validation and for investigation purposes. Significant improvements will be made in the current Kentucky reference product program in terms of operational efficiency, establishing research capability and initiating the proficiency testing program. Information will be presented on the design of the new reference cigarette and the planned changes to the Kentucky reference cigarette program. The schedule for the proficiency testing program and the process used to certify the mean values of the reference cigarettes used in the proficiency testing scheme will be presented.

University of Kentucky, Kentucky Tobacco Research and Development Center, KTRDC Building, 1401 University Dr., Lexington, KY 40546, U.S.A.

CLAYTON P.M.(1); NOTHER K.(1); VERRON T.(2); JULIEN R.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST57

Use of chiroptical spectroscopy to determine the ionisation status of (S)-nicotine in electronic cigarette formulations and snus

A ring trial of the Part-Filter Method, under the auspices of the CORESTA Smoking Behaviour Sub-Group, involving laboratories from the tobacco industry and the contract sector was concluded and the results presented to the Sub-Group in October 2013. A total of eight laboratories returned valid data and the results could be related tentatively to an earlier ring trial reported in 2012, although some details in the standardised method and participating laboratories differed. The aim of the ring trial was to assess the standardised method agreed by the Sub-Group to produce a measure of the repeatability and reproducibility of estimated nicotine and tar yields across the participating laboratories.

The repeatability and reproducibility (as coefficient of variation) for *estimated nicotine* were 4.8 and 9.6%, respectively, averaged across four test product levels. The repeatability and reproducibility (as coefficient of variation) for *estimated tar* were 5.1 and 26.2%, respectively, averaged across the four test product levels. A general rule for the variation introduced by the measurement system is that the methodology is acceptable if the error is below 10%. According to this criterion the method for estimating nicotine yields from nicotine extracted from part filters is under control, while that for estimating tar from the UV absorbance from part-filters requires further attention.

It is the opinion of the Sub-Group that the methodology used in the 2013 ring trial for estimated nicotine yield should be progressed towards becoming a CORESTA Recommended Method.

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

CLAYTON P.M.; VAS C.A.; McADAM K.G.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST49

Use of chiroptical spectroscopy to determine the ionisation status of (S)-nicotine in electronic cigarette formulations

In the vicinity of neutral pH, nicotine is equilibrated between unprotonated and monoprotonated states. The equilibrium is best described by the acid dissociation constant (K_a) and its logarithmic function known as pK_a which in non-aqueous solution is termed p_sK_a. E-cigarette formulations are predominately non-aqueous, typically composed of glycerol, 1,2-propanediol, water and (S)-nicotine, and the p_sK_a of nicotine is influenced by the formulations' concentration, solvent composition and temperature. The objective of this study was to determine the p_sK_a of nicotine in a typical e-cigarette formulation and from that value calculate the distribution between the two states of nicotine. Circular dichroism spectra of (S)-nicotine change with pH, reflecting transitions between unprotonated and monoprotonated forms. Through the addition of acid and alkali solutions we constructed titration curves at 20 °C over the pH range 4-10, whose inflection points yielded the p_sK_a value for nicotine under the specific ionic strength, co-solvent and temperature conditions of the (S)-nicotine/glycerol/water system. The concentration of (S)-nicotine was increased stepwise from 30 µg/mL to 3 mg/mL in each titration curve together with a concomitant reduction in cell path length from 10 to 0.1 mm. From the five p_sK_a values obtained, the p_sK_a value was inferred for 30 mg/mL (a typical e-cigarette formulation level). The p_sK_a at 30 mg/mL in 9% water balanced with glycerol at 20 °C was estimated as 7.24. From this value, using the Henderson-Hasselbalch equation, the proportions of unprotonated and monoprotonated nicotine may be calculated with reference to the pHs (the pH in co-solvent) of the formulation. The methodology described here is advanced as a robust approach for estimating the proportions of the two forms of nicotine that predominate in electronic cigarette formulations.

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

COLARD S.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST02

Optimisation of testing scheme by associating smoking data with cigarette burning model

Tar, nicotine and carbon monoxide (TNCO) cigarette yields determined under different smoking regimes form part of a continuous linear function, with filter ventilation both blocked and open, when linked to the reduction Δt in the smoking time due to puffing (ST09 - CORESTA 2013). This means that the use of a single smoking regime is sufficient to characterise emissions for a particular product if the smoulder rate is known. However, this single smoking regime has to be carefully chosen because the application of intense conditions can produce high water yields leading to trapping issues, to high analytical variability and to over estimation of tar yields. In addition, the smoulder rate determination involved in the calculation of Δt can be difficult for cigarettes with low ignition propensity design which present some tendency for self-extinguishment during measurement.

Experimental issues were overcome in a novel testing scheme involving the determination of smoking times under two smoking regimes and inputting this data into a burning model. Beyond inter-puff smoulder rate determination, the model was used to provide an extensive set of information such as the weight of tobacco burnt during puffs. Good correlations were observed between the mass of tobacco burnt during puffs and TNCO or benzo[a]pyrene yields derived under ISO validated methods.

It was concluded that the application of two smoking regimes is required to provide smoking times and Δt but the analysis of the yields is required from only one of them; taken together, this is an optimal scheme since it provides comprehensive characterisation of products at reduced cost. An appropriate choice of validated smoking regime for yield determination (e.g. ISO regime) could then overcome the limitations observed with the Health Canada intense regime and still fit with the regulatory purposes of product monitoring and characterisation.

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

COLARD S.(1); CHOLET G.(2); TESTUD M.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST25

Leak-based method for measurement of low air permeability of cigarette papers

The air permeability of cigarette paper is assessed currently under the ISO 2965 standard by applying a constant difference of pressure of 1 kPa between the two faces of a paper and by measuring the corresponding airflow.

Lower Ignition Propensity regulations have led tobacco manufacturers to use specific cigarette papers with narrow bands of low air permeability to achieve regulatory compliance. ISO 2965 was revised, then published in 2009 to take into account the specific geometry and characteristics of the bands and to include suitable narrow measuring heads. The consequence was a drastic reduction of the measured airflow levels with banded papers and a need for equipment covering specifically low airflow ranges.

The well-known pressure-airflow relationship across cigarette paper enabled the development of an alternative method to ISO 2965 not requiring direct airflow measurement, and then not requiring expensive airflow meters. In the alternative method, measurements are made of the evolution of the pressure over time after an initial difference of pressure was applied between the two faces, and to analyse consecutively the profile of pressure impacted by the leaks across the paper. The related theoretical aspects were developed for both viscous and inertial airflows, and experimental investigations were conducted with banded and standard cigarette papers.

Results showed very good consistency with ISO 2965 and lower repeatability, demonstrating that a leak-based method could be a simple, reliable and cheaper alternative.

1. *Imperial Tobacco Ltd, Winterstoke Road, Bristol BS3 2LL, U.K.*
2. *Sodim SAS, 48 rue Danton, 45404 Fleury-les-Aubrais Cedex, France*

COLARD S.(1); VERRON T.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST55

Modelling of the effect of indoor intermittent emissions produced by electronic cigarette users on exposure of bystanders

In the context of the regulatory debates about the appropriateness to ban or not e-vapour use in public places, the need to understand and identify the factors affecting bystanders' exposure is particularly useful.

The propagation, dilution and extraction of indoor intermittent, localised emission sources are described by a simplified macro scale modelling based on basic physical principles and reproducing the following steps: puff inhalation, retention in air pathways, exhalation in the environment, aerosol propagation, dilution in air, air renewal, deposition on surfaces, exposure of bystanders and dose inhaled by breathing during a certain period of time. The modelling input parameters were the quantity of constituents inhaled per puff; the retention rate; the puffing session frequency; the number of puffs in each puffing session; the speed of aerosol propagation in the environment; the volume of the room; the air exchange and recycling rates; the speed of deposition on the surfaces; the distance from e-vapour user to bystanders; bystanders' breathing pattern and the time spent in the room. Outputs were then the exhaled constituent concentrations in space and time, and the quantity of constituent breathed.

The model was applied to describe a scenario considering an e-vapour user and a bystander colleague working in the same office. It was possible to simulate the effect of intermittent aerosol constituents' exhalation in environmental air over time and to compare calculations with experimental data.

Even if further experimental studies in real-life conditions need to be conducted, modelling is a good way to understand how indoor air quality can be changed by e-vapour users and for identifying the key factors related to exposure. This also provides elements for discussion on the need or not of a ban in public places.

1. *Imperial Tobacco Ltd, Winterstoke Road, Bristol BS3 2LL, U.K.*
2. *SEITA, Imperial Tobacco Group, 48 rue Danton, 45404 Fleury-les-Aubrais, France*

CUNNINGHAM A.(1); SLAYFORD S.(1); VAS C.A.(1); GEE J.(1); COSTIGAN S.(2); PRASAD K.(1)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST56

Development and validation of a device for measuring puffing topography of e-cigarette users

The study objective was to develop and validate a smoking analyser device able to record the puffing topography of e-cigarette users. The Smoking Analyser Number 7 (SA7) (British American Tobacco) was modified to overcome issues caused by the condensation of humectants in the topography head, which led to inaccurate puff volume determinations using the existing SA7 design.

The device was first calibrated against known pressure and flow rates in the ranges of 0-120 mmWG and 2-120 mL/sec, respectively, using a calibrated pressure meter and A14 syringe driver (Borgwaldt). The calibrated output demonstrated linearity over these ranges.

The modified device demonstrated equivalence to the original SA7 when tested across a range of puff volumes, durations and profiles (sine, square and triangle). Puff volumes were on average within ±0.1 mL of the pre-set volume across the range 20-80 mL, whilst puff durations were within ±0.01 s of the duration measured using the original SA7 device, in the range 1.5-3.0 s.

The performance of the modified device to accurately record puffing topography profiles of e-cigarettes was evaluated using disposable, cartomiser based and modular devices, puffed to exhaustion of the e-cigarette battery or a maximum of 150 puffs. Each e-cigarette was tested in triplicate at three pre-defined puffing regimes across flow rates of 18.3-40.0 mL/s and produced puff volumes within 8.2% (4.5 mL) of the pre-set values. The degree of accuracy observed for puff volume measurements of the e-cigarette aerosols is comparable to the 6.0% level observed for combustible cigarette aerosol measurements.

We propose that the modification to the existing SA7 technology provides a robust system for the measurement of e-cigarette users' puffing topography. We provide examples of users' puffing behaviour, as a first step in providing data to support evaluation of e-cigarettes in a manner reflective of users' behaviour.

1. *British American Tobacco, Group R&D, Regents Park Road, Millbrook, Southampton SO15 8TL, U.K.*
2. *Nicoventures, 22 Tudor Street, London EC4Y 0AY, U.K.*

DEMOUTE A-L.; AL-JOBORI O.; POULEAU B.; SMART D.J.; McHUGH D.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST23

Quantitative evaluation of the mutagenicity induced by tobacco smoke-derived total particulate matter using the mouse lymphoma assay

The mouse lymphoma assay (MLA) is a regulatory *in vitro* test deployed to evaluate the mutagenicity of a test substance via exposure of the thymidine kinase locus in L5178Y mouse lymphoma cells. As part of a proficiency study, we evaluated the mutagenicity of the total particulate matter (TPM) aerosol fraction derived from the mainstream smoke of 3R4F reference cigarettes (3R4F; University of Kentucky, USA). L5178Y $tk^{+/-}$ clone 3.7.2C IVGT (PHE) cells were exposed to TPM derived from 3R4F under 4 h ± S9 and 24 h (-S9) treatment conditions using a study design compliant with the OECD guideline n° 476 and the recommendations of the International Workshop on Genotoxicity Testing (MLA Workgroup). On nine out of 10 independent test occasions, the TPM fraction induced biologically-relevant and concentration-dependent mutagenicity under the 4 h +S9 treatment condition. The lowest observed genotoxic effect level (LOGEL) range for this treatment condition was 30-60 µg/ml at relative total growth (RTG) values of 10-20%. TPM was also found to induce biologically-relevant mutagenicity in the absence of S9 with LOGELs between 12-30 µg/ml although the responses observed under these treatment conditions were less pronounced and occurred less frequently than in the presence of S9, and always arose at the cytotoxic limit of the assay. In conclusion, these data demonstrate the proficiency of the MLA at quantitating the mutagenicity induced by tobacco smoke-derived TPM.

References:

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Philip Morris International, Research & Development, Philip Morris Products S.A., Department of Biological Systems Research, Genetic and Cellular Toxicology Laboratory, Neuchâtel CH-2003, Switzerland

DUROT N.(1); LE BEC L.(1); RIGOULAY C.(1); RAVERDY-LAMBERT D.(1); BENSE T.(2); GIORDANO O.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST90

Effect of reconstitution and tobacco blend components on Hoffmann analytes

The perspective of potential regulatory ceilings on smoke deliveries in the future increase the need to assess the influence of tobacco blend components on smoke deliveries, more particularly the influence of papermaking reconstituted tobacco leaf (RTL), expanded stem (CRES) and expanded lamina (EL).

The effect of papermaking tobacco reconstitution on smoke yields has been assessed with respective contribution of the fibrous and the soluble fractions.

In the ISO smoking regime, and when comparing RTL to the tobacco blend used as feedstock for reconstitution, reductions up to 25% are observed for tar, tar/puff, benzene, nicotine, hydrogen cyanide (HCN), butadiene, toluene, phenols except catechol, carbonyls except formaldehyde. Reduction of ammonia, isoprene, styrene, aromatic amines, poly aromatic hydrocarbons (PAHs), semi volatiles are specific and superior to 40%.

The main precursors of carbon monoxide (CO), aldehydes, volatiles, ammonia, HCN, PAHs are in the fibre part. For other some constituents analysed, the precursors are present both in fibres and solubles (e.g. nitrogen oxide, phenol), or mostly in solubles (e.g. hydroquinone, 1-aminonaphthalene, 4-aminobiphenyl). On the other hand, the effect of blend components (CRES, RTL, EL) level (0-20%) has been tested through a design of experiment (simplex centroid matrix). Smoke deliveries were measured with the Health Canada Intense (HCI) smoking regime. Models were validated at 95% of confidence for puff number, CO, NO, NO_x, ammonia, certain aldehydes and para-cresol and 4-aminobiphenyl. All three components decreased ammonia, aromatic amines, some phenols and PAHs. EL was the most effective to reduce phenols whereas RTL was the most effective for PAHs. The other smoke constituents are more or less stable except formaldehyde.

In conclusion, a majority of smoke constituent precursors are insoluble and reside in the fibrous insoluble portion of tobacco. In addition, the proportions of expanded lamina, expanded stems and reconstituted tobacco will influence smoke deliveries, contributing to reducing some of them, in particular RTL aromatic amines, phenols and PAHs.

1. *SWM Intl, c/o LTR Industries, Usine Le Mans, 72702 Allonnes Cedex, France*
2. *MONTEPAZ, c/o Compania industrial de Tabacos Monte-Paz SA, San Ramon 716, CP 11800 Montevideo, Uruguay*

DUROT N.(1); TARDIF N.(1); ZIMNY S.(1); LE MOIGNE C.(1); LOUREAU J-M.(2); RAVERDY-LAMBERT D.(1)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST20

Evolution of cigarette design versus 10/1/10 cigarettes and LIP regulations

The implementation of a Low Ignition Propensity (LIP) regulation in combination with 10 mg tar/1 mg nicotine/10 mg carbon monoxide ceilings (10/1/10) requires adjustments in cigarette design.

The levers and tools available to comply with both regulations and how they are actually used will be reviewed.

The major tools to reduce smoke yields are based on two principles: the reduction of puff number and the reduction of yields per puff. Main design changes adopted to achieve 10/1/10 were the decrease of tobacco weight and density and the increase of filter ventilation, leading to a decrease of puff number or a reduction of deliveries per puff. Additionally and depending on blend style, filter retention and paper permeability were adjusted.

Compliance to LIP requirements is achieved by switching from an unprinted base paper to an LIP paper with two bands printed per cigarette. The low ignition behaviour is obtained through a shutdown of paper permeability in the band, which ensures ASTM requirement when properly designed on one hand, but on the other hand, the decrease of permeability generates increases in smoke deliveries due to a reduction of diffusion/dilution by cigarette paper. To comply with LIP as well as the 10/1/10 regulations, cigarette manufacturers have to modify the design of their cigarettes. In Europe, a variety of solutions was applied. Increased paper permeability and filter ventilation were the most obvious ones. Some limited tobacco blend adjustments were also observed.

In a majority of cases, several cigarette paper parameters (e.g. permeability, burn additives) have been adjusted to offset effect of band application on smoke deliveries, in particular carbon monoxide.

Tobacco blend components (expanded lamina, expanded stems, reconstituted tobacco) also influence ASTM and FASE performances. Nevertheless, by and large tobacco blend composition has remained unchanged, the achievement of Low Ignition Propensity being achieved through fine tuning of the LIP paper design.

1. *SWM Intl, c/o LTR Industries, Usine Le Mans, 72702 Allonnes Cedex, France*
2. *SWM Intl, c/o Papeteries de Mauduit, Kerisole, 72702 Quimperlé Cedex, France*

EITZINGER B.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST19

Key design parameters of cigarettes for smoke yield reduction

Due to regulations, but also for other reasons, the smoke yields of commercial cigarettes have decreased over the last decades. For the design of cigarettes several options are available to reduce smoke yields. In this study an overview of these options is given. Apart from the filter, the tobacco and the overall cigarette geometry, also the paper components, that is, tipping paper, plug wrap paper and cigarette paper, are discussed with respect to their relative contributions to smoke yield reduction when their main properties such as air permeability, diffusion capacity, burn additives, filler content, fibre composition and basis weight are varied. Typical high-"tar" and low-"tar" cigarettes are explained in view of these design choices. Certain interactions, for example, between the tobacco rod, the filter and the ventilation system, are explained, and also the design limitations are discussed with respect to technical considerations and consumer acceptance aspects, particularly with respect to pressure drop. Regulatory changes such as the introduction of lower ignition propensity cigarettes and alternative smoking regimes are reviewed for their effect on the ability to adjust smoke yields with the available design tools. In summary, it can be concluded from these considerations that, as the number of regulatory requirements increases, the effectiveness of certain cigarette design tools is reduced and the fine-tuning of cigarette properties to develop a legally compliant and consumer acceptable product becomes more difficult.

delfortgroup AG, Fabrikstraße 20, 4050 Traun, Austria

EITZINGER B.(1); GLEINSER M.(2); BACHMANN S.(2); VOLGGER D.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST95

The pore size distribution of naturally porous cigarette papers and its relation to air permeability and diffusion capacity – Part 2

Several publications have shown in the past, on theoretical grounds and by actual measurements, that the perforation of cigarette papers causes a substantial increase in air permeability, while the diffusion capacity increases comparably less. These findings have been simplified and popularised in the probably questionable statement that large pores are responsible for air permeability and small pores for diffusion capacity. It is the aim of this study to investigate the substance of such statements by correlating the pore size distribution of naturally porous cigarette papers with their air permeability and diffusion capacity, respectively. To this end eight cigarette papers were selected that differed in permeability, diffusion capacity, fibre furnish, filler content and burn additive content. The pore size distribution of these papers was measured by mercury porosimetry before and after the papers had been exposed to 230 °C for 30 minutes. The pore size distribution was multiplied with a Gaussian weighting function and integrated to obtain a weighted pore volume. The two parameters of the weighting function, mean value and standard deviation, were chosen to maximise the correlation of the weighted pore volume with air permeability and diffusion capacity, respectively. The results show a good correlation with correlation coefficients greater than 0.9 for the air permeability as well as for the diffusion capacity. The optimal mean values of the weighting functions were at a pore radius of 2.5 µm for air permeability and 1.0 µm for diffusion capacity. These results indicate that in fact large pores are better correlated with changes in air permeability, while small pores are more strongly correlated with changes in diffusion capacity. They also demonstrate the tight relation between pore size distribution, air permeability and diffusion capacity, which makes the pore size distribution a tool to further optimise cigarette papers, for example, with respect to carbon monoxide yields.

1. *delfortgroup AG, Fabrikstraße 20, 4050 Traun, Austria*
2. *Papierfabrik Wattens GmbH & Co. KG, Ludwig-Lassl-Straße 15, 6112 Wattens, Austria*

ERICSSON D.; PATRING J.; LINDHOLM J.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST58

Analysis of volatile aldehydes in smokeless tobacco with a rapid, one-step extraction and derivatisation with UHPLC-MS/MS quantification

The volatile carbonyls formaldehyde, acetaldehyde and crotonaldehyde have routinely been analysed in tobacco smoke for many years, but much lesser in smokeless tobacco. Since 2012 they have been included in the "Draft Proposed Initial List of Harmful/Potentially Harmful Constituents in Tobacco Products" presented by the United States Food and Drug Administration (FDA). Today there is no recommended method for the analysis of these aldehydes in Smokeless Tobacco Products (STPs). Consequently there is a need for a reliable method to be used in a high-throughput manner in tobacco industry labs for analysis of STPs.

Aldehydes need to be derivatised in order to improve the sensitivity and selectivity of both GC- and UHPLC-methods. In this method the commonly used derivatisation agent 2,4-dinitrophenylhydrazine (DNPH) is used together with UHPLC-MS/MS.

The key characteristic of this rapid sample preparation method is that aldehyde extraction, derivatisation and enrichment are performed at the same time during a 1-hour-shaking in a two-phase system, consisting of an aqueous and an organic phase. The extraction and the derivatisation occur in the aqueous ammonium formate buffer and then aldehyde-DNPH derivatives are enriched in the organic isohexane phase. After one hour shaking, a non-measured volume of the isohexane phase is readily transferred to an LC-vial. Besides the enrichment-effect of the derivatives in the organic phase, the most important function of isohexane is stabilisation of the aldehyde-derivative concentration (at least 8 days), which otherwise proves troublesome in aqueous tobacco extracts.

Isotopically labeled internal standards improve the precision and accuracy. UHPLC enables fast separation (5 min/sample) and MS/MS gives excellent sensitivity and selectivity. The limit of quantitation (LOQ) for formaldehyde, acetaldehyde and crotonaldehyde is 0.05, 0.5 and 0.03 ppm, respectively. The method repeatability (RSD) is 6-8%, accuracy 70-110% and the capacity is c. 75 samples/day. Altogether this method has the robustness, sensitivity and capacity to meet the demands at tobacco industry labs.

Swedish Match North Europe AB, Maria Skolgata 83, SE-118 85 Stockholm, Sweden

FARMEN R.H.; NEWLAND K.E.; NACHI R.; KAFONEK C.J.; ISLAM R.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST72

The analysis of isotopically labeled propylene glycol in e-cigarettes

Measurement of the excipients in e-cigarettes has become a hot topic of discussion with the release of the new draft guidance from the United States Food and Drug Administration (FDA). However difficulties in the selective measurement of the bioavailable portion of the excipients have allowed questions regarding the reduced health risk of e-cigarette use to remain open and nebulous to resolve. One of the commonly maligned chemicals is the carrier agent, propylene glycol. Despite the warnings from popular media and less popular politicians, propylene glycol (also found in soft drinks, ice cream, deodorant, cosmetics, and M&Ms) is identified by the FDA as safe for consumption at reasonable levels over long periods of time.

To date, methods validated for the measurement of propylene glycol in blood matrix have been confounded by significant interference of dietary and common lifestyle exposures. Measurements of basal levels of propylene glycol in plasma from healthy non-smokers (and non-vapers) range from 200 to 13,000 ng/ml. With this broad range of exposure there is no measurable difference between vapers and non-vapers.

We have developed a novel approach to the clinical testing of e-cigarettes which includes either propylene glycol or glycerin or both as a carrier for nicotine delivery. By utilizing carbon heavy propylene glycol as the carrier agent in the e-cigarette all dietary and lifestyle related exposures of non-labeled propylene glycol in plasma can be separated during mass spectrometry analysis. With this approach the question of propylene glycol bioavailability from each model, dose and dose period can be answered. Furthermore, this approach will be able to unequivocally determine the second hand exposure to e-cigarettes.

Celerion, Lincoln, NE 68502 U.S.A.
Celerion, Zürich, Switzerland

FLORA J.W.; MERUVA N.; HUANG C.B.; BENNETT D.; BALLENTINE R.; WERLEY M.S.; McKINNEY W.J.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST74

Characterization of electronic cigarette formulations and aerosols

E-cigarettes, also known as e-vapor products and electronic nicotine delivery systems are gaining popularity in the U.S. and global markets. Currently, limited published data exists on the formulations and chemicals that may be formed during aerosol generation. A harmful and potentially harmful constituent (HPHC) list developed for currently regulated tobacco products includes several chemical classes such as carbonyls, aromatic amines, volatile organic compounds, polyaromatic hydrocarbons and tobacco specific nitrosamines (TSNAs). The e-vapor product category, however, is currently not regulated and no specific list of HPHCs exists. Most e-cigarette formulations contain propylene glycol (PG) and glycerin, which are known to produce aldehydes when heated. In addition, ethylene glycol, diethylene glycol and nicotine related chemicals have been previously reported as potential e-cigarette formulation impurities. Our objective was to evaluate NuMark's commercial e-cigarette formulations and aerosols for the chemicals listed above, determine toxicological significance and share results with the scientific community. Aerosols were collected using 4 second puffs, 55 cc puff volumes, and 30 second puff intervals. E-cigarettes were puffed to battery exhaustion to maximize aerosol collection. For carbonyls analysis, aerosols were collected in 20 puff increments to account for analyte instability. TSNAs were measured at levels acceptable in US pharmacopeia grade nicotine. Nicotine related impurities in NuMark's e-cigarette formulations were below levels suggested by ICH guidelines Q3B(R2) (0.5% of the total nicotine concentration). The levels of thermal degradation products from PG and glycerin detected in the aerosol were determined to be toxicologically insignificant.

Altria Client Services Inc., 601 E. Jackson St., Richmond, VA 23219, U.S.A.

FLORA J.W.; MILLER J.; SCHRALL J.; SMITH J.; McFARLANE C.; MERUVA N.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST43

Nicotine and related impurities in electronic cigarette cartridges: stability studies and methodologies

E-cigarettes, also known as e-vapor products and electronic nicotine delivery systems, are gaining popularity worldwide. The nicotine used in e-cigarette fluids is extracted from tobacco and the purity of the nicotine can vary depending upon manufacturer and grade (e.g. US pharmacopeia grade). The US and European Pharmacopoeia make recommendations for the purity of nicotine intended for pharmaceutical products; however, no official purity recommendation for the nicotine used in e-cigarettes has been made. Not only can nicotine contain natural impurities such as other tobacco alkaloids, but it can also degrade and form nicotine-N-oxides, cotinine and myosmine. To date, only a few publications have evaluated the nicotine related impurities in e-cigarette fluids and none have evaluated these fluids during long term storage. Therefore, the objective of this work was to investigate nicotine and nicotine related impurities in disposable NuMark prototype e-cigarette cartridges during long term storage. Sensitive, selective and robust analytical methodologies for quantitation of nicotine (gas chromatography mass spectrometry (GC-MS)) and nicotine impurities (liquid chromatography tandem mass spectrometry (LC-MS/MS)) in e-cigarette fluids were developed and validated. Nicotine and the nicotine impurities listed in the US and European Pharmacopoeia guidelines were quantitatively investigated for 52 weeks. Storage conditions included both "worst case" environmental conditions (e.g., high temperature with high and low humidity) as well as the long-term and accelerated conditions recommended by the International Conference on Harmonisation (ICH) Guidance Q1A(R2). For all products investigated, at ambient (long-term) conditions up to 52 weeks, nicotine related impurities remained below levels suggested by ICH guidelines Q3B(R2) (0.5% of the total nicotine concentration).

Altria Client Services Inc., 601 E. Jackson St., Richmond, VA 23219, U.S.A.

GADOIS-POMMEREUL S.; REY M.; LHERMITE S.; TROUDE V.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST14

Electronic devices – investigation of the direct thermal extraction properties of the e-liquid

The mode of action of a wide range of e-devices on the market is based on the transfer of e-liquid compounds through a heated air flow. The aim of this work was to provide a method to assess the e-device for qualitative screening of the vapour without using a smoking machine. We will investigate the liquid used in the e-device by focusing on the properties of the liquid itself using Gerstel Thermal Desorption (TDS3) or Pyrolysis Module (PM1)-mass spectrometer equipment as compared to e-vapour analysis from the e-device.

A direct thermal extraction and determination of volatile and semi-volatile organic compounds from 300 µL of e-liquid without sample preparation was carried out. Samples were heated at normal thermal desorption temperatures (150-400 °C) and beyond at pyrolysis temperatures (500-1000 °C). The compounds were then cryo-trapped before being directed into the gas chromatography (I presume) column. This allowed the determination of thermal decomposition products.

Operating conditions of key parameters within the analytical system were optimised such as pneumatics (flow and pressure), timing of desorption, temperatures (TDS oven, cryo-trap conditions (CIS) and the transfer to the capillary column).

Several decomposition compounds were studied, for example, from propylene glycol. These were formaldehyde, acetaldehyde, acrolein, acetone, propanal, acetic anhydride, 2-propene-1-ol, acetol, 2,3-butanedione, acetic acid, methylglyoxal and unchanged propylene glycol.

This direct thermal extraction method allowed a qualitative assessment of targeted compounds formed from different liquid solutions. The effect of the temperature during desorption (150-400 °C) up to the pyrolysis (500-1000 °C) on the relative volatile profile of the generated vapour is discussed.

Comparison with an aerosol generated on a VC10 smoking machine (55 ml puff volume, 4 sec puff duration, 30 sec inter-puff duration) on carbonyls and alpha-carbonyls yields is also presented.

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

GALE N.; BYROM D.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST26

Development of a biologically-representative laboratory analysis system for the measurement of emissions from snus during use

The 2014 revision of the European Commission Tobacco Products Directive refers to the reporting of emissions from tobacco products, including substances released during the process of using smokeless tobacco products. We have previously reported one approach to determining extraction of a range of constituents from different snus products during use by consumers and data from studies employing this approach with human subjects. However, such studies can be costly and time-consuming, and as such the availability of a laboratory-based analysis system to model the extraction of constituents from smokeless products to the same extent as that seen in human studies would be beneficial.

The development of the laboratory approach we present here has been guided by human extraction data for a number of constituents, obtained over a typical 60-minute usage period (previously shown to be the median usage duration of a snus pouch by consumers). The method was developed to be biologically representative, utilising a flow system allowing ingress of fresh media into the test product and egress of used media. An extraction period of 60 minutes using artificial saliva was employed and the system was maintained at body temperature (37 °C). The importance of flow rate as well as amount and composition of the material used to represent the buccal mucosa was assessed.

The methodology is shown to be capable of achieving levels of extraction of nicotine (33%), N'-nitrosonornicotine (34%) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (34%) from snus pouches similar to that seen with consumers (33%, 36% and 38% respectively). Additionally the extraction of two sparingly-soluble constituents, linalool and linalyl acetate, is shown to represent human extraction data. The technique appears to be of value from both product development and regulatory perspectives, and validation against further human data for other products and constituents may confirm its potential utility in the reporting of emissions from this product category representative of everyday consumer use.

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

GAO Qian(1); ZENG Wanli(1); ZHU Zhouhai(1); GUAN Ying(1); MI Qili(1); MIAO Mingming(1); LI Xuemei(1); YAO Jianhua(1); LI Ming(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST18

Development and validation of a high content screening *in vitro* micronucleus assay for the assessment of total particulate matter in cigarette smoke

High Content Screening (HCS) is imaging based multi-parametric approaches to cell analysis at the single-cell level, which was originally developed as a complementary technology to traditional biochemical high-throughput screening in drug discovery. It has been applied in a far broader area of the life science as an unbiased method of imaging multiple cellular samples. To investigate the possibility of HCS for *in vitro* micronucleus assay of Total Particulate Matter (TPM) and improvements in the assay efficiency, Chinese Hamster Ovary (CHO) cells were treated with TPM produced from ten types of cigarettes at five concentrations (25-200 μg/ml). The two following methods were used to score the micronucleus (MN) frequency: (a) HCS with DAPI and FITC dyes, which differentially stained micronuclei and cytoplasm to enhance assay reliability; (b) Visual microcopy with Giemsa dye. The test results obtained using the two methods were compared using correlation analysis. The result showed that HCS method was effective for MN identification, the MN frequencies that were measured in the same samples by HCS and visual microscopy were highly correlated (R=0.950), and there were no significant differences (p=0.570). In conclusion, HCS can be used to evaluate the MN frequency induced by TPM.

1. *Yunnan Academy of Tobacco Science of CNTC, Kunming 650106, P.R. China*
2. *China Tobacco Society, 55 South Yuetan Street, Xicheng District, Beijing 100045, P.R. China*

GILLMAN I.G.; BROWN S.S.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST27

Measurement and variability of tobacco-specific nitrosamines content in the 3R4F reference cigarette filler

A reference or monitor product is often employed to assure the acceptable performance of routine analytical methods for the determination of compounds of concern in cigarette filler and cigarette smoke. This is usually accomplished with the use of statistical process controls and control charting of analyte results. The use of control charts allows the laboratory to detect trending data and to reject sample runs that fall outside of an expected range. In our laboratory, we have observed that control charts for the determination of tobacco-specific nitrosamines appear to have greater variability than other assays. The objective of this study is to determine if this variability is due to the natural variability of the reference product or the variability of the analytical method used to determine tobacco-specific nitrosamines. For this study, the concentration of nitrosamines was determined in four unique composites of 3R4F filler. From one case of 5000 cigarettes, the nitrosamines content of 100 individual cigarettes was also determined. To minimize method variability, all samples were analysed in one analytical run. The N-nitrosoanabasine (NAB) and N-nitrosonornicotine (NNN) content of the four composite samples varied by 20% and 10% respectively. The NNN and 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK) content varied from 2024 to 2637 ng/g and 608 to 1546 ng/g respectively, across the 100 individual cigarettes selected for testing. The impact of blend nitrosamine variability on statistical process control methods will also be presented.

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

GLEINSER M.(1); EITZINGER B.(2); BACHMANN S.(1); VOLGGER D.(1)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST94

The pore size distribution of naturally porous cigarette papers and its relation to air permeability and diffusion capacity – Part 1

In the tobacco industry it is well known that porosity is one of the most significant physical properties of cigarette paper. There are indirect as well as direct methods to characterise this parameter. Measurement of the diffusion capacity and the air permeability are indirect methods, where gases are transferred through the paper by concentration or pressure differences. By using mercury porosimetry the porosity is measured directly via the pore volume of the paper.

The aim of this study is to analyse the pore size distributions resulting from mercury porosimetry of eight different cigarette papers with different air permeability, fibre furnish, filler contents and burn additive contents. The pore size distribution has been measured before and after heating the papers to 230 °C for 30 minutes in the presence of air.

For a qualitative analysis, the pore size distributions have been divided into three sections according to the pore radius: 0.04-0.8 µm (section 1), 0.8-3 µm (section 2) and 3-30 µm (section 3). By comparing the pore size distributions for the unheated paper samples with different permeability, it was found that papers with low air permeability show higher pore volumes in section 1, while papers with high permeability show higher pore volumes in section 2. Variation of the filler content causes changes in the pore volume only in sections 1 and 3. As expected the influence of the burn additive content on the pore size distribution is rather small in all sections. By heating the papers before the analysis by Mercury Porosimetry the overall pore volume increases compared to the unheated papers.

The results show how the pore size distribution of cigarette paper can be influenced by various tools available to the cigarette paper manufacturer such as the paper composition and paper properties.

1. *Papierfabrik Wattens GmbH & Co. KG, Ludwig-Lassl-Straße 15, 6112 Wattens, Austria*
2. *delfortgroup AG, Fabrikstraße 20, 4050 Traun, Austria*

GOUJON C.; MAEDER S.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST54

Indoor air chemistry (IAC): comparative study between conventional cigarette and heat-not-burn technology

Philip Morris International (PMI) is developing products with the potential to reduce the risk associated with smoking. The Electrically Heated Tobacco System (EHTS) operates by heating a tobacco stick with a holder at a controlled temperature. By heating rather than burning, it is possible to substantially reduce or eliminate the formation of a large number of Harmful or Potentially Harmful Constituents (HPHCs). In addition to the decrease or elimination of HPHCs from the mainstream aerosol, the EHTS does not produce sidestream aerosol in the same manner as conventional combusted cigarettes do, i.e. the aerosol is only generated when puffs are taken on the system.

Consequently, the potential impact of using the EHTS indoor on the air quality is expected to be very different from lit-end cigarettes. To verify this hypothesis, PMI has built an environmentally controlled furnished room and developed analytical methods to measure potential air pollutants in the room, under diverse simulated indoor environments.

The analytical methods developed so far focused on: (i) ISO measurement standards for Environmental Tobacco Smoke (ETS) to allow a comparison of the EHTS emissions to those of a lit-end cigarette and, (ii) selected carbonyls (acetaldehyde, acrolein, crotonaldehyde, formaldehyde) and volatile organic compounds (acrylonitrile, benzene, 1,3-butadiene, isoprene, toluene).

The presentation will focus on the room technical features and capability, the methods' development and challenges to detect and quantify background pollutants levels, the methods' achieved performances, results obtained during the methods validation and the plans to further extend the portfolio of analytes.

Philip Morris International, Research & Development, Quai Jeanrenaud 5, CH-2000 Neuchâtel, Switzerland

GUPTA A.; HAYDEN B.; ATKINSON M.; BEHRINGER S.; WESTERBERG B.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST29

Characterization of inhalation exposure atmosphere generated from e-cigarettes

Electronic cigarettes (e-cigarettes) are regarded by some as a less hazardous alternative to tobacco burn down products. Characterization of the health effects of individual products may include conduct of meaningful nonclinical investigations. This study demonstrates an approach for generating and characterizing the output from three major e-cigarette products currently available in the market, as part of preparation for the conduct of *in vivo* inhalation studies.

Three tests for each of three products were performed. Testing was performed under the Canadian Intense Regimen (CIR). The e-cigarette output from smoking machine was transported to a rodent nose-only inhalation exposure carousel. The inhalation atmosphere at the nose port was characterized for concentration stability, puff-to-puff variability, particle size and major chemical constituents. Samples for determination of constituents were collected during the beginning, middle and end of each e-cigarette test.

Product 1 showed consistent concentrations during the two hours of test generation (240 puffs). Product 2 showed fairly consistent concentrations, however Product 2 stopped generating output after ~55 minutes (~110 puffs). Product 3 showed a gradual decrease in concentration during two hours of data collection. The output generated by Product 3 was considerably higher than Products 1 and 2. Also, Product 3 produced output for ~3 hours as compared to ~2 hours or ~55 minutes for Products 1 and 2 respectively. Particle size was in the sub-micron range for all three products. The concentrations of nicotine, glycerol and propylene glycol in the inhalation atmosphere were determined from samples obtained from exposure carousel nose port.

Battelle Memorial Institute, 505 King Avenue, Columbus, OH 43201, U.S.A.

HAHN J.(1); ROSE N.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST14

Water pipe tobacco smoking – from the first idea to an international standard

The presentation is divided into two parts:

The first part concerns the requirement for a standardised method in general. It points out the reasons why the German national standardisation body DIN initialised the project within the ISO/TC 126. It also explains why the leadership of the ISO *ad hoc* group "Water pipe smoking" was given to the German regulatory laboratory although smoking of water pipe is not historically linked to the German culture as it is obvious for other countries.

Based on this background, the work of the *ad hoc* group is summarised starting with finding participants for the development work and ending with the organisation of an international collaborative study.

The second part deals with two constitutive method proposals that have been worked out. The first method proposal describes a routine analytical smoking machine for water pipe tobacco while the second proposal describes the determination of nicotine, water and nicotine-free dry particulate matter in the water pipe smoke condensate. It also mentions the technical challenges and discussions around the recommended equipment like puff generator, laboratory shisha or heating system, and their influence on the smoking process to determine suitable standard parameters.

1. *Chemisches und Veterinäruntersuchungsamt Sigmaringen, Hedingerstr. 1, 72488 Sigmaringen, Germany*
2. *Borgwaldt KC GmbH, Schnackenburgallee 15, 22525 Hamburg, Germany*

HAN E.J.; LEE J.; SHIN H.G.; LIM G.H.; KIM H.K.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST53

The effect of the puff duration on the cigarette smoke chemistry

It is well known that puff parameters (puff duration, puff interval, puff volume) used for machine smoking affect cigarette smoke yield and chemistry. Smoking machines are generally operated under three or four smoking regimes to generate and collect smoke from cigarette products. However, puff durations of these smoking regimes are usually the same set at 2 seconds. Based on previous survey data concerning human smoking behaviour, actual puff duration of smokers ranged from 1.2 to 2.4 seconds.

This study has been conducted to describe the change in cigarette smoke yield and composition caused by various puff durations. It was shown that as the puff duration increased, tar and nicotine content of smoke decreased. Various factors including reduced air velocity through a cigarette rod, increased filter removal efficiency and decreased burning temperature seemed to play important roles. These trends were more significant in low tar cigarettes. Also, the yield and composition of cigarette smoke generated under the ISO regime varied according to puff duration more widely than those under the Health Canada intense regime. These data may be very useful in predicting and estimating actual smoke uptake by smokers.

KT&G Research Institute, 302 Shinseong-Dong, Yuseong-Gu, Daejeon 305-805, South Korea

HANNA S.N.(1); MORGAN W.T.(2); ROWE J.M.(2); OGDEN M.W.(1)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST26

Contribution of manufacturing to variability in cigarette constituent levels

A range of tobacco and smoke analytes in cigarettes is of current regulatory interest. Understanding and communicating the variability associated with measurement of those analytes will aid regulators in making science-based decisions. Tobacco and smoke analyte variability arises from multiple sources including agricultural, structural materials, process capability, and analytical testing. The contribution of analytical variability is relatively well-understood for many analytes; however, there is currently a need for better understanding of total manufacturing variability in the regulatory context.

A study was conducted to monitor a series of analytes comprising the Food and Drug Administration's abbreviated list of Harmful and Potentially Harmful Constituents (HPHCs). Classes of compounds in this list include polyaromatic hydrocarbons (PAHs), tobacco-specific nitrosamines (TSNAs), carbonyl compounds, aromatic amines, volatile organic compounds (VOCs), and certain metals of interest.

In this study, 140 commercially available cigarettes were manufactured and collected at four discrete time points over approximately 18 months. All samples were stored, conditioned, and prepared according to ISO standards, and smoked to either ISO or Health Canada Intense (HCI) specifications. Samples were then analyzed for HPHC levels found in the smoke as well as in the tobacco filler.

Variability in the measured levels of HPHCs was assessed across the life of the study. These results will be compared to those from test method monitor (reference) products to begin to understand the relative contribution of manufacturing variability in the context of HPHC variability. This understanding will provide opportunities to engage with regulators in discussions regarding meaningful differences among products manufactured over time.

1. RAI Services Company, 401 N. Main St., Winston-Salem, NC 27102, U.S.A.
2. R.J. Reynolds Tobacco Company, 950 Reynolds Blvd, Winston-Salem, NC 27105, U.S.A.

HELLER W.D.; CZECHOWICZ M.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST51

Collaborative studies, what have we learnt? Some practical examples

One of the key activities in CORESTA is the development of robust methodologies through collaborative work, to help laboratories to obtain and maintain ISO (International Organization for Standardization) accreditation and to provide a forum for dialogue and sharing knowledge and best practices.

Many collaborative studies have been set up relating to TNCO (tar, nicotine, carbon monoxide) smoke yield measurement in Europe, Asia, ISO and CORESTA. These studies include participants from regulatory, manufacturers and independent contract and research laboratories.

This paper documents the generation of TNCO data over ten years in the EU collaborative study and shows how participants have benefited and improved their performance over time.

The paper also compares and contrasts some of the effects observed when smoking under ISO 3308 and the Canadian intense regime by using data from ISO Working Group 10. The use of ISO 5725 statistical methodology on inhomogeneous datasets resulting from linear and rotary machine smoking is considered.

Reproducibility data obtained for TNCO and other smoke emissions from the CORESTA Special Analytes Sub-Group are also presented.

Each of these studies includes a large number of participants and conclusions apply across different studies to both reference and commercially available products.

Institut für Tabakforschung GmbH (IfT), Chausseestr. 51A, 10115 Berlin, Germany

HERDT O.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST04

The influence of smoke path geometry on TCNO results using smoking machines complying with ISO3308

The work within ISO/TC 126 WG10 has shown that the supersaturated smoke generated under intense smoking regimes like Health Canada T115 is partly condensed in an uncontrolled manner within the path from the cigarette end to the filter. The amount of condensed matter on the way to the filter pad results in a loss of TPM on the filter pad. It has been demonstrated that the loss is slightly higher on machines following the rotary principle than the linear principle. It is assumed that this loss is caused by the distance and by the dead volume between cigarette and smoke trap.

This poster discusses the correlation between design features like distance and/or dead volume between cigarette and filter trap and the influence of these parameters on the collected TPM.

Furthermore the poster demonstrates how to develop a different smoke path design for rotary smoking machines in order to generate more comparable TPM data between rotary and linear smoking under more intense smoking conditions.

Beyond the comparison of different smoke path volumes, the filter holder geometry and the smoke flow distribution in the filter pad holder itself has been studied as influencing factors on TNCO results.

Borgwaldt KC GmbH, Schnackenburgallee 15, 22525 Hamburg, Germany

HU Bin; YU Fei; CHEN Li; AI Dan; PAN Lining; LIU Huimin

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST63

Simultaneous determination of 114 pesticide residues in tobacco by LC-MS/MS using multi-walled carbon nanotubes-based dispersive solid phase extraction

A method capable of simultaneously determining the residues of 114 pesticides in tobacco was developed by dispersive solid phase extraction (DSPE), where multi-walled carbon nanotubes (MWCNTs) were used as extraction material, and LC-MS/MS in multiple reaction monitoring (MRM) mode. With its unique middle-hollow structure and huge specific surface area, MWCNT exhibited excellent adsorption ability in pre-treating tobacco samples for pesticide residue detection. The type and addition rate of MWCNT were optimised in experiments, and the purification of the sample was enhanced on the basis of the QuEChERS method. The residues of 114 pesticides in tobacco were determined by LC-MS/MS after the preparation of samples with the method mentioned above. Matrix matched calibration was used for quantification, the calibration curve presented good linearity (r^2>0.999) and the mean recoveries of three spiking levels (0.02~0.2 mg/kg) ranged from 69% to 119% with the relative standard deviations of 1 to 19%. The limits of quantitation (LOQs) were in the range of 0.2-40 µg/kg. The developed method has shown its advantages in high throughput, sensitivity, accuracy, low cost and convenience in sample treatment.

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

HUANG C.B.(1); ADAMS B.(2); WAGNER K.(1); MERUVA N.(1)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST59

Determination of benzo[a]pyrene in smokeless tobacco products using gas chromatography-mass spectrometry

Benzo[a]pyrene (B[a]P) is a Harmful and Potentially Harmful Constituent (HPHC) found in tobacco products. As mandated by the Family Smoking Prevention and Tobacco Control Act, tobacco manufacturers and importers are required to report quantities of HPHCs to the United States Food and Drug Administration (FDA). The objective of this work was to develop a highly sensitive, selective and validated method for the determination of B[a]P in the smokeless tobacco products (STPs). The method consisted of extraction of the tobacco sample using methanol followed by purification of the tobacco extract using solid phase extraction (SPE). The eluent from SPE was evaporated to dryness and the sample was reconstituted using an organic solvent prior to gas chromatography-mass spectrometry (GC/MS) analysis. The use of SPE cleanup and the concentration step followed by selected ion monitoring (SIM) using GC-MS provided a highly sensitive and selective method for determination of trace levels of B[a]P in STPs. CORESTA reference products (CRP1, CRP2, CRP3 and CRP4) and Kentucky reference cigarette filler (3R4F) were used for method validation. All requirements for method validation were met including linearity, accuracy, precision, limits of detection (LOD), limits of quantitation (LOQ), method robustness, and standard and sample extract stability. For example, the linearity was demonstrated with a coefficient of determination of R^2>0.995, the mean recovery for B[a]P was within 93%-107%, and the LOQ was 0.5 ng/g.

1. *Altria Client Services Inc., 601 E. Jackson St., Richmond, VA 23219, U.S.A.*
2. *Eurofins Lancaster Laboratories, 601 E. Jackson Street, Richmond, VA 23219, U.S.A.*

HUANG C.B.; MERUVA N.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST76

Real-time puff-by-puff analysis of electronic cigarette aerosol using GC-MS

With the increasing popularity of e-cigarettes (electronic nicotine delivery systems (ENDS) or e-vapor devices) in the global marketplace, there has been an increasing interest in characterizing the aerosol produced by these devices. E-cigarette formulations and aerosols typically contain propylene glycol and/or glycerin, water, nicotine, and flavors. The objective of this research was to develop an automated puff-by-puff gas chromatography-mass spectrometry (GC-MS) system for real-time analysis of e-cigarette aerosols to better understand how these devices perform during use. The system is equipped with a single port smoking machine, a 6-port automated sampling and switching valve integrated with an Agilent 5973 GC-MS. The system is capable of monitoring nicotine and selected flavors (e.g. menthol) in multiple puffs of an e-cigarette. A portion of the e-cigarette aerosol from each single puff was directly injected onto the GC column for real-time puff-by-puff analysis. This technique used one e-cigarette per measurement and required no sample preparation of the aerosol prior to GC-MS analysis. This novel method provided qualitative data and percent change in the analyte concentration relative to a control product. The puff-by-puff data from analysis of commercial e-cigarettes containing different amounts of nicotine in the formulation showed that nicotine delivery decreases with number of puffs.

Altria Client Services Inc., 601 E. Jackson St., Richmond, VA 23219, U.S.A.

ISHIDA N.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST62

A simultaneous method for analysing phytosterols and phytosterol esters in tobacco leaves using non-aqueous reversed-phase chromatography and atmospheric pressure chemical ionisation mass spectrometry detector

While numerous analytical methods for free phytosterols have been reported, the similarity of chemical structures among phytosterol esters, and, in particular, among esters that include a saturated or an unsaturated fatty acid, have required complicated multistep separation or preliminary hydrolysis. For this reason, a simultaneous method requiring no preliminary processing was developed. Separation was achieved through non-aqueous reversed-phase chromatography using only acetone and acetonitrile. An atmospheric pressure chemical ionisation / mass spectrometry detector configured in the selected ion monitoring mode was hyphenated with the separation system to efficiently detect phytosterols and phytosterol esters. Twenty-four types of these were completely separated and then identified through their authentic components that had been prepared in advance. The calibration curve was drawn in the range of about 5 to 25,000 ng/mL with a regression coefficient over 0.999. The limit of detection and limit of quantification respectively ranged from 0.9 to 3 ng/mL and from 3 to 11 ng/mL. Recovery rates ranged from 80 to 120%. The quantification results were subjected to statistical analysis and hierarchical clustering analysis, and were used to determine the differences in the amounts of phytosterols and phytosterol esters across tobacco leaves. The newly developed method succeeded in clarifying the whole composition of phytosterols and phytosterol esters in tobacco leaves and also the compositional differences across the cultivars of tobacco leaves.

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

ISHIKAWA S.; SUZUKI T.; NAGATA Y.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST19

Comparison of *in vitro* and *in vivo* exposed chemical levels following cigarette smoke exposure

For the risk assessment of airborne chemicals, a variety of *in vitro* direct exposure systems have been developed and applied in the biological evaluation of cigarette smoke. In direct exposure systems, cells are exposed to cigarette smoke as an aerosol at the air-liquid interface. This exposure scenario can be adapted to the situation of cigarette smoke exposure in the human respiratory system. The purpose of this study is to clarify whether the smoke exposure for the cells using the CULTEX® RFS module, which is a recently developed direct exposure system, is consistent with the smoke retained in the human airway. For this purpose solanesol and acetaldehyde were respectively chosen as the particulate and gas/vapour phase representatives of smoke constituents, and their deposition efficiency and balance per unit area of cell culture surface of the RFS module were measured (dosimetry). We also conducted human retention studies to compare with the dosimetry data. We estimated the regional retention efficiency and balance of each representative per unit area of respiratory tract (mouth, bronchi and alveoli separately). The deposition efficiency of solanesol and acetaldehyde decreased dependent on dilution flow rate and ranged from 0.26 to 0.0076%/cm^2. The ratio of deposited acetaldehyde to deposited solanesol ranged from 0.96 to 1.96 in the RFS module. The retention efficiency of solanesol and acetaldehyde in the mouth and the bronchi ranged 0.095–0.0083%/cm^2. The lowest retention efficiency (0.0000063%/cm^2) was observed in the alveoli. The ratio of retained acetaldehyde to retained solanesol ranged from 0.54 to 1.97. From these results, it was concluded that the CULTEX® RFS module can simulate *in vivo* cigarette smoke exposure in terms of the exposed particulate and gas/vapour phase chemical balance. We also found that the exposure efficiency in this module could replicate the retention efficiency in the mouth and the bronchi.

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

JACCARD G.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST07

Manufacture and analysis of CORESTA Ignition Propensity Monitor Test Piece CM IP 2

CORESTA Monitor Test Pieces have been used for many years as monitors for mainstream smoke yields of nicotine, nicotine-free dry particulate matter (NFDPM) and carbon monoxide (CO) in a number of laboratories. The production and release of Monitor Test Pieces is the responsibility of the CORESTA Routine Analytical Chemistry (RAC) Sub-Group.

With the implementation in some countries of regulations to have cigarettes with reduced ignition propensity, the CORESTA RAC Sub-Group was asked to provide a Monitor Test Piece for the ignition propensity test. As a result, the CORESTA Monitor Test Piece CM IP 2 was recently approved by the CORESTA RAC Sub-Group and remains to be qualified by the CORESTA Scientific Commission.

In the present paper, the challenges and issues faced during the development of the ignition propensity Monitor Test Piece will be discussed, as well as details related to its manufacture and the checks performed to ensure a good homogeneity of all cigarettes produced. The results of the ignition propensity collaborative test organised to qualify the CM IP 2 Test Piece will be provided.

Philip Morris International Management SA, Quai Jeanrenaud 56, CH-2000 Neuchâtel, Switzerland

JI H.(1); FANNIN F.(2); CAI B.(2); BUSH L.P.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST88

Quantitative determination of enantiomers of tobacco-specific nitrosamines in tobacco using gas chromatography-mass spectrometry

Different enantiomers of tobacco-specific nitrosamines (TSNAs) have different biological activities. TSNAs exist as either (R)- or (S)-enantiomeric isoforms that differ at the 2'-C position of the pyrrolidine ring. It has been reported that (S)-N'-nitrosonornicotine (NNN) had more biological activity than the (R)- isoform. It was postulated that (S)-NNN had greater carcinogenicity in rat esophagus and this has been confirmed by a more recent rat feeding assay. Recently it has been reported that (R)-NNN was inactive but synergistically enhanced the activity of (S)-NNN. Thus, the enantiomeric ratio of NNN in tobacco becomes more significant. However, methods reported in the literature were found not to sufficiently resolve all the enantiomeric composition of TSNAs in tobacco for valid quantitation.

The objective of this study was to develop and validate a routine assay to quantify the enantiomeric composition of the TSNAs in tobacco. Tobacco was extracted by 100 mM ammonium acetate with shaking. The solvent was filtered to remove the tobacco powder, and then extracted with methylene chloride. The methylene chloride extract was reduced to 1 ml and injected into the gas chromatograph/mass spectrometry (GC/MS). Two columns, an Agilent DB-5 30 m × 0.25 mm × 0.25 μm film column followed by an Agilent cyclosil B 30 m × 0.25 mm × 0.25 μm column, were connected in series. Our experimental data demonstrated that connection of these two columns in sequence does indeed resolve (baseline separation) the enantiomers of TSNAs in tobacco. All (R)- and (S)-NNN, (R)- and (S)-NAT and (R)- and (S)-NAB are totally separated. Several different tobacco tissues with vastly different alkaloid content were evaluated. The (S)- and (R)-enantiomeric TSNAs reflected the (S)- and (R)-enantiomeric composition of the corresponding alkaloid present in the tissue.

1. *University of Kentucky, Kentucky Tobacco Research and Development Center, Lexington, KY 40546, U.S.A.*
2. *University of Kentucky, Department of Plant and Soil Sciences, Lexington, KY 40546, U.S.A.*

JOZA P.; LI D.; MASTERS A.; RICKERT W.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST47

Determination of pyrazine flavourants in electronic cigarette cartridges and liquids by headspace solid phase microextraction-gas chromatography-mass spectrometry (HS-SPME-GCMS)

The purpose of this study was to implement HS-SPME-GCMS, used for profiling volatile and semi-volatile compounds in tobacco products, to electronic cigarettes and e-liquids for the determination of four targeted analytes; 2,3,5-Trimethylpyrazine (TriMPZ), 2,3,5,6-Tetramethylpyrazine (TetraMPZ), 2-Acetylpyrrole (APL) and 2-Acetylpyrazine (APZ). These compounds are alkyl or acetyl-substituted derivatives of pyrazine and pyrrole, which occur as natural flavour constituents in foods and tobacco. The challenge was to minimize the impact of the high levels of the major components of the e-liquid (propylene glycol, glycerine, nicotine) on the repeatability of the analysis for these flavourants.

The test sample was placed into a 10 mL headspace vial. Two (2.0) mL saturated KCl aqueous solution was added to increase the extraction efficiency ("salting-out") of the volatile and semi-volatile components. Ten (10) µL of ISTD solution containing d_5-2-ethylpyrazine (used for TriMPZ and TetraMPZ) and d_7-quinoline (used for APL and APZ) was then added to the vial. Extraction of the headspace onto a DVB/CAR/PDMS fiber occurred for 20 min at 50 °C. The fibre was desorbed onto the GC inlet for 5 min at 260 °C. Chromatographic separation was achieved using a 30 m × 0.25 mm × 0.25 µm DB WAX column. Mass spectrometer was operated in full scan mode (35-400 m/z) with the following ions used for quantitation: 122 for TriMPZ and APZ, 136 for TetraMPZ, 109 for APL.

Matrix matching to the major components of the e-liquid was found to be necessary for the calibration model since propylene glycol demonstrates significant suppression on the sensitivities of all analytes. Linearity ranged from 1-1000 ng for TriMPA and TetraMPZ, and 10-10000 ng for APL and APZ with an r^2>0.999. With the same matrix matched e-liquid spiked onto blank collection pads, calibrations for the analysis of particulate phase aerosol were also achieved with the same procedure.

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

KANEMARU Y.; SAWAMOTO Y.; TANAKA H.; FUKUSHIMA T.; SOFUNI T.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST16

Loss of heterozygosity analysis of *Tk* mutants induced by cigarette smoke condensates and their chemical components in L5178Y mouse lymphoma cells

For the testing of tobacco smoke genotoxicity in mammalian cells, CORESTA *In Vitro* Toxicity Testing of Tobacco Smoke Task Force recommends the micronucleus assay (MN), chromosome aberration assay or L5178Y mouse lymphoma assay (MLA). These three assays detect chromosomal aberrations, while only the MLA also detects gene mutations. Despite the differences in detection endpoints, these assays showed the same ranking in genotoxic potencies for three different cigarette smoke condensates (CSC) derived from flue-cured, Burley, and Kentucky 3R4F (CORESTA Congress 2010). In order to provide further insight into this similarity in ranking, we sought to compare the quantitative contribution of smoke constituents to CSC genotoxicity found in the MN and MLA, and examined the loss of heterozygosity (LOH) in *Tk* mutants induced by CSC and its chemical components to investigate types of mutations detected in MLA. Selected chemicals in cigarette smoke were grouped according to specific structural features; tobacco-specific nitrosamines, phenols (PHEs), polyaromatic hydrocarbons (PAHs), and aromatic amines (AAs). The chemicals in each group were then mixed by mimicking the composition of cigarette smoke, and each mixture was applied to MN and MLA. Among these groups of chemicals, PHEs showed the highest contribution, 10-20%, to CSC genotoxicity in both assays regardless of metabolic activation. PAHs and AAs also gave a clear positive response under metabolic activation but the contributions were approximately 0.1% for each. In addition, the majority of *Tk* mutants induced by CSCs and all groups of mixtures were LOH mutants. In the genotoxicity assessment of CSCs the MLA might show comparable results to the MN assay. Results suggested that the majority of CSC-induced DNA damage detected in the MLA was likely due to a clastogenic mode of action.

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

LAUTERBACH J.H.; LAUTERBACH S.J.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST41

Temperature profiles of e-cigarettes and e-cigars during heating and thermal analyses of e-liquids

The temperature profiles within burning cigarettes are well known. However, very little has been published on the temperature profiles within an e-cigarette during puffing or static heating. There have been reports of bad-tasting aerosol (and potential formation of toxicants) when there has been insufficient e-liquid going to the heater. Thus, knowledge of the temperature at the heater during use and the thermal stability of the e-liquid is needed for good product stewardship. Consequently, the objectives of this study were (1) to determine the range of temperatures encountered in cigarette-like e-cigarettes (disposable and rechargeable) and similarly constructed but much larger diameter e-cigars; and (2) to determine the volatility of e-liquids under the temperature ranges observed. Temperature determinations were made with Type-K thermocouples connected to a digital thermometer or a Measurement Computing USB-2001-TC device connected to a PC with DAQami software for recording the temperatures as the e-cigarettes or e-cigars were puffed. For 510-style products, static heating (up to 6-seconds) without puffing was achieved using a BestEcig 510 battery section with a manual switch. Temperatures in e-cigarettes (diameters under 9 mm) were determined by inserting a thermocouple through the hole in the mouth-end of each product and moving the thermocouple up to the heater coil. Temperatures in the e-cigars (19 mm diameter) were determined by removing the mouth end up to the delivery tube so that the thermocouple could reach the heater and reverse-puffing the cigars. In all cases, temperatures did not exceed 200 °C even under abusive conditions such as rapid puffing or puffing on depleted cartomizers. The thermal stability of several e-liquids was studied using thermogravimetric analysis and all were completely volatile by 300 °C without decomposition. This study showed that for products studied there was good thermal stability under expected conditions of use.

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

LAUTERBACH J.H.; LAUTERBACH S.J.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST48

E-liquid pH as an important element for research and regulation. Are mentholated e-liquids really different?

Very recently, Stepanov and Fujioka reported that the pH-values of mentholated e-liquids were generally higher than those of their non-mentholated counterparts [*Tobacco Control*. 2014 May 14. pii: tobaccocontrol-2014-051540. doi: 10.1136/tobaccocontrol-2014-051540. (Epub ahead of print)]. According to Stepanov and Fujioka, the samples they used were matched on each manufacturer's labeled nicotine strength; however those authors did not report any analytical data that explained the differences they found. Moreover, their findings were not consistent with data we reported at the 67th TSRC (2013, Paper #78). To gain understanding on the possible differences in the pH values between mentholated and non-mentholated e-liquids, pH-values were determined on twenty e-liquids. These were taken from cartomizers, disposable e-cigarettes, purchased e-liquids, and a 25 mg/mL nicotine in propylene glycol solution prepared by NicVape, Inc., that we had obtained over the past eighteen months. The brands that were used included three of the four used by Stepanov and Fujioka. Label nicotine concentrations ranged from 1.2 to 4.5%. Their procedure was followed, and the wadding containing the e-liquid was removed from the cartomizers and disposable e-cigarettes and each sample slurried in 10 mL ASTM Type II deionized (DI) water. For neat e-liquids, we used 0.7 mL of each as typical cartomizers contain about that volume of e-liquid and we added that amount to 10 mL DI water. We had three pairs of menthol/non-menthol cartomizers, two pairs of menthol/non-menthol disposable e-cigarettes, and one pair of menthol/non-menthol e-liquids. Only in the case of the e-liquids (2.4% nicotine) was the pH of the menthol version higher than the non-menthol one (8.90±0.09 vs. 8.19±0.04). Our findings did not support Stepanov's and Fujioka's conclusion that mentholated e-liquids had higher pH-values than their non-menthol counterparts and are also consistent with GC-MS analyses we obtained on these e-liquids.

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

LE BEC L.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST92

Influence of cigarette paper characteristics on the smoke yields in a flue-cured Chinese cigarette design under ISO and Canadian intense smoking regimes

In 2012 at the CORESTA Congress in Sapporo, a presentation was done on the influence of cigarette paper permeability, basis weight and citrate level on the smoke yields in a flue-cured Chinese cigarette design under the ISO smoking regime. This earlier study has been complemented with the Canadian Intense (CI) smoking regime and with two additional cigarette paper parameters, citrate type and filler level. The objective of this study is to evaluate via a Design of Experiment (DoE) the influence of cigarette paper characteristics on the smoke yields and to compare the effects obtained under ISO and CI smoking regimes. Non-ventilated machine-made cigarettes were manufactured using a Chinese flue-cured tobacco, the same mono-acetate filter and at constant tobacco density. Main effects, squared effects and the interactions between the main parameters were calculated. Carbon monoxide (CO) yields were significantly reduced under ISO and CI smoking regimes when the permeability was increased to 80 CU, the citrate level increased to 2% and with the use of potassium citrate as burning agent. The highest reduction of the CO to tar ratio was achieved by the combination of low basis weight (26 g/m^2), the use of potassium citrate and the increase of the permeability of the cigarette paper to 80 CU. The parameters which are significant under the ISO smoking regime are also significant under the CI smoking regime. In this study, the effects of the cigarette paper on smoke yields were lower for the CI smoking regime than for the ISO smoking regime.

SWM Intl, Global Research, Usine Le Mans, 72702 Allonnes Cedex, France

LEE J.M.; MIN H.J.; LEE M.Y.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST70

Determination of volatile nitrosamines in mainstream cigarette smoke by gas chromatography tandem mass spectrometry

Volatile nitrosamines are very important constituents of tobacco and cigarette smoke. Since volatile nitrosamines were placed on the United States Food and Drug Administration (FDA) Harmful and Potentially Harmful Constituents (HPHC) list, there is a need for an analytical approach to quantify volatile nitrosamines in smoke. In this study, a comprehensive analytical method based on gas chromatography coupled with tandem mass spectrometry (GC-MS/MS) was developed for the determination of seven volatile nitrosamines in mainstream cigarette smoke. Volatile nitrosamines were found in the gas phase fraction of mainstream cigarette smoke of Kentucky Reference 3R4F and various commercial cigarettes. The gas phase was trapped by two bubblers containing 1% HCl solution passing through 92 mm Cambridge filter pad, and then extracted with 20% isopropanol in ether. Qualitative and quantitative analyses were carried out for the analytes under the multiple reaction monitoring (MRM) mode after chromatographic separation on a DB-1701 (30 m × 0.25 mm, 0.25 µm) capillary column. The limits of quantification (LOQs) for the seven volatile nitrosamines were in the range from 0.2 to 1 ng/cig. Recoveries of seven volatile nitrosamines in smoke yielded more than 80% and reproducibility was found to be better than 10% RSD. These results demonstrate that the method is accurate, rapid and sensitive, and can be used for the analysis of volatile nitrosamines in mainstream cigarette smoke.

KT&G Research Institute, 302 Shinseong-Dong, Yuseong-Gu, Daejeon 305-805, South Korea

LINDHOLM J.; HEDIN A.; HASSLER T.; RUTQVIST L.E.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST13

The Quality Standard – GothiaTek®

GothiaTek® is a product quality standard established by Swedish Match and applies to the manufacture of the company's Swedish snus products. The principal components of the GothiaTek® are standards relating to constituents, the manufacturing process, and consumer information. The constituent standard includes maximum levels for selected, undesired constituents in the finished products, including Tobacco Specific Nitrosamines (TSNAs). Although Swedish Match's current manufacturing methods for snus build on those that were introduced more than a century ago, the quality of modern Swedish snus is largely due to improvements in production techniques and selection of raw materials in combination with several programmes for quality assurance and quality control that have been successively introduced by the company since the early 1970s. In 2001 these developments formed the basis for a codified, voluntary product quality standard named GothiaTek®. The maximum levels defined by GothiaTek® are pragmatic and based on considerations of what can be consistently achieved in large-scale production, comparisons with levels observed in common food stuffs, comparisons with estimated total daily exposure from food and drinking water, and from observations in Swedish epidemiological studies of no increased risk of oral cancer associated with long-term use of Swedish snus. During 2012 the GothiaTek® standard was updated to meet the World Health Organization (WHO) proposal for product regulation of smokeless tobacco products regarding upper limits for N'-nitrosonornicotine (NNN) plus 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and benzo[a]pyrene (B[a]P).

Currently Swedish Match is developing more detailed and comprehensive performance standards for GothiaTek® that will be discussed in the presentation.

Swedish Match North Europe AB, Research & Development, Maria Skolgata 83, SE-118 85 Stockholm, Sweden

LINDNER M.(1); RAUNIC VADANJEL R.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST93

Plasma perforation of tipping paper – a novel method to generate ventilated filter cigarettes

Perforation of tipping paper is essential for adjusting the ventilation of cigarettes to achieve specific smoke deliveries. Hereby, the type of tipping paper perforation plays an important role and can be separated into online-laser perforation directly inside the cigarette machine and offline or pre-perforation performed by tipping paper manufacturers. Until recently, offline perforation could only be carried out mechanically and by means of pulsed laser radiation or electrostatic discharge. In this study, plasma perforation will be introduced as an advanced technology to realise pre-perforated tipping paper. Plasma perforation is performed with a so-called low-temperature plasma within an inert gas environment which implies the generation of small perforation holes and high hole density via local material evaporation. The comparison of plasma perforation with conventional perforation methods regarding the stability of the air permeability, filter ventilation and draw resistance is the first step to gain profound knowledge about this latest development. As a second step, smoke analysis is carried out in order to demonstrate the effect of plasma perforation on the control of the basic smoke yields with the emphasis to optimise the carbon monoxide (CO)/tar and nicotine/tar ratios. Physical and geometrical parameters of this perforation type serve as a basis for the third step which is to determine the quasi-diffusion effect accompanying regular filter ventilation processes and reducing the CO output. The findings confirm that plasma perforation is a smart way to create natural-like permeabilities with the excellence of outstanding cigarette product quality parameters.

1. *TANNPAPIER GmbH, Johann Roithner-Strasse 131, A-4050 Traun, Austria*
2. *TDR d.o.o., Obala Vladimira Nazora 1, 52210 Rovinj, Croatia*

LIU Baizhan; WANG Ying; XIE Wenyan

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST69

Qualitative analysis of free radicals in cigarette gas-phase smoke by ultra performance convergence chromatography/Q-TOFMS

A novel analytical strategy composed of a spin-trapping method and ultra performance convergence chromatography with high-resolution time-of-flight mass spectrometry (UPC^2/Q-TOFMS) was developed for the separation and qualitative analysis of free radicals in the gas phase of mainstream cigarette smoke. By reacting with a trapping agent PBN (N-tert-butyl-α phenylnitrone), the free radicals in the gas phase of mainstream cigarette smoke were trapped effectively due to the high efficiency of PBN to trap various free radicals and the high stability of PBN derivative products. Each component in the complicated matrix of PBN-radical adducts trapped from mainstream cigarette smoke was effectively separated by ultra performance convergence chromatography. By comparing the mass spectrum data of PBN-radical adducts (PBN-Smoke) derived from Q-TOFMS with those of the control cigarette smoke sample (Smoke), 10 characteristic compounds were targeted by orthogonal partial least-square discriminant analysis (OPLS-DA), and their molecular formulas and molecular structure were inferred by referring to PBN spin trapping mechanism. The method developed is efficient and accurate for qualitative analysis of free radicals in the gas phase of mainstream cigarette smoke. It completes an analytical cycle in less than seven minutes and offers an accurate analytical result. It also provides an effective means for further investigating the formation and reaction of gas phase free radicals during cigarette smoking or tobacco burning.

Shanghai Tobacco Group Co., Ltd of CNTC, Technology Center, Shanghai 200082, P.R. China

LIU X.; JOZA P.; MASTERS A.; RICKERT W.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST71

Determination of ethylene oxide in mainstream cigarette smoke using hydrobromic acid derivatization and gas chromatography-mass spectrometry

Ethylene oxide is identified in the United States Food and Drug Administration (FDA) publication list of harmful and potentially harmful constituents (HPHCs) found in mainstream cigarette smoke. Most methods, directly analyze the gas phase cigarette smoke trapped into cryogenic methanol, or captured into a Tedlar bag, by gas chromatography-mass spectrometry (GC-MS) using a single quantitation ion (m/z = 44) for its determination. Direct analysis using this ion is vulnerable to matrix interferences which may not be chromatographically separated and bias yields.

In this study, ethylene oxide was determined using a hydrobromic acid (HBr) derivatization to convert ethylene oxide to 2-bromoethanol. Cigarette smoke, passed through a glass fibre filter disc (pad), was collected into cryogenic traps containing methanol. An aliquot of the trapping solution was dried with anhydrous sodium sulphate, derivatized with hydrobromic acid, and neutralized with sodium carbonate. The solution was spiked with d_4-2-bromoethanol (internal standard), centrifuged, and injected onto the GC-MS using a single ion (m/z = 124) for quantitation and ion 107 as a qualifier.

Validation studies showed good calibration linearity with a regression coefficient of r^2=0.999. Spike recoveries ranged from 81.1 to 94.4%. The limits of detection and quantification were 33.7 ng/cig and 112 ng/cig, respectively. Yields from Kentucky Reference 3R4F cigarette were found to be 8.37 ± 1.74 µg/cig (n=12) under the ISO smoking regimen and 26.03 ± 5.02 µg/cig (n=65) under the "Health Canada intensive" smoking regimen. These yields are less than 50% of those observed by the direct analysis of the cryogenic solution using ion 44. The selectivity of the derivatization, and ion used for quantification, provides chromatography more suitable for low yield products.

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

LUSSO M.(1); LION K.(1); ADAMS A.(1); LEWIS R.S.(2); MORRIS W.(1); DAVIS G.(1)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST87

From seed to smoke: N-nitrosonornicotine levels in blended cigarettes containing Burley or flue-cured tobacco stable for low nornicotine content

N-nitrosonornicotine (NNN) is a tobacco specific nitrosamine (TSNA) identified in cured tobacco leaf and smoke. Previous work demonstrated that by stabilizing a low level of the precursor nornicotine in Burley tobacco, the level of NNN in cured leaf and smoke of Burley cigarettes can be reduced by up to 75%. Through backcross breeding, novel genetics that stabilize low nornicotine levels have been introduced into Burley and flue-cured tobacco cultivars. These low nornicotine cultivars were grown in the 2012 and 2013 crop years and the resulting cured leaf was used to produce machine-made blended cigarettes using a blending formulation similar to 3R4F reference cigarettes. International Organization for Standardization (ISO) smoking conditions were used for evaluations and constituent analyses reported here on a per cigarette basis. Cigarettes containing low nornicotine Burley cultivars at inclusions of 0%, 8%, 15% or 23% (w/w) of the total blend formulation were produced. For tobacco produced in 2012, cigarettes containing low nornicotine Burley at 8%, 15% or 23% (w/w) inclusion showed a reduction in NNN levels of 16%, 17% and 38%, respectively, when compared to the control cigarette containing 0% leaf from low nornicotine cultivars. The same blend formulations showed reductions in NNN levels of 8%, 19% and 31%, respectively, for tobacco produced in 2013. Additionally, cigarettes containing the low nornicotine flue-cured cultivars at inclusions of 0% or 35% (w/w) of the total blend formulation were produced in 2013 and evaluated. Preliminary data for other tobacco constituents will also be presented.

1. *Altria Client Services Inc., Research, Development & Engineering, 601 E. Jackson St., Richmond, VA 23219, U.S.A.*
2. *North Carolina State University, Crop Science Department, Raleigh, NC 27695, U.S.A.*

MARANO K.M.(1); MORGAN W.T.(2); OGDEN M.W.(1); SWAUGER J.E.(1)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST91

Quantitative risk assessment of cigarette products from the US market, 2012 and 2013

Quantitative risk assessment, including incremental lifetime cancer risk (ILCR) and hazard index (HI) calculations, was applied to 160 cigarette products marketed in the United States (US) in 2012 and 2013. ILCR and HI values, incorporating both estimates of toxicity and concentration for individual harmful and potentially harmful tobacco constituents (HPHC), were calculated overall and for six cigarette sub-categories including three ISO "tar" categories (i.e. <6 mg, 6-13 mg, >13 mg), each with a menthol and non-menthol sub-category. For determination of HPHC yields, cigarettes were machine-smoked using both the ISO regimen and the Health Canada Intense (HCI) regimen. For non-cancer and cancer toxicity estimates, values established by US regulatory authorities or values derived from more recent dose-response data were used. Overall, for cigarettes smoked using the ISO regimen, ILCR values ranged between 4.17E-4 (minimum) and 6.87E-3 (maximum), and HI values ranged between 238 and 3632. For cigarettes smoked using the HCI regimen, ILCR values ranged between 3.37E-3 and 1.33E-2, and HI values ranged between 4720 and 9065. These results provide a range of non-cancer hazard and cancer risk estimates for current market cigarette products overall and by six sub-categories. In the context of a toxicological risk assessment, it is suggested that a new cigarette product, with ILCR and HI estimates falling within the relevant sub-category ranges for current market products, does not raise different questions of public health.

1. *RAI Services Company, 401 N. Main St., Winston-Salem, NC 27102, U.S.A.*
2. *R.J. Reynolds Tobacco Company, 950 Reynolds Blvd, Winston-Salem, NC 27105, U.S.A.*

MARTIN A.; WILLIAMSON F.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST75

Selected primary aromatic amines (PAAs) in e-cigarette vapor by GC-MS

Primary aromatic amines (PAAs) are compounds of interest to a number of tobacco regulators and are routinely found in mainstream (MS) tobacco smoke. Six of the 93 compounds currently included on the United States Food and Drug Administration (FDA) established list of harmful and potentially harmful constituents (HPHCs) in tobacco products and tobacco smoke (HPHCs) are primary aromatic amines. Health Canada and ANVISA Brazil also require the reporting of PAAs. With the increasing interest in e-cigarette and other vapor products, there is need for sensitive methods of measurement for PAAs, which are expected to occur at much lower levels in these types of products. The method described here is a modification of a method used routinely in a high-sample throughput laboratory for PAA analysis in MS smoke and demonstrates the successful analysis of four PAAs in e-cigarette vapor: 1- and 2- aminonaphthalene and 3- and 4-aminobiphenyl.

E-cigarette vapor is collected using a 44-mm Cambridge filter pad (CFP). Once the vapor collection is complete the CFP is extracted with methylene chloride for 30 minutes using mechanical shaking. A portion of the extract is evaporated to approximately 2.5 mL, dried with sodium sulfate and derivatized with pentafluoropropionic acid anhydride (PFPA). The final sample is analyzed by GC-MS using negative chemical ionization.

The calibration range for the aminonapthalenes is 10 pg/mL to 500 pg/mL and the range for the aminobiphenyls is 5 pg/mL to 250 pg/mL. It should be noted that, even with a lower limit of quantitation (LLOQ) of 50 pg/pad, no quantifiable PAAs have been observed in the vapor from any e-cigarettes tested to date.

Arista Laboratories, 1470 East Parham Road, Richmond, VA 23228, U.S.A.

MARX F.P.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST10

Fine-cut tobacco smoke analysis: learnings from collaborative studies

In the late 1980s, European regulators asked for a measurement method for fine-cut tobacco smoke yields. As a consequence CORESTA decided in 1989 to establish a Task Force in order to study the various issues associated with the use of fine-cut tobacco. These included investigations into consumer making preferences as well as into developing measurement methods. The work was finalised with a Technical Report and formed the basis of four ISO Standards published between 2001 and 2003. The overall principle of these ISO Standards is that 'tar' and smoke nicotine yields from fine-cut tobacco are measured on the basis of two types of rolling papers and two amounts of tobacco used.

Since 2004, ESTA (European Smoking Tobacco Association) has offered interested laboratories the opportunity to participate in annually conducted collaborative studies on ISO fine-cut tobacco smoke analysis using different ESTA Monitor Tobaccos (EMTs). The purpose of these studies is to establish ‘tar’ and smoke nicotine yields of the respective EMT blend and to provide a regular overview on repeatability and reproducibility based on a recognised statistical methodology.

This presentation will share insight into the studies and results in terms of repeatability and reproducibility values for tar and nicotine over a longer time period.

Although an improvement of method precision was observed over time, these studies have continuously highlighted greater variability values compared to those normally seen for ISO cigarette smoke analysis, likely due to the making of the Fine-Cut Smoking Articles (FSCAs) as part of the related standard.

Further annual studies will be performed in order to monitor trends of method and laboratory performances. Such studies also offer laboratories an opportunity to prove their competence in ISO FCSA smoke analysis as required by ISO 17025.

European Smoking Tobacco Association (ESTA), Rond Point Schuman 9, Box 1, B-1040 Brussels, Belgium

MASON T.J.P.; COLE A.O.; VINCENT J.H.; TINDALL I.F.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST07

A comparison of selected aerosol trapping mechanisms for use in the analysis of electronic cigarettes

Much of the current research on the analysis of e-cigarette aerosols has assumed that the testing apparatus suitable for conventional cigarettes can be readily adapted for this purpose. In particular, it is common to utilise glass fibre filters (Cambridge filters) for the trapping of the particulate matter contained within the aerosol.

The objective of this study was to compare a selection of alternative trapping options under different vaping regimes and to determine which glass fibre trap is the most suitable.

Tests were carried out on a group of locally available e-cigarette brands using a dedicated e-cigarette vaping machine. Samples from each brand were vaped onto standard 44 mm Cambridge pads, 55 mm Cambridge pads and electrostatic traps. Analysis was by weight difference before and after vaping, both of the trapping system and the product under test. Test were done using two puffing regimes (square wave, 70 ml volume, 3 s duration and square wave, 55 ml volume, 3 s duration).

Mass balance comparing the weight gain of glass fibre pads against the weight loss from the product showed significant differences. For example, after 350 puffs the weight of the collected material was on average 41 mg lower than the average of 883 mg lost by the products, roughly 5%. Mass balance comparing the weight gain of single pad against a series of pads weighed at intermediate stages showed a significant difference in measured yield. Differences were also dependent on puff regime.

Glass fibre trapping is suitable provided that losses due to evaporation are considered in the design of the experiment. The large liquid volume provides technical challenges for experiment designers in trying to ensure efficient capture of substances under analysis.

Cerulean, Rockingham Drive, Linford Wood East, Milton Keynes MK14 6LY, U.K.

MASUGI E.(1); DUNKLE M.(2); t'KINT R.(2); MITSUI M.(1); OCHIAI N.(3); DAVID F.(2); SANDRA K.(2); SANDRA P.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST45

Comprehensive analysis of lipid compounds in tobacco leaf

In recent years, lipidomics has been a vastly expanding field of research due to the development of new technologies in liquid chromatography and from recognising the role of lipids in metabolism.

Tobacco leaf consists of thousands of chemical compounds that differ in molecular weight and polarity. A comprehensive analysis combined with a statistical approach is of interest in revealing differences between tobacco samples for which further information is not available. Focusing on the higher molecular weight fraction, lipid compounds are especially of interest because they provide information on the lipidome and may help to understand changes during the curing process. However, there are only a few reports available which are related to the comprehensive and comparative measurement of lipids in tobacco leaf.

The purpose of this study was to develop a comprehensive analysis for lipid compounds in tobacco leaf.

The sample preparation consists of two steps: 1) Lipid extraction, 2) Lipid enhancement/fractionation. Lipids were extracted from tobacco leaf by the Folch method (2:1 Chloroform/Methanol), and solid-phase extraction (SPE) was performed to separate and collect fractions of neutral lipids, fatty acids and polar lipids. These three fractions were analysed using reversed-phase liquid chromatography (RPLC) with high resolution time-of-flight mass spectrometry (TOF).

As a result, more than 500 lipids could be identified in tobacco leaf. In conclusion, the entire workflow from sample preparation down to data analysis was performed in a highly automated manner.

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

MAYR M.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST05

Comparison of Full Length Burn testing according to ISO 12863 and the proposed alternative by NIST Laboratories, USA

NIST Laboratories USA proposed to use a thin stainless steel substrate plus one layer of filter paper instead of the 10 layers, as defined in ASTM E.2187-09 and ISO 12863 for measuring the percentage of Full Length Burn on Lower Ignition Propensity (LIP) / Fire Standard Compliant (FSC) cigarettes. Some previous studies, performed by NIST showed an interchangeability of the two test methods. However it has been mentioned that LIP/FSC cigarettes have been used, that gave a reading for Full Length Burn of close to 0%. It was described that these results are comparable to the standard procedure mentioned in ISO 12863 / ASTM E.2187-09. The objective for performing this study was to compare LIP/FSC cigarettes, showing different pass rates with the two test methods, first according to ISO 12863 and secondly with the alternative set-up as proposed by NIST. Samples have been chosen with a Full Length Burn rate below and above 25%. The study design also included an evaluation of the impact on the variation of the results when performing the test on the metal sheet. The observations from our study were that LIP cigarettes which failed the test under ISO 12863 will pass the test when performing according to the proposed alternative. There were no significant differences when comparing the results of LIP cigarettes which passed ISO 12863 with 0% Full Length Burn and the proposed NIST alternative. A further topic in our study was to test if the results of the proposal from NIST were more comparable to ISO 12863 when increasing the layers of filter papers on the metal sheet. We called it "*progressive testing*". It is described in ISO 12863 – Annex C (normative) C3. – *Procedure for selection of substrate assemblies for testing*. In conclusion it means that cigarettes which will not pass the test today will pass the test when analysed with the metal sheet plus one layer of filter paper. Results with the metal sheet plus one layer of filter paper are misleading, with the risk of non-conformity products on the market having Full Length Burn above 25%.

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

MAYR M.(1); CORESTA PTM Sub-Group and Diffusivity Working Group Members(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST08

CORESTA Recommended Method No. 77

In 2010, the Physical Test Methods Sub-Group of CORESTA launched the Diffusivity Working Group with the objective to develop a CORESTA Recommended Method (CRM) for the "*Determination of Diffusion Capacity by Measurement of CO_2 Transfer Rate Through Materials Used as Cigarette Papers and Cigarette Papers having an Oriented Zone of Reduced Diffusion Capacity*". During eight meetings from January 2010 until September 2012 the Working Group developed a draft of the method. In the Working Group it was decided to have a method available which covers all instruments commercially available at that time. In January 2012 a test for the robustness of the method was conducted, involving current commercially available types of diffusion capacity measurement instruments and a broad selection of conventional and lower ignition propensity cigarette papers from various paper suppliers. Over five days the influence of laboratory conditions, conditioning of the paper, and instrument set-up and configuration, such as head size and differential pressure, on the measured diffusion capacity was checked. These results were discussed within the Working Group and were used to identify and define important parameters for the diffusion capacity measurement in the draft of the CRM. In 2013 a first inter-laboratory study for the determination of repeatability and reproducibility according ISO 5725-2:1994 involving 15 laboratories and five paper samples was conducted. After adding these results to the final draft of the CRM, the drafted CRM was approved by the Scientific Commission and the Board of CORESTA and was published as CRM No. 77 in April 2014.

1. *Papierfabrik Wattens GmbH & Co. KG / delfortgroup AG, Ludwig-Lassl-Straße 15, 6112 Wattens, Austria*
2. *Various origins*

MEGER M.(1); CLEVA BERSAN D.(1); WEBER E.(2); CAMERON P.(3); MILLER-HOLT J.(1); JONES I.(1)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST79

Annual monitoring of a range of cigarette products from the Canadian market and their *in vitro* biological responses over time (from 2005 to 2013)

In vitro toxicity assays have been recognized as valuable tools to assess the biological activity of tobacco products and their emissions. Such data can also be used to compare relative differences in *in vitro* biological activity between tobacco products.

The influence of tobacco type/blend and design features, such as cigarette diameter, filter and ventilation, on *in vitro* activity has been extensively investigated. The present study compares *in vitro* toxicity data over time (2005-2013) for products from the Canadian market, covering a range of standard design features.

Data are taken from annual regulatory reporting to Health Canada, which includes Ames (bacterial mutagenicity), Neutral Red Uptake (cytotoxicity) and Micronuclei (clastogenicity) results. Cigarettes are classified by design and biological activity data are normalized to total particulate matter (TPM) mass. Summary statistics are provided for all biological endpoints.

Assay response rates, levels of variability, and correlations between different product classes across the years are broadly consistent with recently published data (Wright et al., 2007; Belushkin et al., 2014). The results suggest that observed differences between years are attributable to long-term assay variability rather than product changes. The change of tobacco sourcing had no effect on *in vitro* responses.

The present study reveals the limited value of repeated data generation for products which remain within a standard set of design features.

1. *JT International S.A., 1 rue de la Gabelle, CH-1211 Geneva 26, Switzerland*
2. *Ökolab Gesellschaft für Umweltanalytik GmbH, Hasnerstrasse 127, A-1160 Vienna, Austria*
3. *JTI Macdonald Corp., 1 Robert Speck Parkway Suite 1601, Mississauga, ON, L4Z 0A2, Canada*

MIN H.J.; LEE J.M.; LEE M.Y.; JANG G.C.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST66

Development of a fast screening method for crop protection agents in tobacco by solid sorptive extraction-thermal desorption-gas chromatography mass spectrometry

Simultaneous determination of crop protection agents (CPAs) in food is done with multi-residue methods, which are composed of sample clean-up, concentration, chromatographic separation and detection. The Solid Sorptive Extraction (SSE) technique is used for sample preparation of various analytes in several fields.

The aim of this study was to develop a sensitive and fast method based on SSE followed by thermal desorption-gas chromatography-mass spectrometry (TD-GC/MS) to determine CPAs in tobacco samples.

For the analysis of tobacco samples prior to the SSE method, solvent extraction or ultrasound-assisted solvent extraction was performed. Acetonitrile was used as the extraction solvent. The extract was then diluted with water. Finally, the sample was subjected to SSE.

A method for fast screening of CPAs in tobacco using SSE-TD-GC/MS has been developed. About 30 CPAs including organochlorine, organophosphorous and others were identified and quantified. This method showed good linearity and high sensitivity for most of the target CPAs. The method was applied to the determination of CPAs at ng/mL levels in tobacco samples. This method is simple, rapid and may be applied to other components.

KT&G Research Institute, 302 Shinseong-Dong, Yuseong-Gu, Daejeon 305-805, South Korea

MORTON M.J.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST28

Within laboratory variance outlier detection

The interpretation of Harmful and Potentially Harmful Constituent (HPHC) test results and the results of other analytical testing is often guided by the inherent variability of the associated test method. A common approach to characterize that variability is through interlaboratory collaborative studies. One of the standard guides for the conduct and analysis of interlaboratory collaborative studies is described in ISO 5725-2:1994(E). One of the recommendations of ISO 5725-2:1994(E) is the use of Cochran's test to determine laboratories that have excessively large replicate-to-replicate variation. One potential problem with employing Cochran's test is that it is very sensitive to the assumption that the data follow the Gaussian probability distribution. A simulation analysis showed that the probability of falsely eliminating a laboratory as an outlier is approximately 30% for some common distributions instead of the stated probability of 1%. This false rejection of outliers has the potential to result in a misleading underestimation of method variation. An alternative approach, derived from Levene's test, was evaluated that is much less sensitive to the Gaussian assumption. This approach falsely eliminates laboratories as outliers much less frequently than Cochran's test. In the cases where Cochran's test has a false error rate of 30%, simulation studies showed this approach to have an error rate of a little less than 5%. This approach thereby has the potential to more accurately represent the variability within and between analytical laboratories.

Altria Client Services Inc., 601 E. Jackson St., Richmond, VA 23219, U.S.A.

MOSTOVOJUS V.; TUCINSKAS G.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST02

Influence of different filtration efficiency of new design split dual filter

Paper filters were extensively used a few decades ago. Filters containing a paper segment are more environment-friendly due to much faster biodegradation compared to cellulose acetate tow filters. Today there are crepe papers available suitable for filter production with different physical and chemical properties. Besides the environmental aspect, paper filters can remove certain amounts of harmful components from tobacco smoke.

In this study new design split dual filters were used with different filtration media: paper, charcoal paper, cellulose acetate and charcoal with higher carbon tetrachloride (CTC) activity. The main focus was to study the effect on benzene, toluene, carbon monoxide and nicotine.

Cigarettes with the above described filters were prepared using CORESTA Monitor 6 (CM6) tobacco columns without tipping ventilation. Smoking was done according to ISO using a Borgwaldt RM20D smoking machine. Compound analyses were performed using Agilent 5973N GC-MS equipment according to CORESTA Recommended Methods (CRM) Nos. 5, 7 and 70.

A comparative analysis of new design filters was performed aimed at identifying the filtration efficiency of new design filters.

Nemuno Banga LLC, Kestucio Str.1, Lentvaris, Lithuania

MUELLER C.(1); COLARD S.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST11

Investigations on cigar burning and yields under different smoking intensities

In order to better understand the effect of smoking parameters on the cigar burning process and mainstream smoke yields, a pilot study was conducted. A 'large' commercial cigar (weight about 6.5 g) was investigated for the generation of 'tar', nicotine, and carbon monoxide in mainstream cigar smoke. Nicotine and water were also determined in the cigar butts after smoking.

Four segments corresponding to 25%, 50%, 75% and 100% of the cigar, measured down to the butt length, were smoked under five smoking conditions using a constant puff volume of 33 mL with puff frequencies of 10, 20, and 40 seconds. A constant puff frequency of 40 seconds was then applied with puff volumes of 10 mL and 40 mL. Four replicate measurements were performed for each set of parameters.

Different mainstream smoke yield changes were observed relating to different smoking intensity. For carbon monoxide a linear correlation was found between smoke yields and length smoked. For other smoke emissions, an increase was observed during the course of smoking. Tobacco analyses associated with yields suggest a gradual build-up of tar and water in the cigar body and an increase of the release efficiency of nicotine.

Although the cigars smoked in this study have been pre-selected to reduce variations in some physical characteristics, a considerable variability was observed between replicates for smoke emissions. Compared to yields from cigarettes, cigars are much more variable and behave fundamentally differently. These observations might be due to the inherent characteristics of large cigars e.g. more variability in weight, density, pressure drop, length and circumference, as well as thickness, texture, porosity and combustibility of the processed leaves.

Considering the analytical and the natural human smoking behaviour variability, this study raises the question of the value of cigar yield determinations above analysis of physical characteristics and tobacco content.

1. *Imperial Tobacco Group, Reemtsma Cigarettenfabriken GmbH, Albert-Einstein-Ring 7, D-22761 Hamburg, Germany*
2. *Imperial Tobacco Ltd, Winterstoke Road, Bristol BS3 2LL, U.K.*

MUREYA C; MUSUNA-GARWE C.C.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST65

Analysis of seven strobilurins in tobacco using gas chromatography equipped with electron capture detector (GC-ECD)

Pesticide residues in tobacco may be harmful to the consumer and the environment hence the need to monitor and ensure residues on the leaf are within acceptable levels. Strobilurins are being introduced for use on tobacco in Zimbabwe. The aim of this study was to develop and validate a method that uses Gas Chromatography Electron Capture Detection (GC-ECD) for monitoring strobilurin residues in tobacco. Ground tobacco samples were macerated using ethyl acetate and sodium sulphate. An aliquot of the extract was eluted on activated florisil with ethyl acetate:cyclohexane mixture. The eluant was concentrated on a rotary evaporator and analysed for seven strobilurins using GC-ECD (kresoxym-S-methyl, azoxystrobin, picoxystrobin, dimoxystrobin, trifloxystrobin, orysastrobin, and fluoxastrobin). The method was validated by spiking blank ground tobacco with mixed standards solved in acetonitrile (0.25-0.5 mg/kg of each analyte). Recoveries (from seven replicates) ranged from 70-120% except for dimoxystrobin and picoxystrobin where more work is needed. The relative standard deviations for all analytes were acceptable with ≤20%. Linear calibration was used with a correlation coefficient of >0.95 and a limit of detection (LOD) at 0.01 mg/kg and a limit of quantification (LOQ) at 0.1 mg/kg. The method has been validated for five strobilurins in tobacco.

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

NELSON P.R.; CHEN P.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST81

Clinical trial to compare smoking cessation rates with Camel SNUS and a nicotine lozenge – Part 1: Study Design

R.J. Reynolds conducted a multicenter clinical trial to compare smoking cessation rates when healthy smokers with the intent to quit were switched to Camel SNUS or a nicotine lozenge.

The primary objectives of the study examined whether SNUS was superior to a nicotine lozenge (4 mg Nicorette® Lozenge) for smoking cessation as determined by four different criteria that were based upon the Society for Research on Nicotine and Tobacco (SRNT) working group recommendations. Criteria evaluated included: prolonged smoking abstinence; repeated point prevalence smoking abstinence; and two measures of continuous smoking abstinence. The effect of providing one-time smokeless tobacco relative risk information on cessation rates with SNUS was also examined. Study product and cigarette usage was evaluated throughout the course of the study.

The study consisted of three cohorts: SNUS with one-time smokeless tobacco relative risk information; SNUS without smokeless tobacco relative risk information; and Nicorette Lozenge. The study was powered to detect a difference of 14.5% (OR of 0.57) for an average abstinence of 50% between two cohorts with a sample size of 200 subjects per cohort. This powering allowed detection of smaller differences in cessation if the true abstinence rate was different than 50%.

Following enrolment, subjects were randomized to a cohort and provided with either SNUS or Nicorette Lozenges for a 12 week period. The subjects were followed for up to 12 months beyond their target cessation date. Abstinence was confirmed by measurement of exhaled CO and blood cotinine levels.

A description of the study design, study endpoints, and cohort demographics will be provided in this presentation.

R.J. Reynolds Tobacco Company, P.O. Box 1487, Winston-Salem, NC 27102-1487, U.S.A.

NELSON P.R.; CHEN P.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST82

Clinical trial to compare smoking cessation rates with Camel SNUS and a nicotine lozenge – Part 2: Results

R.J. Reynolds conducted a multicenter clinical trial to compare smoking cessation rates with Camel SNUS, with and without smokeless tobacco health-related background information, and a nicotine lozenge. Cessation was evaluated using Fisher's exact test and logistic regression across study visits for prolonged smoking abstinence, repeated point prevalence smoking abstinence, and two measures of continuous smoking abstinence and by survival analysis.

At twelve months, overall quit rates were low for all cessation endpoints (1-5%, depending on the endpoint) and there were no statistically significant differences among quit rates for any cohort as measured by any cessation criterion. Survival analysis results with or without a grace period (total abstinence from quit date) showed no statistically significant difference between the Nicorette and Snus with/without information cohorts. Additionally, there was no statistically significant difference in cessation rates between SNUS with and without limited, one-time risk information at any time point.

Product usage among participants in each cohort was also examined. For subjects who reported use of study product and cigarettes (dual users), regardless of their status as a treatment success or failure, the number of cigarettes smoked per day was determined. During the period of the study during which product was provided to the subjects (through week 11), those subjects who used both study product and cigarettes concurrently greatly reduced ($p<0.05$) their consumption of cigarettes. During the period of the study when subjects provided their own SNUS or Nicorette, dual users of study product and cigarettes also experienced a statistically significantly ($p<0.05$) reduction in their number of cigarettes smoked.

R.J. Reynolds Tobacco Company, P.O. Box 1487, Winston-Salem, NC 27102-1487, U.S.A.

NEWLAND K.E.; FARMEN R.H.; ISLAM R.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST36

Do you have a validated biomarker for this compound?

There are many ways to answer the question "Do you have a validated biomarker for this compound?" Often this question cannot be answered unless you have the following information about the intended assay:

- Is this for a small molecule or a large molecule?
- What is the species? Is this the right biomarker for this species?
- What is the intra-subject and inter-subject variability?
- Is this data going to be used for a Pharmacokinetic/Pharmacodynamic (PK/PD) plot or for a statistical comparison?
- Is this an endogenous biomarker? Will this biomarker be present in the matrix? Do different disease states affect the concentration of this biomarker?
- Is this biomarker present in the environment?
- Is reference material available for this biomarker?
- What is the cost you are willing to spend for this assay?

Since the tobacco industry has moved into the area of regulated bioanalysis, we have noted that there is confusion surrounding the regulatory standards required for analytical methods to be used for testing biomarkers in biofluids. This presentation will focus on demonstrating the benefits of using a fit-for-purpose approach concerning the validation of biomarkers. In conclusion, a properly constructed and validated Bioanalytical Tobacco Assay (BTA) that has the appropriate selectivity, sensitivity and improved analytical precision has a direct impact upon statistical analysis of the study. Specifically, these parameters will have a direct impact upon the number of subjects that you have to dose in order to achieve the same statistical power.

Celerion, Lincoln, NE 68502 U.S.A.
Celerion, Zürich, Switzerland

O'CONNELL G.(1); COLARD S.(1,2); VERRON T.(2); CAHOURS X.(2); PRITCHARD J.D.(1)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST28

Assessment of nicotine in the ambient air before, during and after the use of e-cigarettes in an office

E-vapour products are gaining acceptance with consumers as potential alternatives to traditional tobacco products. Both regulators and public health organisations are beginning to examine potential implications that exposure to e-vapour exhalate (exhaled vapour) may have on non-users in workplaces and enclosed public spaces. A critical review of the current scientific literature reveals that there is insufficient evidence from which to assess the impact of exhalate on indoor air quality and thus the air breathed by non-users.

In the few studies that have considered this issue, a number of methodological issues have been identified and are discussed. Despite an absence of robust scientific evidence on this issue, there are calls, including some by government bodies, to prohibit the use of e-vapour products in workplaces and enclosed public spaces.

There are few published scientific studies that adequately assess the indoor air quality in an environment encountered in everyday life (e.g. car, home, office) during and after e-vapour product use. In order to provide evidence from which robust conclusions may be drawn, we present an experimental study which could be employed to evaluate indoor air quality in 'real-life' conditions following use of e-vapour products.

Physicochemical characterisations are proposed for air quality assessment before, during and after use of an e-vapour product in a typical meeting room. Chemical analyses of indoor air have been considered including screening for volatile organic compounds, carbonyl compounds, heavy metals, polyaromatic hydrocarbons, nicotine and nitrosamines. Monitoring techniques for the mass of particles in the air and their size distribution have also been considered. Studies conducted in this manner may be useful in the development of evidence based regulation.

1. *Imperial Tobacco Ltd, Winterstoke Road, Bristol BS3 2LL, U.K.*
2. *Imperial Tobacco Group, SEITA, 48 rue Danton, 45404 Fleury-les-Aubrais, France*

OTTE S.; ELSTER L.; INTORP M.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST13

Method development and validation of a multi-compound method for the quantification of selected volatile analytes in gas phase of cigarette smoke by GC-MS: an experience report

The increasing demand from regulatory bodies for the characterisation of tobacco and tobacco smoke products creates a need to generate reliable results in short time frames.

A multi-compound method for the quantification of volatile organic compounds (VOC), selected carbonyls, hydrogen cyanide and further volatile organic compounds mentioned in the harmful and potentially harmful constituents (HPHC) list published by the United States Food and Drug Administration (FDA) and in the gas phase of cigarette smoke was developed and presented during the CORESTA Congress and Joint Study Groups Meeting in 2010 and 2013 [SSPT 11, CORESTA 2010; SSPT 29, CORESTA 2013]. It was concluded that results obtained by the multi-compound method and other alternative methods were at comparable concentration levels [SSPT 11, CORESTA 2010].

Here an extended and fully validated method is presented which covers 20 volatile organic compounds, such as isoprene, toluene, benzene, butadiene, acrylonitrile, acetone, acrolein, acetaldehyde, hydrogen cyanide, ethyl benzene, furan, vinyl chloride, vinyl acetate, ethylene oxide, and propylene oxide, methanol, acetonitrile, nitromethane, 2-nitropropane, and styrene in cigarette smoke. This method is based on the same collection as previously described for the determination of selected volatile Hoffmann Analytes, but features a different chromatography system [SSPT 29, CORESTA 2013]. Calculation of results was carried out using different deuterated internal standards.

During the method development, the reactivity of volatile compounds in a gas matrix and their stability in the gas phase of cigarette smoke were carefully investigated.

In this poster, detailed information and observation obtained during the method development and validation, e.g. impact of different gas bag materials, stability of calibration standards over a longer time period, and the influence of different internal standards on data evaluation, will be presented.

Imperial Tobacco Group, Reemtsma Cigarettenfabriken GmbH, Albert-Einstein-Ring 7, D-22761 Hamburg, Germany

OUAHIOUNE M.; DUMAS J.; JOYEUX T.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST01

Taste impact of different LIP band material

LIP (Low Ignition Propensity) cigarettes extinguish due to low diffusivity bands, which are coated onto cigarette paper. Different coating materials may be used to produce these bands. Different coating materials have different taste impact.

To investigate this effect in more detail, we carried out a pyrolysis study. We studied different coating compounds in comparison to the pyrolysis products of normal cigarette paper. For this, standard cigarette paper was band coated with different LIP additives. All LIP bands were matched to the same diffusivity level of 0.14 cm/s.

The pyrolysis conditions tried to simulate the temperature profile during actual smoking. Hence we used two pyrolysis temperatures:

- 200 °C to simulate evaporation/distillation and the onset of the thermic decomposition
- 450 °C to simulate the decomposition close to the char line

Additionally, the reaction with TMAH (tetra methyl ammonium hydroxide) was used to further elucidate the chemical entities. As TMAH reacts with many of the chemical functionalities, it was also used as a "chemical probe" to further characterise the thermal reaction products.

All pyrolysis products were analysed via GC-MS (gas chromatograph/mass spectrometer). All experiments were run in triplicates.

The decomposition products of the different band materials were compared in relation to normal cigarette paper. The results were ranked according to their similarities with the pyrolysis products of standard cigarette paper.

Papeteries du Léman, PDL Cigarette Papers, BP43, 74202 Thonon-les-Bains Cedex, France

PANG Tao; LI Yong; SHI Junli; LI Junying; DENG Jianhua; LU Xiuping

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST64

Structure identification of flavonoids in tobacco based on liquid chromatography mass spectrometry method

A structure identification method of flavonoids in tobacco was developed based on liquid chromatography mass spectrometry (LCMS). The two-step precursor ion scan method was used to identify flavone aglycones and glycosidic conjugates. The substitutional position of glycosidic conjugates was deduced by retention rules in high performance liquid chromatography. The identification was confirmed by MS^2 spectrum and authentic standards. With this method, 18 flavonoids were identified from tobacco leaf and flowers and 10 of them were identified from tobacco for the first time. The extraction solvent and mobile phase of liquid chromatography method were optimised. The best extraction result can be obtained by using water-acetonitrile solvent (1:3, v: v) and the best separation result can be obtained by using both formic acid and ammonium acetate as additive agent. The method was then applied in the determination of flavonoids in different types of tobacco and the correlationship between flavonoids and flavour related compounds in flue-cured tobacco. The result showed that Turkish tobacco has the highest flavonoid content while Burley has the lowest content. The content of dihydroquercetin has a significant positive correlationship with flavour related compounds such as maillard reaction product, phenylalanine degradation product and carotenoid degradation product, which prove the opinion that flavonoids are the precursors of flavour related compounds.

Yunnan Academy of Tobacco Agricultural Sciences, No. 14 Nanxiang Road, Yuxi, Yunnan, P.R. China

PANI J.; HERWIG S.; MAYER-HELM B.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST61

Determination of ethyl carbamate in tobacco and smokeless tobacco products by HPLC-APCI+-MS/MS

Ethyl carbamate (urethane) occurs in a wide range of alcoholic beverages and fermented foods. Although its acute oral toxicity is low, it has been reclassified from "possibly carcinogenic to humans" (IARC group 2B) to "probably carcinogenic to humans" (IARC group 2A).

So far, many different protocols have been successfully applied to analyse ethyl carbamate.

In this work, we show a highly efficient purification technique of ethyl carbamate from smokeless tobacco.

After extraction using an aqueous buffer, a polymeric reversed phase material, especially designed for the binding of hydrophilic substances (Strata-X; Phenomenex), is used in a solid phase extraction step to extract the highly polar analyte from the extract. Afterwards, the addition of 10% methanol is sufficient to quantitatively elute the analyte while keeping interfering matrix components bound to the column. By saving large amounts of organic solvents, this purification procedure is advantageous compared to liquid-liquid extraction. Final determination of ethyl carbamate is performed using reversed phase HPLC-APCI+-MS/MS. After quantification with external standard calibration, an isotope-labeled standard (ethyl carbamate-D5) is used to determine the recovery of the analyte and to correct the results.

A comparison of both commonly used ionisation techniques, APCI und ESI, showed that APCI gives a significantly improved signal to noise ratio and therefore much lower limits of quantification and detection for ethyl carbamate analysis. CORESTA reference products (CRP1-4) were analysed but only CRP2 (moist snuff) showed detectable levels of 38 ng/g. Recoveries ranged from 80% in CRP2 to 45% in CRP3. Furthermore, extensive validation was performed.

Our proposed clean-up method results in highly purified samples which are a prerequisite to establish a robust method and to improve column lifetimes. Our results could not confirm the high levels of ethyl carbamate found in tobacco by Schmeltz et al. (*J. Anal. Toxicol.* 1978) 2 (6): 264-268). However, our findings are consistent with the data published by Faizi et al. (SSPTPOST14; 2010 CORESTA Congress, Edinburgh) who tested various smokeless tobacco matrices.

JTI-Ökolab, Hasnerstrasse 127, 1160 Vienna, Austria

PANI J.; WERNETH M.; MAYER-HELM B.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST46

Determination of N-nitrosarcosine (NSAR) in tobacco

The determination of NSAR in tobacco or smokeless tobacco products has been reported several times in the literature, mainly using gas chromatography coupled with a thermal energy analyser. A different technique, liquid chromatography combined with electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS), is advanced and more convenient but involves difficulties regarding (i) the retention mechanism of the polar compound NSAR and (ii) unequal ESI-MS/MS responses of the syn and anti conformers of NSAR.

Thorough investigations by LC-ESI-MS/MS and NMR spectroscopy will be presented, as well as validation data of a method for highly sensitive NSAR quantification in smokeless tobacco products. LC-ESI-MS/MS measurements were carried out using a Liquid Separation Cell Technology Column in Hydrophilic Interaction Liquid Chromatography (HILIC) mode coupled with a triple quadrupole MS in negative ionisation mode. Sample preparation was performed according to Wu et al. (*Anal. Methods* 4 (2012) 3448): extraction with 2% aqueous formic acid, clean-up with solid supported liquid-liquid extraction and concentration by reconstitution of the evaporated eluate in mobile phase starting conditions. For quantification, a single-point external calibration and internal standard-correction using the isotope labeled standard NSAR-D3 was applied. NMR spectroscopy was used to assign the syn and anti conformers and to determine their ratios.

Two different LC gradient methods have been developed: one that separates the syn from the anti conformer and another that combines the two conformers. Using these two gradient methods for the determination of NSAR in standard solutions and smokeless tobacco samples, the following observations have been made: (i) the conformers show different ESI-MS/MS response, (ii) the conformer ratios differ in real samples and in standards of different ages and (iii) the conformer ratio is altered during sample preparation. As a consequence, for proper quantification the conformer ratio in the external standard solution needs to be adjusted to the same ratio as in the samples, which is accomplished by heating.

The LC-ESI-MS/MS method described has been applied to several tobacco products and has been fully validated for CORESTA Reference Product 2 (CRP2, moist snuff) and CORESTA Reference Product 3 (CRP3, dry snuff): NSAR concentrations of 31 ng/g and 63 ng/g in CRP2 and CRP3, respectively, have been determined with expanded measurement uncertainties of 21% and 16%, respectively. Recoveries of the overall method are low (average 17% for CRP2 and 9% for CRP3). Nevertheless, internal standard recoveries are consistent with analyte recoveries and therefore high intra-day and inter-day repeatabilities of the final results (5-8% RSD) are accomplished by internal standard correction. Limits of quantification are matrix dependent: 14 ng/g for CRP2 and 28 ng/g for CRP3.

The presented method is advantageous to conventional techniques in terms of selectivity, sensitivity, trueness and straightforwardness of handling. It overcomes the difficulties of LC retardation and conformer dependent ESI-MS/MS response by applying a special stationary phase and by adjusting the conformer ratio in sample and external calibration standard.

JTI-Ökolab, Hasnerstrasse 127, 1160 Vienna, Austria

PLUYM N.; GILCH G.; SCHERER G.; SCHERER M.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST37

Analysis of 18 urinary mercapturic acids by two multiplex-LC-MS/MS methods

Mercapturic acids (MAs) are metabolic end-products formed from conjugates between glutathione (GSH) and electrophilic compounds. MAs are, therefore, suitable biomarkers of exposure to toxicants, which are either electrophiles by themselves or are metabolised to electrophilic intermediates. We developed and validated two LC-MS/MS methods which allow the complementary, rapid and sensitive determination of MAs derived from acrolein, acrylamide, acrylonitrile, benzene, 1,3-butadiene, crotonaldehyde, *N*,*N*-dimethylformamide, ethylene, ethylene oxide, vinylchloride, propylene oxide, styrene, toluene, methylating and ethylating agents.

Since separate determinations of single or small groups of MAs are time-consuming and expensive, we multiplexed several different methods into only two LC-MS/MS methods. Method validation according to the United States Food and Drug Administration (FDA) guidelines showed excellent results in terms of sensitivity, reproducibility and robustness. Moreover, the use of a minimal, simple and straightforward sample clean-up accelerated the analytical workflow, which allows us a time- and cost-efficient analysis of up to 18 MAs derived from the toxicants mentioned above.

The methods were applied to urine derived from a diet-controlled clinical study including 25 smokers and 25 non-smokers. For smokers, MA concentrations correlated significantly with the smoking dose for the majority of analytes (14 of 18 MAs), except for the MAs of methylating/ethylating agents, ethylene/ethylene oxide and toluene ($p>0.05$; Pearson). Furthermore, a significant increase was observed in smokers as compared to non-smokers for the MAs of acrolein, acrylamide, acrylonitrile, benzene, 1,3-butadiene, crotonaldehyde, *N*,*N*-dimethylformamide and styrene ($p<0.05$).

In conclusion, the newly developed assays represent a powerful tool for the analysis of MA conjugates in clinical studies, which allows the fast and reliable quantification of biomarkers for eight relevant toxicants in tobacco smoke.

ABF Analytisch-Biologisches Forschungslabor GmbH, Goethestr. 20, 80336 Munich, Germany

POULEAU B.; SMART D.J.; McHUGH D.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST21

Evaluating the genotoxicity of tobacco smoke-derived aerosols using the flow cytometry-based *in vitro* micronucleus assay

The *in vitro* micronucleus (MN) assay is used to assess the genotoxic potential of a test substance by measuring the induction of micronuclei in cultured mammalian cells. The flow cytometry-based assay (*in vitro* MicroFlow® kit, Litron Laboratories, USA) coupled with a high-throughput sampler permits the analysis of thousands of nuclei over a short period of time. Furthermore, clastogenic or aneugenic mechanistic signatures can be discerned using this high content approach. Using this version of the assay, the genotoxicity of the total particulate matter (TPM) fraction and the saline-soluble portion of the gas-vapour phase (GVP) derived from 3R4F reference cigarettes (University of Kentucky, USA) along with several prototypical genotoxins was evaluated using a study design compliant with OECD guideline No. 487 and recommendations from Litron Laboratories. In order to maximise the possibility of detecting a genotoxic response, three different treatment conditions were deployed in CHO-K1 cells: extended (24 h), short-term without metabolic activation (4 h -S9) and short-term in the presence of a S9 metabolic activation system (4 h +S9). In order to understand cytotoxicity vis-à-vis genotoxicity in the assay, three different cytotoxicity indices were explored, namely relative population doubling (RPD), relative increase in cell counts (RICC) and relative cell counts (RCC) all of which were calculated from absolute cell counts measured by flow cytometry. Known clastogens, e.g. methyl methanesulphonate, and aneugens, e.g. colchicine, were shown to induce marked genotoxicity as well as clear clastogenic and aneugenic mechanistic signatures, while several non-genotoxic cytotoxins, e.g. phthalic anhydride, were confirmed as non-genotoxic in the assay. 3R4F-derived TPM and GVP induced clastogenic responses but there was no detectable evidence of aneugenicity. Cytotoxicity was underestimated by RCC for most of the substances tested, whereas RICC appeared to be the most sensitive index and RPD typically yielded levels of cytotoxicity midway between RCC and RICC. In conclusion, the flow cytometry-based *in vitro* MN assay is appropriate to evaluate the genotoxic potential of tobacco smoke-derived aerosols.

Philip Morris International, Research & Development, Philip Morris Products S.A., Department of Biological Systems Research, Genetic and Cellular Toxicology Laboratory, Neuchâtel CH-2003, Switzerland

PRASAD G.L.; JONES B.A.; CARAWAY J.; BORGERDING F.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST16

Inflammatory cytokines in tobacco consumers as potential biomarkers of tobacco effect

Chronic cigarette smoking causes inflammation and some reports indicate that generally healthy smokers (SMK) exhibit changes in the inflammatory cytokines versus non-smokers. However, it remains to be established whether consumers of non-combustible tobacco, such as moist snuff consumers (MSC) and dual users of cigarettes and moist snuff (DU-SKMS), also experience altered inflammation. Recently in a biomarker discovery study (Study 1), we observed that in contrast to SMK, the MSC exhibited cytokine profiles similar to non-tobacco consumers (NTC). Here, we present the levels of selected inflammatory cytokines from several cohorts of natural adopters of various tobacco products participating in a different study (Study 2).

Cytokine profiles of Human InflammationMAP®v1.0 panel (Myriad RBM) were generated from plasma and saliva samples collected in the Study 2 from cohorts of generally healthy, adult SMK, MSC, DU-SKMS and NTC who fasted overnight from food and tobacco. While several analytes from both plasma and saliva were found to be significantly different among the cohorts ($p<0.05$), 11 analytes from plasma were found to be highly significantly different ($p<0.02$). The SMK cohort had the highest mean values of all 11 analytes compared to the other cohorts, followed by the DU-SKMS which had cytokine profiles similar to SMK, reflecting the level of smoking in DU-SKMS. MSC and NTC cohorts had lower mean values. Cytokine profiles were similar between MSC and NTC, consistent with the previous findings from the biomarker discovery study. Although the demographics of the two studies were notably different, six analytes, fibrinogen, ICAM-1, VEGF, MMP-9, ferritin and complement component 3, emerged as potential biomarkers that distinguish tobacco consumers. These data suggest that smoking is the likely agent driving inflammation. Overall, the inflammatory cytokine levels suggest that inflammation is increased among combustible tobacco consumers relative to MSC and NTC, with few differences detected between MSC and NTC.

R.J. Reynolds Tobacco Company, R&D Department, Winston-Salem, NC 27102, U.S.A.

PRASAD G.L.(1); JONES B.A.(1); SCHMIDT E.(1); KENNEDY A.D.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST17

Global profiling of metabolites from saliva of tobacco consumers

The long-term health effects associated with cigarette smoking have been shown to be more harmful compared to those associated with the consumption of non-combustible tobacco products, such as moist snuff. In order to investigate the long-term health effects of tobacco exposure, we evaluated the saliva metabolomic profiles of 40 smokers (SMK), 40 moist snuff consumers (MSC), and 40 non-tobacco consumers (NTC) using UHPLC-mass spectrometry based metabolomic profiling. Saliva samples were collected from adult male subjects who abstained overnight from both food and tobacco. Metabolomic profiling and data analyses performed at Metabolon Inc., (Durham NC) indicate that a total of 407 biochemicals (310 known and 97 unnamed metabolites) were detected. While the known metabolites included three biochemicals from nicotine metabolism, the rest of them belonged to diverse metabolic pathways.

Smokers exhibited a larger number of statistical ($p<0.05$) differences relative to the non-smoking groups. For example, 94 metabolites were significantly different in SMK compared to NTC, 91 changes were detected in SMK versus MSC and 46 biochemicals were different between MSC and NTC. These biochemicals fit into distinct metabolic pathways such as oxidative stress, inflammation, macromolecular rearrangement/tissue remodeling, xenobiotic biochemistry, amino acid, cellular energetics and microbial metabolism. Random Forest analyses revealed that SMK profiles were distinct from the MSC and NTC cohorts, while MSC profiles showed more subtle changes and were difficult to distinguish from NTC. In summary, these data present changes in global saliva biochemical profiles in generally healthy cigarette smokers and moist snuff consumers. These differences in the metabolite profiles may be useful in understanding the relative risks associated with smoking compared to consumption of smokeless tobacco products.

1. *R.J. Reynolds Tobacco Company, R&D Department, Winston-Salem, NC 27102, U.S.A.*
2. *Metabolon, Durham, NC, U.S.A.*

PRASAD G.L.(1); ZACHARIAS W.(2); ARIMILLI S.(3)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST17

Differentiating the effects of exposure to combustible and non-combustible tobacco product preparations using *in vitro* and *ex vivo* models

Exposure to cigarette smoke or its constituent phases induces a range of adverse cellular responses that include cytotoxicity, genotoxicity and inflammatory responses in several human cell culture models. The cellular effects of exposure to smokeless tobacco (ST), however, are less clear and appear to vary based on experimental conditions. Here we summarise some of our recent research efforts to address this critical gap. The Tobacco Product Preparations (TPPs) in our studies included total particulate matter (TPM) and whole smoke conditioned medium (WS-CM) from 3R4F reference cigarettes, and extracts of 2S3 moist snuff in complete artificial saliva (ST/CAS). Oral cavity cells and peripheral blood mononuclear cells (PBMCs) were utilised to assess local and systemic effects, respectively. We used the nicotine content of the TPPs (termed equi-nicotine units) as reference to compare the responses elicited by TPPs.

The combustible TPPs (TPM and WS-CM) caused significant cytotoxicity, apoptosis, elicited inflammatory responses and DNA damage (as measured by SRB assays, FACS, ELISAs, Comet assays, and histone H2AX phosphorylation and immunofluorescence) in oral and hematopoietic cells. Treatment of PBMCs with TPM and WS-CM predominantly quenched the Toll-Like Receptor (TLR)-mediated cytokine secretion and functional measures such as target cell killing. These responses were muted or not evident when cells were treated with ST/CAS at equi-nicotine concentrations, or were measurable only at significantly higher doses. Nicotine-elicited responses were measurable only at millimolar doses. These findings show that TPPs differ in their cytotoxic, genotoxic effects and inflammatory responses in the following order: WS-CM>TPM>ST/CAS>nicotine. Collectively, these findings a) support the epidemiological evidence that combustible tobacco products are more harmful compared to the non-combustible products, b) support evidence of a risk continuum across tobacco product categories and c) suggest that *in vitro* and *ex vivo* models may be useful in evaluating combustible and non-combustible tobacco products and nicotine.

1. *R.J. Reynolds Tobacco Company, R&D Department, Winston-Salem, NC 27102, U.S.A.*
2. *University of Louisville, Louisville, KY, U.S.A.*
3. *Wake Forest University Baptist Health, Winston-Salem, NC, U.S.A.*

PRAT M.(1); BRAECKMAN H.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST52

Proficiency studies, what have we learnt? A review of the Proficiency Tests conducted by the CORESTA Agrochemical Analysis Sub-Group

The objectives of the CORESTA Agrochemical Analysis Sub-Group are to perform regular proficiency testing of multi-residue methods for the analysis of Crop Protection Agents in tobacco, to undertake joint experiments to resolve identified issues, and to publish and review a series of Technical Notes and Guidelines for method development and improvement.

Annual studies include more than 20 participants from regulatory, manufacturers, and independent commercial and research laboratories.

This paper reviews the results from nine Proficiency Tests conducted since 2005 using the FAPAS testing scheme. The results indicate that continuous ring trials improve the quality of participating laboratories significantly over time. Big differences in improvement were found between regular and occasional participants. The paper also discusses evaluation issues which arise when participating laboratories do not provide data for the full set of test articles. However, when calculating the weighted average of squared z-scores for all studies, a decrease from 4.5 to 2.5 is observed indicating significant progress in quality through joint experiments, scientific dialogue and exchange of available knowledge.

1. *JT International Germany GmbH, Diedenhofener Strasse 20, D-54294 Trier, Germany*
2. *FYTOLAB cvba, Technologiepark 2/3, B-9052 Zwijnaarde, Belgium*

PURKIS S.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST05

A cautionary note: making reference to ISO standards is not a sufficient means of providing validation for non ISO methods

ISO3308 and ISO4387 standards for smoking cigarettes were published in 1977 and 1987 respectively. They have since been reviewed at least every five years, updated and improved with major changes thoroughly evaluated, with development and validation based on the puffing regime defined in ISO3308.

In 1999, Health Canada published an intense regime referencing ISO standards but with increased puffing intensity and filter ventilation 100% blocked. It has recently been incorporated into a WHO TobLabNet standard operating procedure. These documents assume that procedures and equipment, specified in ISO standards and validated for the ISO regime, can equally apply to this more intensive regime. However, this assumption has never been properly checked.

Under intense smoking, smoke properties may not be sufficiently similar to have no impact on smoke collection and measurement. For example, smoke temperature can impact on the stability of the smoke aerosol. Puff-by-puff measurements show that temperature increases near the mouth end of the cigarette filter during smoking and the increase is magnified by increasing puffing intensity.

Smoke yields increase puff by puff under all regimes. However, under intense smoking, water concentration increases radically whilst those for NFDPM and nicotine remain almost unchanged. This can be explained by the known change in saturated vapour density with temperature that allows the smoke to carry over greatly increased quantities of water vapour from the cigarette, much of which condenses on the surfaces of the collection system as it cools. Since the Cambridge Filter Pad and Holder are designed specifically for collecting particulates, their efficiency is compromised by high water vapour content which results in inaccurate yield measurements. Also, the measurement variability increases relative to ISO due to machine differences specifically harmonised for use under ISO conditions.

It is concluded that it is a misuse of ISO standards ISO3308 and ISO4387 to specify them for use with intense regimes unless substantive validation work is carried out.

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

RAABE H.; BARNES N.; HILBERER A.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST15

Evaluation of the EpiOral™ reconstructed human oral buccal tissue model as a testing platform for determining the oral irritation potential of tobacco products

There is an increasing need by the tobacco industry to evaluate the oral/buccal irritation potential of tobacco products both to support product development goals and for sound product stewardship. The use of *in vitro* human cell and tissue-based test methods to replace *in vivo* animal models addresses the need for more human-relevant predictive tools, and is consistent with many corporate animal welfare policies. Although monolayer cell-based cytotoxicity assays have been used, three-dimensional tissue constructs provide distinct advantages since tissue exposures and pharmacokinetics more closely resemble the *in vivo* events. This study was conducted to evaluate the EpiOral™ human oral/buccal tissue model (MatTek Corporation) for determining oral irritation of tobacco products. We applied a dilution series of tobacco extracts onto EpiOral™ tissues for various exposure times (2 to 16 hours) and measured cell viability and the synthesis/release of the inflammatory mediators IL-1α and IL-8, relative to an Artificial Saliva-treated control. We measured tobacco extract concentration-related decreases and exposure-time related decreases in viability for the highest tobacco extract concentrations (relative viability was reduced to 32% for the 100% tobacco extract after 16 hours exposure). We also found that increases in IL-1α release (up to 19-fold) generally correlated with reductions in viability, confirming that the exposures likely induced cytotoxic or cytolytic effects and consequent loss of cell membrane integrity. Exposure time-related increases in IL-8 release were generally observed in tissues treated with the three lower tobacco extract concentrations where relative viabilities were generally similar to negative control levels. However, IL-8 release values were below control levels at the highest tobacco extract concentrations where cytotoxic effects inhibited the cells' ability to synthesize proteins. These IL-8 expression results demonstrate that secondary inflammatory cytokine synthesis can be induced in oral epithelium tissue constructs, particularly at exposures that would not be expected to cause overt cytotoxic effects.

Institute for In Vitro Sciences, Inc., 30 W. Watkins Mill Road, Suite 100, Gaithersburg, MD 20878, U.S.A.

ROBERTSON R.M.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST31

CelFX™ Matrix Technology super slim filter comparison with commercial carbon filters

Small diameter or super slim cigarettes (5.0-5.5 mm) have approximately two times the smoke velocity and about half of the filter residence time of large diameter cigarettes (7.6-7.8 mm). This limitation, coupled with lower active ingredient loadings, results in a significant challenge for smoke constituent reduction in small diameter cigarettes. The CelFX™ super slim platform has been evaluated for its potential to offer improved constituent reduction of 20% and greater, without sacrificing pressure drop. Smoke results compare commercial super slim carbon filter designs with super-slim CelFX™ carbon filter designs with 5 to 6 mg/mm carbon loadings.

Celanese, 3520 Virginia Drive, Narrows, VA 24124, U.S.A.

ROSE N.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST12

E-cigarette vaping machine vs. conventional smoking machine – a compendium of differences in requirements and technology in regards to ISO 3308

E-products are a rapidly growing market. Starting with disposable or rechargeable products looking similar to conventional cigarettes, the market changed to more complex products. These so called second and third generation products offer a technical platform of much higher flexibility and customisation to the user.

Beside the fundamental discussions on how to regulate these products as a medical device or as a tobacco product, questions were raised about the presence of ingredients in the liquid and in the aerosol.

Certain studies have been performed and published, but it became obvious that nearly all of the publications were based on data that have been generated in different ways.

To get a better understanding of the products and the way they have to be analysed, CORESTA, with 9% members linked to e-products, launched a Task Force in 2012 to support harmonisation of nomenclature and to define the relevant categories of products with a view of making recommendations for product testing.

This presentation will summarise specific technical behaviours and design features of the e-products influencing the requirements of the design of a standard aerosol generator ("vaping machine“) in comparison to the existing conventional smoking machines. Physical properties such as size, weight, pressure drop or activation systems are discussed as well as their impact on the used puff generator, cigarette holder, termination system or aerosol traps. Finally the outlook to a first harmonised method is presented and some first results are shared.

Borgwaldt KC GmbH, Schnackenburgallee 15, 22525 Hamburg, Germany

RUFENER C.; BENSE T.; RUNGA N.; TAROCO E.; BONILLA T.; UMPIERREZ E.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST34

Effects of acetate and charcoal sector position in Hoffmann analytes of dual and triple filter cigarettes

It is known that activated charcoal has a good general efficiency in the adsorption of components of the vapour phase of cigarette mainstream smoke. Many studies have been published concerning the different factors involved in the filtration efficiency of carbon filters.

This work studied the influence of the position of the carbon section in the filter and the removal efficiency under an intensive smoking regimen for eight carbonyls, ten polyaromatic hydrocarbons (PAHs), five organic volatile compounds (VOCs) and seven phenols. The study was done by using two dual filters with a “Dalmatian” sector of 60 mg of activated carbon inverting the order with the acetate sector; and by using a triple (acetate-carbon-acetate) and a dual filter (carbon-acetate) with a carbon sector constituted by a polymer matrix supporting a content of 150 mg of activated carbon. A reference filter of mono acetate was also analysed to compare retention of carbon filters. All filters were attached to the same American blend tobacco column.

No significant differences in retention were found related to the position of the charcoal sector in the filter but significant differences were found between the reference acetate filter and the Dalmatian filter and between acetate filter and polymer matrix filter for carbonyls, VOCs and three PAHs. The average reduction percentage for these compounds in the Dalmatian filters was 23% and for the polymer matrix filters 45%. No significant differences in retention were found for the majority of PAHs and phenols.

The results corroborate that the weight, rather than the position, of the carbon within the filter is the dominant factor affecting retention, and that a high retention level of carbonyls, VOCs and even PAHs could be obtained with polymer matrix filters.

Compañia Industrial de Tabacos Montepaz S.A., San Ramon 716, Montevideo, Uruguay

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

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST39

Untargeted metabolomic profiling in saliva of smokers and non-smokers by a validated GC-TOF-MS method

Smoking is a major cause of several diseases. However, the underlying pathophysiological mechanisms are still not completely understood. To investigate endogenous alterations in the metabolome as a consequence of smoking, a gas chromatography hyphenated to time-of-flight mass spectrometry (GC-TOF-MS) method was developed and validated for analysis of the metabolic fingerprint in saliva of smokers and non-smokers. The method was validated by spiking 37 different metabolites and six internal standards into saliva samples, covering a wide range and polarity of chemical classes. Biological samples were obtained from a 24 h, strictly controlled clinical study, including 25 smoking and 25 non-smoking subjects.

Sample preparation was carried out by a combined derivatisation procedure including a methoximation and silylation step. Metabolite separation was conducted with a non-polar dimethyl polysiloxane column with a total run time of 30 minutes. For data processing and statistical analysis the software tools MZmine, Metaboanalyst and PSPP were applied, allowing for baseline correction, automated peak selection, integration and partial least square discriminant analysis (PLSDA; group separation). Thirteen significantly altered metabolites were identified in smokers' saliva ($p<0.05$), from which tyramine showed by far the strongest association to smoking. Since tyramine is a physiological and neurological active compound, a targeted Hydrophilic Interaction Liquid Chromatography (HILIC)-MS/MS methodology was developed and validated. The method is characterised by a simple sample preparation procedure, short run time and good precision. The tyramine level elevation in smokers (≈100 fold) could be confirmed by using the quantitative targeted HILIC-MS/MS assay.

It was demonstrated that saliva, as a non-invasively accessible body fluid, is a suitable biological matrix for investigating the human metabolome. Nevertheless, further research in this direction is required to verify and extend these findings.

ABF Analytisch-Biologisches Forschungslabor GmbH, Goethestr. 20, 80336 Munich, Germany

SHAH N.; FLORA J.W.; MERUVA N.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST09

Improved method for determination of tobacco-specific nitrosamines (TSNAs) in tobacco and tobacco smoke by UPLC-MS/MS

Tobacco-specific nitrosamines (TSNA) are harmful and potentially harmful constituents (HPHCs) found in tobacco products. In an effort to standardize quantitative analytical methods for TSNA analysis and reporting, CORESTA has published reference methods (CRM) for the determination of TSNAs in tobacco (CRM No. 72) and tobacco smoke (CRM No. 75) utilizing liquid chromatography tandem mass spectrometry (LC-MS/MS). The objective of this work was to develop an improved method for the determination of TSNAs in tobacco and tobacco smoke using ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). The application of a UPLC system combined with the new generation mass spectrometers facilitated improved resolution, more rapid analysis, and greater sensitivity. This method utilized a UPLC column with sub 2-micron particle size that offered higher chromatographic resolution for TSNAs, minimized sample matrix effects and reduced analysis time. All requirements for method validation were met including linearity, accuracy, precision, limits of detection (LOD), limits of quantitation (LOQ), method robustness, and standard and sample extract stability. For example, the linearity was demonstrated for all analytes with a coefficient of determination of R^2>0.995. The recoveries for all analytes were within 85%-115%. For tobacco analysis, the LOQ (based on lowest calibration standard) for N-nitrosonornicotine (NNN), N-nitrosoanatabine (NAT), and 4-(N-Methyl-N-Nitrosamino)-1-(3-Pyrridyl)-1-Butanone (NNK) was 162 ng/g and the LOQ for 4-nitrosoanabasine (NAB) was 40 ng/g. For smoke analysis, the LOQ for NNN, NAT and NNK was 8 ng/cig and the LOQ for NAB was 2 ng/cig. We also established criteria for the migration of the TSNA methods from LC-MS/MS to UPLC-MS/MS. It was also demonstrated that there is no need for sample clean up using solid phase extraction (SPE), a costly and time-consuming step. Results for this improved TSNA method using UPLC-MS/MS correlated well with results obtained from CORESTA recommended methods (CRM Nos. 72 and 75).

Altria Client Services Inc., 601 E. Jackson St., Richmond, VA 23219, U.S.A.

SHAH N.; JIN C.; AVERY K.; FLORA J.W.; MERUVA N.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST10

Determination of heterocyclic aromatic amines in cigarette smoke by UPLC-MS/MS

As stated in the Federal Register (Vol. 77, No. 64 / Tuesday, April 3, 2012) Docket No. FDA-2012-N-0143, Heterocyclic Aromatic Amines (HAAs) are included in the "Established List of the Chemicals and Chemical Compounds Identified by the United States Food and Drug Administration (FDA) as Harmful and Potentially Harmful Constituents [HPHCs] in Tobacco Products and Tobacco Smoke." Currently no standardized method exists for determination of the listed HAAs (AC, MeAC, PhIP, IQ, Trp-P-1, Trp-P-2, Glu-P-1 and Glu-P-2) in tobacco smoke. Therefore, the objective of this work was to develop a highly sensitive, selective and validated method for the determination of these HAAs in tobacco smoke using ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). For this new analytical method, five cigarettes were smoked under both ISO and Health Canada Intense conditions and the mainstream smoke was collected using a 44 mm Cambridge filter pad. The filter pad was extracted using an aqueous acidic solution (0.1 N hydrochloric acid) for 30 minutes and the smoke extract was subjected to solid phase extraction (SPE) clean up using an Oasis MCX cartridge to remove potential interferences prior to UPLC-MS/MS analysis. The chromatographic separation and the MS/MS parameters were optimized for the eight analytes and four isotopically labeled internal standards to achieve accurate quantitation of low levels of HAAs in cigarette smoke. The method was validated for linearity, accuracy, precision, limits of detection (LOD), limits of quantitation (LOQ), robustness and sample extract stability. The method exhibits good linearity (coefficient of determination of R^2>0.995) in the concentration range of 4-200 ng/cigarette for all analytes, with a LOQ of 4 ng/cigarette. This validated method was used to determine the levels of HAAs in 3R4F, 1R5F and CORESTA monitor (CM7) reference tobacco products.

Altria Client Services Inc., 601 E. Jackson St., Richmond, VA 23219, U.S.A.

SHAH N.; SMITH T.; FLORA J.W.; MERUVA N.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST03

Determination of tobacco specific nitrosamines (TSNAs) in tobacco and tobacco smoke by GC-MS/MS

NNN (N'-nitrosonornicotine), NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone), NAB (N'-nitrosoanabasine) and NAT (N'-nitrosoanatabine) are the most common tobacco specific nitrosamines (TSNAs) measured in tobacco and tobacco smoke. While CORESTA Recommended Methods (CRMs) exist for the determination of TSNAs in tobacco (CRM No. 72) and tobacco smoke (CRM No. 75) using liquid chromatography-tandem mass spectrometry (LC-MS/MS), the objective of this study was to develop and validate a more sensitive, selective and higher throughput method for TSNAs using gas chromatography-tandem mass spectrometry (GC-MS/MS). This new method involved extraction of the tobacco samples using an organic solvent followed by solid phase extraction (SPE) to reduce matrix interferences and to concentrate the sample extract. Samples were then analyzed by GC-MS/MS in the chemical ionization (CI) mode using multiple reaction monitoring (MRM). All requirements for method validation were met including linearity, accuracy, precision, limits of detection (LOD), limits of quantitation (LOQ), method robustness, standard and sample extract stability. For example, the linearity for the GC-MS/MS method was demonstrated with a coefficient of determination of R^2>0.995. For tobacco analysis, the LOQ (based on the lowest calibration standard) for NNN, NAT, and NNK was 162 ng/g and the LOQ for NAB was 40 ng/g. For smoke analysis, the LOQ for NNN, NAT and NNK was 8 ng/cig, and the LOQ for NAB was 2 ng/cig. The analytical performance of GC-MS/MS method was compared to the conventional LC-MS/MS method based on the analysis of CORESTA tobacco reference products (CRP1, CRP2, CRP3 and CRP4) and two Kentucky reference cigarettes (3R4F and 1R5F) where both methods showed comparable quantification of TSNAs. However, this new GC-MS/MS platform provided lower LOQs, higher selectivity, and higher throughput compared to the LC-MS/MS method.

Altria Client Services Inc., 601 E. Jackson St., Richmond, VA 23219, U.S.A.

SIMMS L.(1); WIECZOREK R.(2); TRELLES STICKEN E.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST80

The CORESTA recommended *in vitro* test battery alongside the National Research Council vision for toxicity testing in the 21st Century

The CORESTA *In vitro* Toxicity Testing of Tobacco Smoke Task Force recommended a test battery in 2004 which has been subsequently used by the tobacco industry. A similar *in vitro* test battery has been used by the International Community on Harmonisation (ICH) for the genotoxicity testing of pharmaceuticals for human usage since 1994. These assays have been used predominantly in product stewardship risk assessments to measure the effects of the addition of new or increased levels of ingredients added to tobacco.

In 2007, the USA National Research Council (NRC) released the landmark document, “Toxicity testing in the 21st Century - a vision and a strategy”, to provide recommendations for improved assessments of environmental chemicals. Its aims are to increase the use of *in vitro* models for investigating perturbations in toxicity pathways which should be validated with targeted testing (including the collection of human exposure data). It recommends a move away from *in vivo* (animal) high dose toxicity studies; to an increased use of multiple *in vitro* assays. The aims of the presentation are to look at possible modifications and improvements to the current CORESTA testing battery. Also, the implementation of potentially more intelligent testing strategies, to support the *in vitro* genotoxicity data generated will be discussed.

1. *Imperial Tobacco Ltd, Winterstoke Road, Bristol BS3 2LL, U.K.*
2. *Imperial Tobacco Group, Reemtsma Cigarettenfabriken GmbH, Biological Labs Hamburg, Albert-Einstein-Ring 7, D-22761 Hamburg, Germany*

SMITH T.; MERUVA N.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST15

Simultaneous determination of nicotine, propylene glycol, glycerin, menthol, ethanol and water in electronic cigarettes by gas chromatography

As e-cigarettes (electronic nicotine delivery systems (ENDS) or e-vapor devices) are gaining popularity in global markets, there is an increasing need for quantitative analytical methods to measure e-cigarette fluid compositions for quality assurance (QA) testing and commercial product investigations. The objective of this work was to develop a highly efficient quantitative method that simultaneously determines the amount of major formulation and aerosol components of e-cigarettes including nicotine, propylene glycol (PG), glycerin, menthol, ethanol and water. The sample extracts of e-cigarette formulations and aerosols were analyzed using gas chromatography (GC) equipped with a flame ionization detector (FID) and a thermal conductivity detector (TCD). As this method quantitated six different formulation components of interest from a single sample preparation on a single instrument, it was referred to as the "6-in-1 method." Results are reported as percent by weight for each component (weight %), milligrams per cartridge (mg/cartridge), milligrams per puff count (mg/puff count), or milligrams per e-cigarette (mg/e-cig). All requirements for method validation were met including linearity, accuracy, precision, limits of detection (LOD), limits of quantitation (LOQ), method robustness, and standard and sample extract stability. For example, the linearity was demonstrated for all analytes with a coefficient of determination of R^2>0.995. The recoveries for all analytes ranged between 88-105%. The LOQs were 1.25 mg/cartridge for nicotine, PG, glycerin, menthol, and ethanol and 12.5 mg/cartridge for water. The integrated 6-in-1 method provides the following benefits: lower cost of analysis, reduced sample requirements, and improved laboratory productivity.

Altria Client Services Inc., 601 E. Jackson St., Richmond, VA 23219, U.S.A.

SPOKES M.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST04

Determination of fifteen primary and heterocyclic aromatic amines in mainstream cigarette smoke using liquid chromatography-mass spectrometry (LC-MS/MS)

The levels of potentially harmful compounds in mainstream cigarette smoke such as aromatic amines (AAs) have been of interest for many years. Gas chromatography-mass spectrometry (GC-MS) has previously been the most popular method for determination of AAs in cigarette smoke however the extraction and derivatisation procedure is complex and time-consuming. LC-MS/MS, with higher selectivity and sensitivity, permits a simplified clean-up procedure to be used. With increasing legislation more aromatic amines may be added to existing regulations and a simpler LC-MS/MS procedure has more flexibility to accommodate this requirement.

Four aromatic amines (1-AN, 2-AN, 3-AB, 4-AB) are required for Brazilian ANVISA and Health Canada regulations and o-anisidine, o-toluidine, 2,6-dimethylaniline and eight heterocyclic aromatic amines (HAAs: A-α-C, IQ, MeA-α-C, Trp-P-2, Trp-P-1, PhIP, Glu-P-1, Glu-P-2) have been cited by the United States Food and Drug Administration (FDA) as harmful and potentially harmful constituents (HPHC) in tobacco smoke. A single smoking and extraction method followed by LC-MS/MS methods for the analysis of all fifteen aromatic amines will be presented.

Some HAAs have not been detected in reference cigarettes so transfer rates were evaluated by spiking components into the tobacco rod and collecting the smoke condensate on a Cambridge filter pad.

The procedure has also been shown to be applicable to sidestream smoke and e-cigarette vapour. Data will be presented for cigarette and e-cigarette products.

Essentra Scientific Services, Shaftesbury Avenue, Jarrow, Tyne and Wear NE32 3UP, U.K.

STAAF M.; HODIN F.; LUNDIN J.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST60

A more accurate estimation of free-base nicotine in moist snuff

The term "free-base" nicotine (FBN) refers to the unprotonated or un-ionised form of nicotine which is readily available for uptake through the mucous membrane. Commonly, the availability of FBN in moist snuff is estimated by the Henderson-Hasselbalch equation (HHE) using the pH-value of an aqueous extract and the dissociation constant for nicotine at 25 °C and infinitely dilute solution (pK_{a2}≈8.02).

However, it is well known that both temperature and ionic strength (I) influence pK_a-values, and since the standard estimation does not take into account the prevalent ionic strength in the products or temperature during use, this may give misleading results. Furthermore, the pH of a dilute aqueous extract has little relevance to FBN during oral usage.

The purpose of this study was to present a new approach to estimate the FBN by using liquids obtained by compressing moist snuff and an adjusted pK_{a2}-value for nicotine.

The pH of liquids from different moist snuff were determined at 35 °C and the ionic strength was measured to be around 1 M. Titration of a model liquid consisting of an aqueous solution of nicotine and sodium chloride (I=1 M) at 35 °C was conducted. The pK_{a2} was estimated using two approaches: 1) displacement of the HHE to fit the titration curve; 2) curve fitting of the titration data using a sigmoidal equation.

The pH of the snuff liquids were lower compared to the aqueous extracts and the differences varied between different types of snuff. The pK_{a2} at I=1 M and 35 °C was estimated to 8.20. Using the above methodology, the estimated amount of FBN for a typical Swedish snus is only up to approximately 50% of the value compared to using the current approach for determining the FBN levels.

Swedish Match North Europe AB, Research & Development, Maria Skolgata 83, SE-118 85 Stockholm, Sweden

TAO Wenmei(1,2); SHI Jinwen(1); DUAN Meng(1); LU Yi(1); LI Jun(1); LI Qinghua(3); WEI Qing(1,2); LIU Weijuan(1,2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST35

Removal of toxicants using reconstituted tobacco sheet with novel plant fibre

The China Institute of Tobacco has encouraged the pursuit of a potential method or process to reduce toxic products in cigarettes, to reduce the risk of one or more specific diseases or other adverse health effects. Reconstituted tobacco sheet (RTS), used as an important cigarette blend component, has attracted more attention to reduce exposure to toxic materials in the cigarette smoking process. RTS is mainly composed of tobacco flavour, cellulosic fibres, calcium carbonate and polymers. Today, the cellulosic fibres in the RTS are mainly derived from tobacco fibres and softwood pulp (SP). All these fibres can reduce toxicants in the combustion of RTS. One potential approach was to replace some SP with porous plant fibres (PPF) in RTS. SP and PPF were characterised using Scanning Electron Microscopy (SEM). SEM images showed that compared with SP, the selected PPF have soft and loose structures, which can support the cellulosic paper with a "skeletal structure". Also, RTS materials with SP and PPF, separately, were characterised using various techniques, such as tensile strength, filling power test, air permeance test, and SEM. Compared with RTS containing SP, the RTS with selected PPF had higher filling power, air permeance and adsorptive rate of liquid tobacco extracts, but with a lower tensile strength, which can be demonstrated by SEM images. Mainstream smoke yields of four toxicants were measured from RTS cigarettes containing porous plant fibres. The process reduced levels of total particular matter (25-39%), nicotine (18-23%), tar (35-43%), and carbon monoxide (20-30%). Toxicants adsorption values demonstrate that RTS with selected porous plant fibres can reduce exposure to toxic products in cigarettes.

1. *Yunnan Reascend Tobacco Technology (Group) Co., Ltd, Kunming, Yunnan, 650106, P.R. China*
2. *Yunnan Tobacco Reconstituted Tobacco Sheet Co., Ltd, Kunming, Yunnan, 650106, P.R. China*
3. *China Tobacco Yunnan Industrial Co., Ltd, Kunming, Yunnan, 653100, P.R. China*

TAYLOR M.J.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST21

The influence of cigarette filter design on the yield and composition of cigarette smoke

Since the first paper filters were used commercially in the mid-1930s, filters have become more integral to the overall design of the cigarette to help modify the yield, composition and taste of cigarette smoke. Cellulose acetate is currently the material of choice for most cigarette filters but other materials and granular additives are available with different filtration properties. Although filters are often used to control the overall tar and nicotine yield of cigarettes, more recently, there has been a growing interest in the selective removal of harmful compounds from smoke. The availability of important smoke compounds for selective removal will be reviewed. The performance of a range of filter materials and granular additives are discussed both in terms of overall performance and the ability to selectively reduce harmful compounds. Consideration will be given to key parameters that can influence filter effectiveness such as smoke velocity and contact time which are in turn dependent on smoking regime and product diameter being much higher and shorter for intense regimes and superslim products when compared to the ISO smoking regime and standard diameter products. The effect of temperature on the adsorption and potential desorption of compounds on granular additives will also be discussed.

Essentra Scientific Services, Shaftesbury Avenue, Jarrow, Tyne and Wear NE32 3UP, U.K.

TEILLET B.(1); VERRON T.(1); CAHOURS X.(1); COLARD S.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST86

Smoke analysis of fine-cut tobacco – a predicting model and other challenges

The ISO 15592-1 to 3 standards specify methods for the sampling, conditioning and determination of nicotine and "tar" in the mainstream smoke generated by the combustion of fine-cut tobacco. As originally requested by several European government chemists, four articles were smoked using two weights of tobacco corresponding to two diameters, and two types of wrappers with different burning properties. This leads to a matrix of four data points providing an indication of the influence of wrapper and tobacco weight on tar and nicotine yields.

Laboratories can face difficulties to perform the measurement on some matrix points especially for small diameters and for testing of high expanded blends. Herein, we present a study to investigate the effects of the paper and the diameter on the tar and nicotine yields. We also evaluate whether the multiple data points in the matrix can be determined from a single set of experimental values.

A model was developed based on the article characteristics and the yields published in the ISO standard in order to estimate any of the matrix points from one reference matrix point. These parameters are diameter, type of paper, and the interaction diameter/paper. This was subsequently validated using datasets of 53 samples corresponding to 212 yields taking into account the variability of the analytical measurement.

Using this modelling approach, the results of the three calculated matrix points from one reference matrix point are in line with the corresponding experimental results from the laboratory. Nevertheless, experimental challenges remain and need to be investigated.

1. *Imperial Tobacco Group, SEITA, 48 rue Danton, 45404 Fleury-les-Aubrais, France*
2. *Imperial Tobacco Ltd, Winterstoke Road, Bristol BS3 2LL, U.K.*

TEILLET B.(1); VERRON T.(1); CAHOURS X.(1); COLARD S.(2); PURKIS S.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST12

Challenges on HPHC analysis – the limit of quantification

In the framework of growing regulations regarding tobacco products, increasing requirements for reporting of analytical figures are observed (e.g. FDA Draft guidance for Reporting Harmful and Potentially Harmful Constituents (HPHC) in Tobacco Products and Tobacco Smoke).

However, the determination of some constituent levels remains challenging when the levels are below the limit of quantification of the testing methods. Blend and smoke constituents of commercial fine cut tobacco and cigarette brands sold on the US market were analysed according to the full HPHC list published by the United States Food and Drug Administration (FDA). In this poster, attention is paid to the constituents where levels were systematically below the limit of quantification (LOQ).

30-40% of the constituents listed were determined below the limits of quantification for the analytical methods typically used. This proportion was observed for both fine-cut and cigarette blends as well as smoke emissions under both ISO and Intense smoking regimes.

Values systematically below the LOQ can be related to the occurrence or not of such constituents in tobacco blend/smoke and/or to the performance of the analytical method. In such cases the number of replicates could be reasonably reduced to two replicates to provide sufficient information.

In this poster, the identification of such non quantifiable constituents, and the limitations of the analytical methods as well as the relevance of performing seven replicates, are discussed.

1. *Imperial Tobacco Group, SEITA, 48 rue Danton, 45404 Fleury-les-Aubrais, France*
2. *Imperial Tobacco Ltd, Winterstoke Road, Bristol BS3 2LL, U.K.*

THIELEN A.(1); HAHN J.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST22

The "Technical Report DIN SPEC 10133 – Toxicological Assessment of Additives for Tobacco Products – A Guidance"

Since 2001 the Tobacco Products Directive 2001/37/EC has requested manufacturers of tobacco products to provide the "available" toxicological data of the additives used for the production of tobacco products. The national implementation of this Directive in Germany was the reason for the elaboration of a report by the German Institute for Standardization (DIN) Working-Group "Toxicology of additives": "DIN Technical Report 133 – Toxicological Assessment of Additives for Tobacco Products – A Guide" in 2004.

Since that time, scientific publications on results of toxicological investigation of tobacco additives have increased significantly. Besides that, the Tobacco Products Directive was revised and was published as Directive 2014/40/EU on April 29, 2014. After the implementation and a transition period there will be an obligation for the manufacturers of tobacco products to accompany their reporting data with "the relevant toxicological data regarding the ingredients in burnt or unburnt form."

The DIN working group recompiled the base material for toxicological methods and reviewed under which basic conditions already existing methods are transferable to the field of tobacco. In the absence of globally recognised standard procedures for toxicological evaluation of tobacco additives, this revised report "Technical Report DIN SPEC 10133 – Toxicological Assessment of Additives for Tobacco Products – A Guidance" will propose an appropriate test battery based on published strategies and methods. Besides guidance, key assessment criteria are proposed. The revised guide is regarded as a basis for the developing of standards and may serve as an aid for the preparation and evaluation of toxicological data for additives in tobacco products.

This poster is intended to give an overview of the present state of the art methods in this field and a recommended test strategy described in the report.

1. *DIN-Working Group "Toxicology of additives", Berlin, Germany*
2. *Convenor of DIN-Working Committee "Tobacco and tobacco products", Berlin, Germany*

THORNE D.(1); KILFORD J.(2); HOLLINGS M.(2); DALRYMPLE A.(1); BALLANTYNE M.(2); MEREDITH C.(1); DILLON D.(1)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST20

The assessment of the mutagenic potential of 3R4F mainstream cigarette smoke using multiple Ames strains

The Ames methodology is governed by clear international regulatory guidelines (e.g. OECD 471), which recommend the use of at least five bacterial strains in an *in vitro* test battery. *Salmonella typhimurium* strains TA1535; TA1537 or TA97 or TA97a; TA98 and TA100 between them detect frameshift and base-pair substitutions. Either strain TA102 or an *Escherichia coli* (*E. coli*) strain (WP2 *uvrA* or WP2 *uvrA* (pKM101)) are accepted as a fifth strain and between them detect certain hydrazines, oxidising mutagens and cross-linking agents.

In this study we have modified the Ames assay, using a spread plate methodology, to allow exposure to cigarette smoke at the air-agar interface, which facilitates the assessment of the complete cigarette smoke aerosol. In total, nine *S. typhimurium* strains and one *E. coli* strain were investigated using varying dilutions of cigarette smoke (12.0, 8.0, 4.0 and 1 L/min), generated from a VC 10 Smoking Robot. Of the assessed strains, five tested positive (TA98, TA100, YG1024, YG1042 and TA104), four were negative (TA102, WP2 *uvrA* pKM101, TA97 and TA1535) and one strain (TA1537) was deemed incompatible with this scaled-down methodology and was not assessed with cigarette smoke. A response was considered positive if greater than a two-fold increase over background spontaneous revertants was observed, combined with statistical differences ($p<0.01$). In the case of a negative response, smoke exposures were increased from 24 to 64 minutes to further assess mutagenic activity. Finally, to support *in vitro* exposure and to quantify dose, deposited particulate mass ($\mu g/cm^2$) was assessed using Quartz Crystal Microbalance technology *in situ* of exposure.

In conclusion, we have assessed an OECD acceptable battery and additional Ames strains for their responsiveness to mainstream cigarette smoke. Based on these data, we propose that a selection of these strains could be appropriate to assess the genotoxicity of current and future aerosol-based tobacco products.

1. *British American Tobacco, Group R&D, Regents Park Road, Millbrook, Southampton SO15 8TL, U.K.*
2. *Covance Laboratories Ltd, Harrogate, North Yorkshire, U.K.*

TRELLES STICKEN E.; WIECZOREK R.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST25

Increasing the *Salmonella typhimurium* reverse mutation assay's sensitivity following exposure to fresh aerosols

For the *in vitro* toxicological assessment of current and next generation products, including e-vapour products, the standard *in vitro* test battery developed for conventional combustible products needs further refinement in terms of sensitivity to ensure that the toxicological potential of such products can be reliably compared and is not underestimated. In particular, the trapping of any particulate matter from such products is more difficult due to increasing trapping times, concomitant ageing and increased water levels trapped. Consequently direct exposure-to aerosol technologies for different biological test systems have been developed.

Here we present a methodology developed to enhance the sensitivity of the OECD recommended *Salmonella typhimurium* TA100 reverse mutation assay (Ames test) for the assessment of e-vapour aerosols but also applicable to fresh cigarette smoke. In our standard exposure protocol the bacteria suspension is exposed directly to the aerosol which is lead through the bacteria suspension located in an impinger. Following the logic of former Ames test developments (e.g. micro-suspension version or liquid pre-incubation assay protocols), the probability of bacteria / fresh whole smoke contact was increased by increased cell density during exposure.

Using the CORESTA Monitor test piece CM7 and the Kentucky Reference cigarette 3R4F a correlation of bacteria cell density and the test sensitivity, as measured by the slope of the linear part of the dose response curve, could be shown. For example, for CM7 we found that a five-fold increase in cell density led to a 18-fold slope increase (from 0.2 rev./puff up to 3.7 rev./puff).

Due to low or missing responses to e-vapour products under standard Ames test conditions, a vapour-specific positive control was developed and tested. Hence, we present an adjusted product assessment for bacterial mutagenicity with increased test sensitivity for aerosol products.

Imperial Tobacco Group, Reemtsma Cigarettenfabriken GmbH, Biological Labs Hamburg, Albert-Einstein-Ring 7, D-22761 Hamburg, Germany

VERRIER J-L.(1); DESTRUHAUT S.(2); COLARD S.(3)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST01

Stalk position effect on harmful and potentially harmful cigarette smoke constituents in Burley tobacco: a case study

Cured leaf chemistry varies sharply according to stalk positions and variations of smoke yields of harmful and potentially harmful constituents (HPHCs) are also expected.

To quantitatively assess the effects on HPHC yields, Burley leaves of the cultivar TN 90LC were produced in a replicated agronomic trial in Bergerac (France), according to standard practices and gathered by stalk position (from bottom to top: lugs, cutters, leaves, tips). The tobaccos were made into cigarettes which were mechanically smoked under the Canadian intense smoking conditions according to WHO's testing proposal. The mainstream smoke (MSS) contents of 32 HPHCs were determined using in-house and recommended methods.

Presenting HPHC yields by mass of burnt tobacco during puffs, and from bottom to top plant position, carbonyls and tobacco specific nitrosamines tended to decrease while some volatile organic compounds (VOCs) (1,3 butadiene, benzene, toluene) and benzo[a]pyrene remained at similar levels. Some VOCs (isoprene, acrylonitrile) and carbon monoxide increased. Nicotine, phenols, hydrogen cyanide, aromatic amines and ammonia increased sharply. In contrast, due to higher nicotine yields in the upper positions, the ratio to the MSS nicotine decreased or stayed constant from bottom to top for all compounds except ammonia.

If the HPHC ratio to nicotine were regulated, this would create a dilemma: increasing the share of upper stalk positions in the Burley part of blends may bring the nicotine level above regulatory and consumer acceptance limits, whereas decreasing it may exceed ceilings for some ratios. If alternatively absolute HPHC yields were regulated, another dilemma would arise, between constituents and stalk positions.

Blending is an expert exercise of selection to find the optimal balance between sensory profiles, regulations and naturally variable raw tobaccos. Regulatory scenarios impact differently on this exercise and clarification from regulators will be needed in order to determine the direction for new tobacco variety developments.

1. *Imperial Tobacco Group, Institut du Tabac Bergerac, Domaine de la Tour, 24100 Bergerac, France*
2. *Imperial Tobacco Group, SEITA, 48 rue Danton, 45404 Fleury-les-Aubrais, France*
3. *Imperial Tobacco Ltd, Winterstoke Road, Bristol BS3 2LL, U.K.*

VERRON T.(1); CAHOURS X.(1); COLARD S.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST29

Product comparison: the risk associated with multiple testing

Manufacturers are increasingly being asked by regulatory authorities to report data on their new products. For example, the United States Food and Drug Administration (FDA) requires that these data are used to compare a new tobacco product to a predicate tobacco product in order to prove substantial equivalence. In 2013, we showed[1] the importance firstly to have validated and standardised methods with known precision, and secondly to use the appropriate statistical methods (critical differences as recommended by the ISO 5725 part 6) to compare results from different laboratories in order to avoid misleading conclusions. However, if the comparison of products involves several analytes simultaneously, then the use of critical differences separately for each analyte, using the same level of significance ($p<0.05$), could lead to wrong conclusions. This issue, well known in statistics, is still a very active topic of research with many challenges to be taken into account, such as the notion of independence and statistical power.

In this presentation we will define the multiple testing issue and concepts and we will introduce some existing methods for addressing multiple testing.

[1] B. Teillet, X. Cahours, T. Verron, S. Colard, S. Purkis. Comparison of Smoke Yield Data Collected from Different Laboratories. *Beitr. Tabakforsch. Int.* 25 (2013) 663-670.
[2] ISO5725-6, 2001. Accuracy (trueness and precision) of measurement methods and results. Part 6. Use in practice of accuracy values.

1. *Imperial Tobacco Group, SEITA, 48 rue Danton, 45404 Fleury-les-Aubrais, France*
2. *Imperial Tobacco Ltd, Winterstoke Road, Bristol BS3 2LL, U.K.*

VINCENT J.H; COLE A.O.; MASON T.J.P.; TINDALL I.F.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST42

Characterisation of e-cigarette aerosol generation behaviour

Disposable e-cigarettes generate aerosol by vaporising ‘e-juice’ carried by a wick to a heating element activated by a flow sensor. The onset of aerosol production depends on flow rate (Connor, 2013) and can substantially lag initiation of the puff, sufficient to perturb yield determination in a way that is variable from product to product.

The objective of this work was to characterise aerosol generation in e-cigarettes using simultaneous determination of aerosol temperature rise, pressure drop, air volume flow rate into the e-cigarette and into the puff engine, and opacity of the drawn puff. The aim is to provide a useful tool for product development and to assess consistency of construction and lifetime performance.

Temperature of the drawn puff was measured using a thermocouple mounted close to the product outlet and aerosol opacity was monitored using a photo-diode/receiver. The pressure drop and inlet and drawn volume flow rate through the products were logged on a time base of 0.01 s. Video capture assisted interpretation, in particular to calibrate the optical detector. Flow activated rechargeable and disposable product types were characterised over life under puffing regimes from 35 to 150 ml volume.

It is shown that the pressure drop of e-cigarettes is non-linear with flow rate, which is significant at the high flows associated with e-cigarette vaping and perturbs the (nominally) square puff profiles likely to be recommended for standardisation of e-cigarette vaping. Information from the puff engine is not in itself sufficient to fully characterise the onset trigger flow.

Development of e-cigarettes and monitoring of production consistency will be enhanced by full characterisation of the aerosol generation function during puffing. This must include initial design of the pressure drop vs. flow characteristics and consideration of how this changes over product life.

Cerulean, Rockingham Drive, Linford Wood East, Milton Keynes MK14 6LY, U.K.

VINCENT J.H; COLE A.O.; MASON T.J.P.; TINDALL I.F.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST06

Detection of e-cigarette aerosol generation over life

E-cigarettes generate an aerosol by vaporising 'e-juice' that is carried by a wick to a heater. Yield and end-of-life are conventionally determined by measuring the mass lost from the product or gained in a trap, but either is labour-intensive and has limited resolution, for example, to detect the final active puff of a product.

The objective of this work was to investigate temperature rise and opacity of the aerosol as real-time methods to complement the absolute yield information provided by mass balance. Manually or flow activated, rechargeable and disposable product types were assessed under puffing regimes from 35 to 150 ml volume.

Temperature of the drawn puff was monitored over product life (defined by expiry of the battery or of the e-juice) using a thermocouple mounted close to the product outlet and aerosol opacity was monitored using a photo-diode/receiver. Video capture assisted interpretation.

It is shown that temperature and opacity are complementary in detecting e-cigarette aerosol generation. Puffs exhibit a distinct temperature profile with an initial temperature rise of the aerosol of about 1 °C, which typically halves in the final 5-10% of the battery life, with the first non-active puff corresponding exactly to onset of flashing of the 'lit-end' diode, where this functionality is enabled. The appearance of aerosol is shown to lag the thermal onset by times that are dependent on brand and puffing regime. E-juice expiry, which is undesirable in products, is clearly distinguished from battery expiry.

Aerosol production can be detected by both temperature rise and opacity of the drawn puff. Combining these methods describes product status in ways that neither alone can manage. They are readily integrated into automated vaping machines and, together with conventional mass balance determination, provide complementary means to monitor performance over life.

Cerulean, Rockingham Drive, Linford Wood East, Milton Keynes MK14 6LY, U.K.

VOISINE R.(1); ERRINGTON G.(2); McADAM K.G.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST09

Impact of reducing the number of analysis replicates prescribed by the Canadian Tobacco Reporting Regulations on the level of information obtained from cigarette emission testing

Before the introduction of the Canadian Tobacco Reporting Regulations (TRR), the Regulator stated a need to obtain more information on tobacco product emissions to support, amongst other things, "better program design" and the "right to know". The TRR requires seven analysis replicates to be conducted per product sample when measuring 37 smoke analytes under three smoking conditions. Seven replicates is an unusually large number to stipulate, and the objective of this study was therefore to establish whether three replicates are sufficient to measure the 37 smoke analytes under the three smoking conditions. We studied smoke emission data generated for regulatory purpose covering 308 cigarette products and the Canadian Monitor cigarette (CM8). When possible, a power calculation was used to mimic the original approach of the Regulator and to establish the minimal number of replicates that would be required to see a set difference. Various other statistical analyses were used to compare results obtained with seven replicates to results calculated from the first three replicates taken from the full seven measured replicates. For CM8, we found that the average coefficient of variation (COV) and the range of COVs for the measured analytes were inferior to the original assumption. The power calculation indicated that three replicates would be sufficient to see a 15% relative difference between measurements (99% confidence level). For all-products, three analysis replicates provided results that are statistically indistinguishable from those obtained with seven replicates. Year-on-year variation in the measurement of emissions was far greater than any differences in the mean emission values of the measured analytes introduced by the use of three analysis replicates. We conclude that the use of three testing replicates for the analysis of the studied smoke analytes will generate the same level of information on tobacco product emissions as the use of seven testing replicates.

1. *Imperial Tobacco Canada Ltd, 3711 St-Antoine Street West, Montréal, QC, H4C 3P6, Canada*
2. *British American Tobacco, Group R&D, Regents Park Road, Millbrook, Southampton SO15 8TL, U.K.*

WANG Hongbo; LI Xiang; XIE Fuwei; PENG Bin; LIU Kejian; WANG Sheng; QIN Yaqiong; NIE Cong; LIU Huimin

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST84

Distribution of toxic chemicals in the different sized particles from mainstream cigarette smoke

It is important for the estimation of risk of cigarette smoke that the distribution of toxic chemicals is accurately measured in the different sized particles of cigarette smoke aerosol. An electrical low pressure impactor (ELPI) directly coupled with a single channel smoking machine was used for the measurement. The different sized particles of mainstream cigarette smoke were collected with 12 polyester films. Then, the nicotine, tobacco-specific N-nitrosamines (TSNAs, including NNN, NAT, NAB and NNK), polycyclic aromatic hydrocarbons (PAHs, including Benzo[a]pyrene (BaP), Benzo[a]anthracene and Chrysene) and heavy metals (including Cr, As, Cd and Pb) in the different sized particles were analysed by GC, HPLC-MS/MS, GC/MS and ICP-MS, respectively, in addition to weighing the particle matter (PM). The results showed that the nicotine, TSNAs, PAHs and heavy metals in mainstream cigarette smoke mainly existed in the particles size ranging from 0.1 μm to 2.0 μm, and the concentration of toxic chemicals increased firstly and declined with the increase of particle size. The largest release of toxic chemicals was observed in the particle size of 0.261 μm. For PM in the particles of sizes less than 0.1 μm, "0.1 to 1.0 μm" and "1.0 to 2.0 μm" fractions, the release of nicotine was uniform, TSNAs and heavy metals of PM in the particles of size less than 0.1 μm is more abundant, and PAHs in PM of the fine size particles is more abundant.

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

WANG Xiaoyu; GUO Jizhao; XIA Qiaoling; DING Li; XIE Fuwei; ZHANG Xiaobing; LIU Huimin; WANG Hongbo

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. STPOST08

Determination of 16 polycyclic aromatic hydrocarbons in mainstream cigarette smoke by using a graphene coated solid-phase micro extraction (SPME) technique followed by gas chromatography-mass spectrometry (GC/MS)

A new method to determine the levels of 16 polycyclic aromatic hydrocarbons (PAHs) in mainstream cigarette smoke simultaneously and rapidly was developed. The method used SPME-GC/MS, wherein an in-house prepared graphene-coated fibre was used in SPME. Compared with commercial SPME fibre, the in-house prepared graphene-coated SPME fibre was superior in thermal stability and extraction efficiency. Due to the strong π-π interaction between graphene-coating and PAHs, the in-house prepared fibre was highly selective to PAHs and more effective in the clean-up of smoke samples compared to either 100 μm PDMS SPME fibre or cyclohexyl solid phase extraction. By collecting the particulate matter from five cigarettes, the limits of detection (LODs) and quantitation (LOQs) for the 16 target PAHs reached from 0.05 to 0.1 and from 0.2 to 0.3 ng/cig, respectively; the linear correlation coefficients were above 0.995, and the precisions measured by six repeated tests ranged from 1.3% to 10.5%. At three addition rates, the recoveries for the PAHs were between 83.6-110.1%. Moreover, the measurements of PAHs concentrations in particulate matter of mainstream smoke of 1R5F Kentucky reference cigarettes were close to the results recently reported in the literature.

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

WANNA J.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST24

Carbon monoxide (CO) diffusion through cigarette paper

Cigarette paper plays a critical role in carbon monoxide (CO) diffusion during the smoking process. Introducing bands with low diffusion to comply with LIP regulations reduce CO diffusion through these bands. However, there are no tests that measure CO diffusion through the bands and paper during the smoking process. SWM contacted Arista Labs and inquired about developing a method using a CO analyser to measure CO diffusion through cigarette paper at various positions along the tobacco column during cigarette puffing. A new technique was successfully developed that measures CO diffusion through the band and base paper. The method will be presented.

Cigarettes from seven brands were selected for testing. Band and base paper diffusions were measured. Band diffusion ranged from 0.034 cm/sec to 0.100 cm/sec and base diffusion from 1.05 cm/sec to 1.319 cm/sec. CO diffusion was measured from the first and second band, in-between the bands and after the second band. CO diffusion through the base paper was 30% higher than CO diffusion through the bands. This paper will discuss the technique and preliminary CO diffusion results from each band.

SWM Intl, 100 Northpoint Center East, Suite 600, Alpharetta, GA 30022, U.S.A.

WEN Jianhui(1); DU Wen(1); PENG Bin(2); LI Bin(2); ZHANG Xiaobing(2); XIE Fuwei(2); LIU Huimin(2); ZHONG Kejun(1)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST33

Filtration and retention characteristics of crotonaldehyde in cigarette filters

In order to study the filtration and retention characteristics of crotonaldehyde in cigarette filters, the filtration efficiencies of crotonaldehyde were investigated in regular acetate filter under ISO and Health Canada Intense (HCI) smoking regimes, and in outer grooved acetate filter, inner grooved acetate filter, paper/acetate composite filter, ventilation cavity acetate filter under ISO smoking regime. The influences of smoking regime and filter structure on crotonaldehyde retention space distribution pattern were investigated. By on-line monitoring of the temperature of smoke flowing through the filters during the whole smoking process and the study of puff-by-puff crotonaldehyde retention longitudinal concentration distribution patterns, the filtration and retention characteristics of crotonaldehyde in filters were explained. The results showed that: 1) The filtration efficiency of crotonaldehyde in different filters under the ISO smoking regime from high to low order is: regular acetate filter > outer grooved acetate filter ≈ inner grooved acetate filter > paper/acetate composite filter > ventilation cavity acetate filter. The filtration efficiency of crotonaldehyde in regular acetate filter reduces remarkably under the HCI smoking regime. 2) The retention concentration distribution pattern of crotonaldehyde in filters is distinctly different from that of nicotine, phenol and other smoke components. Filter structures also have a notable effect on crotonaldehyde retention concentration distribution pattern in filters. 3) The puff-by-puff crotonaldehyde retention longitudinal concentration distribution pattern and smoke temperature on-line monitoring results showed that there was an adsorption-desorption-adsorption effect for crotonaldehyde in the smoking process, and this is the reason why the retention concentration distribution pattern of crotonaldehyde is significantly different from that of other smoke components.

1. *China Tobacco Hunan Industrial Co., Ltd, Technology Center, Changsha 410007, P.R. China*
2. *Zhengzhou Tobacco Research Institute of CNTC, No. 2 Fengyang Street, Zhengzhou Hi-Tech Development Zone, Zhengzhou, Henan 450001, P.R. China*

WEN Siru; CHEN Yuxiang

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST22

Cellulosic fibres for cigarette paper production: thermal characteristics and their implications pertaining to cigarette smoking

As the essential component of cigarette paper, cellulosic fibres can be produced from different sources. The flavour of cigarettes pertaining to smoking may be dependent upon the nature of cellulosic fibres. In this study, the thermal characteristics of different bleached chemical pulps (softwood, hardwood, and flax fibres) were evaluated by thermogravimetry-differential thermal analysis and pyrolysis. For flax fibres, the content of non-carbohydrate components (e.g., pectin and lignin) was lower in comparison to softwood/hardwood fibres, which may be indicative of a favourable impact related to smoking. As shown from thermogravimetry-differential thermal analysis profiles, there were two exothermic peaks for the pulps. Noticeably, in the case of flax fibres, the characteristic temperature for the second peak was lower in comparison to other pulps. This lower temperature may be indicative of less toxic compounds releasable from smoking. A high temperature may result in complex pyrolysis reactions. The pyrolysis analysis was conducted based on three temperatures, i.e., 300, 600, and 900 °C. Interestingly, there were less types of pyrolysis products from flax fibres than from softwood/hardwood fibres. Flax fibres were found to be more advantageous in terms of flavour related to smoking. All of these results supported the conclusion that in terms of flavour related to smoking, flax fibres can be a better choice for the production of cigarette paper. These findings would add to the basic knowledge of cigarette products, which may provide useful implications for process/quality control.

Mudanjiang Hengfeng Paper Co., Ltd, Mudanjiang 157013, P.R. China

WERLEY M.S.; JEROME A.M.; LANGSTON T.B.; OLDHAM M.J.; LILLY P.D.; McKINNEY W.J.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST18

Subchronic rodent inhalation study of e-vapor formulations

An electronic cigarette and three formulations were evaluated in a 90-day inhalation study with a 42-day recovery period, mimicking earlier cigarette smoke studies. Male and female Sprague-Dawley rats (210/gender) were randomly assigned to Low (L), Medium (M), High (H) treatment groups and exposed to vehicle (mixture of glycerin and propylene glycol), vehicle and 1.5% nicotine by weight (NBW), and vehicle, 1.5% NBW and flavor formulation. Daily targeted aerosol doses of 3.2, 9.6 and 32.0 mg/kg/day in the L, M, and H groups were achieved by exposure to 1 mg/L aerosol for 16, 48, and 160 minutes, respectively and confirmed with gravimetric sampling of the exposure atmosphere. Pre-study evaluations included indirect ophthalmoscopy, virology and bacteriological screening. Weekly monitoring for biological effects included body weights, clinical observations and weekly food consumption. During exposure weeks 4 and 13 serum nicotine and cotinine and carboxyhemoglobin levels were measured and after weeks 4, 8 and 13 pulmonary function measurements were obtained. Biological endpoints after exposure and the recovery period included clinical pathology, urinalysis, bronchoalveolar fluid (BAL) analysis, necropsy and histopathology. A subgroup of rats was evaluated following 28-days of exposure. Exposure related effects following 90-days of inhalation exposure included treatment related decreases in body weight and gains, decreased food consumption, and decreased respiratory rate for all formulations. Non-specific organ weight changes and BALF differences from control were noted. Histopathology evaluations revealed non-dose related increases in epithelial hyperplasia and vacuolization in the nose. All effects completely reversed or nearly so following a 42-day recovery period.

Altria Client Services Inc., 601 E. Jackson St., Richmond, VA 23219, U.S.A.

WILLIAMSON F.; MARTIN A.

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST73

A new method for the analysis of carbonyl compounds in e-cigarette liquids

Carbonyl compounds (carbonyls) are organic molecules that contain a carbon atom double-bonded to an oxygen atom. Specifically these include compounds known as aldehydes and ketones that are usually found in traditional mainstream tobacco smoke and are of interest to many regulatory bodies including the United States Food and Drug Administration (FDA), Health Canada and ANVISA (Brazil). The increasing popularity of e-cigarette and vapor products carries with it an increasing interest in the measurement of many of the Harmful and Potentially Harmful Constituents (HPHCs) identified by the FDA.

E-cigarettes and electronic vapor products typically contain a nicotine solution (e-liquid) with high levels of vegetable glycerin (VG) or propylene glycol (PG) that can potentially decompose into various carbonyl compounds at high temperatures. Due to the wide concentration ranges of carbonyls likely to be observed in e-cigarettes and electronic vapor products, the standard tobacco emissions carbonyl assay using 2,4-dinitrophenylhydrazine (DNPH) derivatization followed by HPLC separation may not provide the sensitivity and specificity needed for this analysis.

The purpose of this presentation is to demonstrate a new analytical method for testing carbonyl compounds from e-liquids. The sample is dissolved in acetonitrile, derivatized with o-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (PFBHA), then extracted with hexane. The hexane extract is analyzed by single quadruple gas chromatography-mass spectrometry (GC/MS) using negative chemical ionization.

The validated method works for a wide range of carbonyl containing compounds and provides ng/g detection with good specificity, robustness and reproducibility. The potential application of the method to analysis of carbonyls in e-cigarette vapor will also be discussed.

Arista Laboratories, 1470 East Parham Road, Richmond, VA 23228, U.S.A.

YANG Ji; YANG Liu; WU Yiqin; MIAO Mingming; ZHE Wei; CHEN Yongkuan

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST44

Investigation of pyrolysis behaviour of lutein in air by thermo gravimetry-single drop micro extraction-gas chromatography-mass spectrometry

To investigate the pyrolysis behaviour of lutein in air, an automatic single drop micro extraction (SDME) device for trapping the released fractions was designed and coupled with thermo gravimetry-gas chromatography-mass spectrometry. According to the results of thermogravimetric - differential thermal analysis (TG-DTA), the pyrolysis process was divided into 16 temperature sections in the range of 155-400 °C. The released fractions from thermo gravimetric analyser were automatically extracted with ethanol by SDME. The extract effect of SDME was analysed by comparing the mass loss in each temperature section with the total peak area of pyrolysis products. The results indicated that: 1) The optimised conditions for the thermo analyser were: temperature ascending rate 5 °C/min and carrier gas flow rate 400 mL/min. 2) SDME was excellent in the extraction of volatile components in pyrolysis products. 3) The probable mechanism for the formation of major aromatic substances in lutein pyrolysis was inferred on the basis of monitoring the dynamic changes of relative contents among 43 main pyrolytic components. 4) The main components identified by GC/MS were ketones, aldehydes, alcohols, alkenes and aromatic hydrocarbons, the major pyrolysis products were ß-damascenone megastigmatrienone and 4-oxoisophorone. The pyrolysis behaviour of lutein in air provided a reference for analysing the pyrolysis behaviour of tobacco components during smoking.

China Tobacco Yunnan Industrial Co., Ltd of CNTC, R&D Center, Kunming 650024, P.R. China

YANG Panpan(1); WANG Chao(2); WANG Yu(1); HOU Ying(1); YANG Yingming(2); YANG Shihua(1); LI Wei(1); YUAN Tianjun(1); CHENG Changxin(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST89

Aroma compounds in flue-cured tobacco from Baoshan: distribution and relationships to climate and geographical factors

Baoshan is a city in the southwest of China. Tempered by the low latitude and moderate elevation, Baoshan has a mild subtropical highland climate with short, mild, dry winters, and warm, rainy summers. Baoshan is a tobacco growth region for flue-cured tobacco.

The following study investigates the influence of climate factors on the development of aroma compounds in flue-cured tobacco.

102 samples were taken from five different geographical areas (Changning, Longyang, Tengchong, Longling, Shidian) and profiled by using gas chromatography-mass spectrometry (GC-MS) coupled with multivariate data analyses.

Partial least-squares-discrimination analysis (PLS-DA) showed that samples from five regions could be subdivided into three groups of aroma compounds of flue-cured tobacco, which is in agreement with a cluster analysis of climate and geographical factors. 24 aroma compounds were screened to evaluate possible relationships with climatic factors by comparing the value of variable importance in the projection (VIP).

The amount of degradation products of lutein and ß-carotenoids increased with total sun exposure time and total rainfall. PLS-DA could provide potential indicator aroma compounds which showed significant relationships to climate and geographical factors. This method can provide important information on tobacco plants.

1. *Yunnan Reascend Tobacco Technology (Group) Co., Ltd, No. 1699 Haiyuanbei Road, Kunming National Hi-Technology Industry Zone, Kunming, Yunnan Province, 650106, P.R. China*
2. *Hongyun-Honghe Tobacco (Group) LLC, No. 367 Hongjin Road, Wuhua Zone, Kunming 650231, P.R. China*

YIN Donghong; LUO Wei; DING Duo; CHEN Zeliang; ZHONG Kejun; REN Jianxin

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST23

Effect of pore structure characteristics of cigarette paper on carbon monoxide release in mainstream smoke during cigarette burning process

Two kinds of cigarette papers and a control sample with the same grammage and permeability but different pore structures were designed and prepared. The effect of pore structure characteristics of the cigarette paper on the delivery of carbon monoxide (CO) in the mainstream smoke was investigated. To study the pore structure near the char line, the cigarette papers were baked at a temperature of 250 °C to simulate the charring state near the char line during the burning process. The changes to the diffusivity as well as the permeability of the cigarette papers before and after baking were measured with paper diffusivity and permeability instruments. The pore size distribution between 0.01-200 μm and the pore volume of the cigarette papers before and after baking were detected by Mercury Porosimeter. The cigarette paper pore structure characteristics during pyrolysis completely changed in the burning cone area and incompletely in the area within 2 mm of the char line. This was observed using a Scanning Electron Microscope (SEM). The delivery of CO in the mainstream smoke and sidestream smoke and the temperature distribution of the burning cone were tested by the ISO puffing regime. Compared to the control sample, the diffusivity and permeability of the designed cigarette paper after baking at 250 °C increased significantly. In particular, the pore volume of the cigarette paper in the pore size range from 0.1-8.0 μm increased obviously, which lead to the decrease of CO delivery in the mainstream smoke. According to the pore structure of the cigarette paper and the delivery of CO in the mainstream smoke and sidestream smoke, it was found that the amount of micropores near the cigarette paper char line increased. This benefits CO diffusion from mainstream smoke to sidestream smoke, and therefore the delivery of CO in the mainstream smoke is decreased during the cigarette burning process.

China Tobacco Hunan Industrial Co., Ltd of CNTC, Research and Development Center, No. 426 Laodong Road, Changsha, Hunan 410007, P.R. China

YOSHIDA S.(1); KOBAYASHI K.(2)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST68

Roles of activated benzene species in the formation of polycyclic aromatic compounds in cigarette mainstream smoke

At the CORESTA Congress in 2012, we reported that activated species of monocyclic aromatic hydrocarbons (MAHs), such as benzene, toluene and styrene, formed when phenylalanine applied to tobacco was involved in the formation of 4-aminobiphenyl in cigarette mainstream smoke (MSS).

MAHs were also reported as precursors of various polycyclic aromatic compounds (PACs) in other combustion systems such as a gas combustion reactor (temperature: 1155–1467 K). However, their contribution to the formation of PACs in MSS is still unclear due to lower combustion temperatures inside the burning cone of a cigarette (ca. 870–1170 K). The aim of this study was to investigate the contribution of MAHs to the formation of PACs in MSS. Eight benzenoid PACs (e.g. naphthalene, anthracene), 4 cyclopenta-fused PACs (e.g. 9H-fluorene, fluoranthene) and 4 phenyl-substituted PACs (e.g. biphenyl, 2-phenylnaphthalene) were investigated. The results of PACs showed that the yields of phenyl-substituted PACs in MSS were notably increased by the addition of phenylalanine to tobacco, while those for other PACs did not significantly change. These results indicate that the activated species of MAHs generated from phenylalanine would work as the intermediates for only phenyl-substituted PACs in MSS. Concerning the estimated intermediates for PACs in previous findings, it was proposed that activated alkynyl or cyclopentadienyl species were key intermediates for the growth of PACs structure during cigarette combustion.

1. *Japan Tobacco Inc., 6-2 Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan*
2. *Yokohama National University, Graduate School of Engineering, 79-5, Tokiwadai, Hodogaya-ku, Yokohama 240-8501, Japan*

ZIMMERMANN R.(1); HERTZ-SCHÜNEMANN R.(1); EHLERT S.(1); LIU C.(2); McADAM K.G.(2); BAKER R.R.(2)†; COBURN S.(2); STREIBEL T.(1)

CORESTA Congress, Québec, 2014, Smoke Science/Product Technology Groups, abstr. ST85

Highly time-resolved two-dimensional mapping of molecular combustion and pyrolysis product concentrations: looking into a burning cigarette during puffing

Combustion and pyrolysis are complex and dynamic chemical processes that are by far not fully understood. This is in particular true when the highly dynamic cigarettes smoking process is considered. The gas phase chemistry of tobacco combustion in its voids and pores is extremely difficult to address by conventional analytical methods. In the last years, photo ionisation mass spectrometry (PIMS) was established as a fast on-line analytical technique to analyse the chemical signature of highly dynamic combustion and pyrolysis processes in cigarettes during puffing[1]. Recently a new combination of PIMS (laser based single photon ionisation, 118 nm) and a capillary microprobe sampling system (µ-probe) was developed, allowing direct examination of the composition of organic vapours even in the centre of the cigarette's combustion zone[2]. This µ-probe-PIMS approach now is further developed to a spatial- and temporal resolving mapping method. Repetitive smoking experiments with a reproducible smoke machine and standard reference cigarettes were performed and different sampling positions in the cigarette rod were multiply addressed by µ-probe PIMS measurements. The time resolved PIMS sequences were later combined to spatially resolved, time-dependent "maps" for the different compounds. This new imaging technique was used to measure quantitative distributions of e.g. nitrogen monoxide, benzene and oxygen in the burning tip of a cigarette during a 2 second lasting puff (~200 ms time resolution). The different formation and destruction zones of the investigated compounds in the reaction region and their dynamic changes were observed during the puff, and space-resolved kinetic data was obtained. For example, the classical formation and destruction mechanisms of NO during the puff (fuel-NO formation and re-burn in hydrocarbon rich zones) could be observed in a space- and time-resolved manner. In addition applications of the PIMS technology to better understand heat-not burn or e-cigarette devices are discussed.

[1] Adam, T. et al., *Analytica Chimica Acta*, 657 (2010) 36-44;
[2] Hertz, R., et al., *Analytica Chimica Acta*, 714 (2012) 104-113

1. *University of Rostock, Chair of Analytical Chemistry, Dr.-Lorenz-Weg 1, D-18059 Rostock, Germany*
2. *British American Tobacco, Group R&D, Regents Park Road, Millbrook, Southampton SO15 8TL, U.K.*

† *Deceased*