ABSTRACTS OF PRESENTATIONS MADE AT THE 2012 CORESTA CONGRESS IN SAPPORO, JAPAN

SMOKE SCIENCE AND PRODUCT TECHNOLOGY

(in alphabetical order of first authors)

BACHMANN S.; GLEINSER M.; MÖHRING D.; ROHREGGER I.; VOLGGER D.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. PT16

Puff-by-puff analysis of mainstream smoke constituents of non-LIP/FSC and LIP/FSC cigarettes

Cigarette base paper parameters like permeability and burn additives have a large impact on mainstream smoke yields of cigarettes. Especially CO and potentially also CO_2 and O_2 yields could change by applying LIP/FSC-bands on cigarette papers. Other analytes may show similar trends. Physical band parameters like permeability, diffusivity as well as the band design have an influence on those smoke yields.

The sample set involved cigarettes with permeabilities in a range of 50-130 CU and burn additive levels between 1-2%. Cigarettes have been produced with base papers containing bands with different diffusivities and band designs. The sample cigarettes were smoked according to ISO 3308, with fully blocked filter ventilation. Changes in mainstream smoke yields of CO, CO_2 and O_2 and other analytes will be presented.

A single channel smoking machine (Borgwaldt RM1) and a mass analyser (Airsense Compact) were used for the determination of mainstream smoke yields. A specially designed interface was applied to split a fraction of the mainstream smoke. The smoking machine triggered the interface in such a way that smoke can only enter the mass analyser during a puff. Electron impact ionisation was used for detecting CO_2 and O_2 whereas CO and other analytes were determined by ion molecule reaction ionisation.

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

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

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

BAO M.; WU J.; RICKERT W.; MASTERS A.; JOZA P.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SS18

Analysis of heterocyclic aromatic amines in mainstream cigarette smoke condensate by solid phase extraction and ultra-performance liquid chromatography tandem mass spectrometry

A rapid and sensitive method to measure the levels of twelve heterocyclic aromatic amines (HAAs) in mainstream tobacco smoke condensate has been developed. Mainstream smoke collected onto a glass fibre filter pad is extracted into 0.1M of hydrochloric acid (HCl), with isotope-labelled HAAs added as internal standard (IS). Sample matrix effects are reduced by solid-phase extraction (SPE) using a mixed mode (non-polar and cation exchange) polymeric sorbent. The purified extract is analyzed using an ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) system operating in selected reaction monitoring (SRM) mode. The combination of the SPE and UPLC system provided sensitivity improvements compared to other published LC-MS/MS methods. Most of target HAAs can be analyzed at levels below 0.5 ng/cig.

For the HAAs investigated, average recoveries obtained from spiked reference cigarette smoke matrices ranged from 100 to 118% with relative standard deviations (RSDs) under 14.5%. The precision of the method was evaluated by analyzing HAAs in the mainstream smoke of Kentucky Reference cigarette 3R4F smoked under two smoking regimens: ISO (35mL puff volume, 60 second puff frequency, no vent blocking) and Health Canada Intense (55mL puff volume, 30 second puff frequency, 100% vent block). Seven of twelve target HAAs including IQ, norharman, harman, Trp-P-1, AaC, Trp-P-2, and MeAaC, were detected in mainstream smoke of 3R4F cigarettes. The RSD values obtained were below 10.1% for norharman, harman, AaC, MeAaC, and ranged from 9.8 to 20.0% for IQ, Trp-P-1, Trp-P-2. The levels of Glu-P-1, MeIQ, Glu-P-2, MeIQx, and PhIP in mainstream smoke of 3R4F cigarettes smoked under both smoking conditions were all below the method detection limit.

Yields of HAA's determined by this method are in agreement with those obtained using a validated GC/MS method and the published LC-MS/MS method.

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

BITO R.; MINAMI N.; KAKEHI A.; YUKI D.; ONOZAWA M.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT18

Estimation of mouth level smoke exposure in cigarettes with different tar content using filter analysis

Filter analysis for evaluating the mouth level exposure (MLE) of smokers is non-invasive, allowing ordinary smoking behaviour with simple sample collection. We investigated 1) estimated MLEs to 36 smoke constituents using filter analysis, 2) MLEs of Japanese smokers smoking cigarettes with different tar content, and 3) the relationship between their MLEs and index values obtained by machine smoking.

A low tar cigarette (LTC, 6 mg ISO tar) and an ultra-low tar cigarette (ULTC, 1 mg ISO tar) were smoked using a smoking machine (18 regimes). The nicotine content in the filter butts and 36 constituents such as tar, nicotine, and CO were measured. Except for formaldehyde, a strong correlation was found between the filter nicotine content and each of the 35 constituents in both cigarettes (R^2 = 0.78 - 0.99). This result shows that the MLEs to 35 constituents could be estimated from quantification of the nicotine content in filters.

Japanese male smokers (LTC smokers: 105, ULTC smokers: 105) aged 21-49 years were recruited. Subjects were permitted to smoke their regular use cigarettes *ad libitum* for 7 hours in the examination site. After collection, the nicotine content in each filter was measured to estimate the MLEs. The estimated MLEs to 35 constituents in ULTC smokers were significantly lower than in LTC smokers.

The relationships between the estimated MLE to each constituent from LTC and ULTC smokers and five index values obtained by machine smoking (ISO and Canadian Intense) – "each constituent", "tar", "nicotine", "each constituent/tar", and "each constituent/nicotine" – were evaluated. The results showed that relationships varied among the indexes.

Japan Tobacco Inc., R&D Group, Product Science Division, 6-2 Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan

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

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST16

THEME-SEER: Multidimensional exploratory technique to explore the generation process of smoke compounds

In 2011, we presented at the 2011 CORESTA SSPT Meeting (Graz)[1] a new approach to path modelling based on system extended multiple covariance criteria. We showed why THEME-SEER may be preferred to current methods using either criteria based on covariance of components (such as Partial Least Square Path Modelling) or based on residual sums of squares (such as Generalised Structured Component Analysis). In 2012, we described the main theoretical characteristics (system extended multiple covariance, model local nesting, maximisation programme and algorithm and convergence properties) relating to the THEME-SEER method[2].

We propose now to describe in more details the results obtained from the application of the THEME-SEER model to cigarette data. The aim is to model smoke component yields from 52 physical and chemical variables describing 19 cigarettes. The variables are partitioned into seven thematic groups, linked through two structural model equations.

- Equation 1 predicts smoke yields under the intense smoking regime from the characteristics of the cigarette.
- Equation 2 establishes the relationship between smoke yields derived under the ISO smoking regime and those under the intense smoking regime and the effect of filter ventilation.

A description of the complementary effects of tobacco composition, tobacco type, combustion chemical enhancers or inhibitors, filter retention and ventilation on smoke yields will be presented.

[1] Bry X.; Redont P.; Verron T.; Cahours X. Using a Structural Model based on a Class of Generalised Covariance Criteria, to explore the generation process of smoke compounds. Meeting Smoke Sci.-Prod. Techno Groups, Graz, 2011, abstr. ST 24
[2] Bry X.; Redont P.; Verron T.; Cazes P. THEME-SEER: a multidimensional exploratory technique to analyze a structural model using an extended covariance criterion. *J. Chemometrics*, 2012

1. *I3M, UM2, Place Eugène Bataillon, 34095 Montpellier, France*
2. *SEITA, Imperial Tobacco Group, 48 rue Danton, 45404 Fleury-les-Aubrais, France*

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

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SS10

Comparison of analytical data provided by different laboratories

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

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

[1] ISO5725-6, 2001. Accuracy (trueness and precision) of measurement methods and results. Part 6. Use in practice of accuracy values.
[2] M. Intorp, S. Purkis, M. Whittaker and W. Wright, 2009. Determination of Hoffmann Analytes in cigarette mainstream smoke. The CORESTA 2006 Joint Experiment. *Beiträge zur Tabakforschung Int.* 23, 161-202

1. *SEITA, Imperial Tobacco Group, 48 rue Danton, 45404 Fleury-les-Aubrais, France*
2. *Imperial Tobacco Limited, PO Box 525, Winterstoke Road, Bristol BS99 1LQ, UK*

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

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT34

Product compliance mapping

The joint working group of the WHO Study group on Tobacco Product Regulation (TobReg) have recommended a list of selected toxicants for regulation: N'-nitrosonornicotine (NNN); 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK); acetaldehyde; acrolein; formaldehyde; 1,3-butadiene; benzene; benzo[a]pyrene (B[a]P) and carbon monoxide (CO). TobReg has also recommended ceilings to be established on the levels of these nine selected constituents per milligram of smoke nicotine and prohibition of sales or imports of cigarette brands on the highest levels (typically above some factor related to the median). In our study, smoke constituents have been measured for different cigarette types using our own in-house testing methodologies. The yield values expressed per milligram of nicotine obtained with intense smoking conditions have been compared to the ceilings proposed by WHO and a multivariate analysis has been conducted and represented as a map. This map provides a simple and innovative visual representation of the number of exceeded ceilings per product and a risk acceptability based on tolerance (method variability or arbitrary variation). The conclusion of our study highlights that i) it is fundamental to take into account the measurement variability before deciding whether a product is compliant or non-compliant; ii) an approach based on multiple independent ceilings would affect the vast majority of cigarette types.

1. *SEITA, Imperial Tobacco Group, 48 rue Danton, 45404 Fleury-les-Aubrais, France*
2. *Imperial Tobacco Limited, PO Box 525, Winterstoke Road, Bristol BS99 1LQ, UK*

CAMACHO O.M.; PRASAD R.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SS09

Data standardisation: examining its applicability to the tobacco science

Many industries, for example the pharmaceutical industry, have made efforts towards data standardisation. For regulated industries, the requirement for exchanging data with regulatory agencies has made data standards an essential part of data handling processes. Regulatory authorities have different requirements for the submission of data for evaluation. The Japanese Ministry of Health, Labour and Welfare requires the use of MedDRA/J for clinical data. In the USA, the FDA has published recommendations for data submission which have encouraged organisations to implement data standards such as those proposed by the Clinical Data Interchange Standards Consortium (CDISC).

Data standards are a set of rules guaranteeing interoperability, exchangeability and reusability of data. For example, controlled terminology for data entries and the agreement of formats promotes semantic and functional interoperability, which translates into more efficient processing of data and enhanced sharing capability. Data can be collected as result of processes. When the processes are recorded they also become data with their corresponding administrative aspects. Data are exchanged within institutions and between organisations with more data being generated. In the absence of data standards, organisations are likely to suffer time-consuming and expensive data transformations, increasing the probability of errors.

Implementation of standardisation models in tobacco science would promote transferability in tobacco-related research and also give assurance over conclusions yielded by analyses of these data. Standardisation would also support meta-analyses, given that data standards promote clarity of data set contents. Standardisation of formats improves the efficiency for merging datasets and preparation of data for analysis. In light of this, we have developed a Data Dictionary containing almost 3000 frequently-used terms. In conjunction with rules for data formats and structures, this constitutes our first steps towards data standardisation. We will describe this dictionary and discuss the use and applicability of a wider-scale approach across tobacco research.

British American Tobacco, Group R&D, Regents Park Road, Southampton SO15 8TL, UK

CAO Yi; ZHUANG Ya-dong; ZHU Huai-yuan; WAN Min; LIU Xian-jun; SHEN Xiao-chen; ZHANG Yuan; ZHANG Ying; XIONG Xiao-min

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT21(Poster)

Analysis of stimulation on oral cavity caused by three kinds of volatile compounds in mainstream cigarette smoke

Cigarette smoke stimulation is mainly evaluated by sensory evaluation. In order to analyse smoke stimulation on the oral cavity qualitatively and quantitatively, a set of oral simulators was designed by taking three kinds of stimulating compounds (volatile carbonyl compounds, ammonia and volatile phenolic compounds) in mainstream cigarette smoke as research objects. The relationship of these stimulating compounds in the mainstream smoke of different type cigarettes with the stimulation on tongue and palate in oral cavity was analysed by regulating the moisture content in cut tobacco. The results showed that: 1) The stimulation on tongue and palate caused by main volatile carbonyl compounds in mainstream smoke was basically similar, while ammonia and volatile phenolic compounds provided stronger stimulation on the tongue than on the palate; 2) With the decrease of moisture content in cut tobacco, the contents of main volatile carbonyl compounds and phenolic compounds in mainstream smoke and their stimulation on the oral cavity increased; 3) The stimulation caused by ammonia in mainstream smoke was dependent on cigarette type, with the decrease of moisture content in cut tobacco, the content of ammonia in Virginia type cigarette and its stimulation reduced, while the content of ammonia in blended type cigarette and its stimulation increased.

Research and Development Center of China Tobacco Jiangsu Industrial Co., Ltd., No. 28, Mengdu Road, Nanjing 210019, Jiangsu, P.R. China

CHEUNG J.; KRIMMER-QUENDLER S.; WOLF J.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST33

A comparison between different tobacco product ingredient reporting systems worldwide – experiences from practical use in key countries

Much debate and research has taken place on what and how to generate data in order to satisfy requirements for tobacco manufactures to disclose product information to regulatory authorities. Rather less attention has been paid to the mechanisms by which this data can be provided to regulators in a credible and accessible form. Furthermore, Article 10 of the WHO FCTC is likely to propose that regulators make such data available to the public, raising concerns of misinterpretation of data, and that trade secrets and other confidential information may become commonly available. This presentation compares and contrasts systems currently in use in key communities worldwide (Canada, USA, Taiwan and the European Union) in terms of their reporting requirements, utility to both regulator and industry and protection of trade secrets. Finally the EMTOC (Electronic Model Tobacco Control) system is described. EMTOC has already been accepted for use in several European countries, allowing the industry to satisfy its reporting obligations through a system which is straightforward to use and provides suitable formats for public disclosure.

Table 1: Tobacco Product Ingredient Reporting Systems currently in use in key countries.

	Canada	*US*	*Taiwan*	*EU*
Level of Product Information	high	high	high	high
Frequency of Reporting	quarterly	baseline	annually	annually
Reporting System	paper	eSubmitter	BHP System	EMTOC
Trade Secret Protection	low	medium	low	high
Publication of Data	no	no	yes	yes
Utility of the System	low	medium	low	good

JT International S.A., 1 rue de la Gabelle, CH-1211 Geneva 26, Switzerland

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

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT08

Cigarette burning and alternative smoking regime

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

The calculated number of puffs has been compared to the measured values from a ventilated cigarette smoked under 32 different smoking regimes, with filter ventilation holes blocked or opened. The close values of the measured and calculated number of puffs have validated the proposed model.

Several parameters can be deduced from this approach such as the length and weight burnt during the puffs or the burning time. The results compare well with other published models.

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

1. *Imperial Tobacco Limited, PO Box 525, Winterstoke Road, Bristol BS99 1LQ, UK*
2. *SEITA, Imperial Tobacco Group, 48 rue Danton, 45404 Fleury-les-Aubrais, France*

COULOT A.; RAJNOCH C.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST20

A demonstration of effective ventilation in a designated smoking area

Measurements were made to assess the efficacy of a ventilation system in removing tobacco smoke from a newly installed designated smoking area (DSA) and in preventing the migration of smoke to an adjacent non-smoking area in a café. Prior to the installation of the DSA, a computer simulation was employed to select ventilation parameters and system layout, including air flows in the non-smoking and smoking areas. These air flows were confirmed after installation by the use of visual smoke tests.

Air samples were then collected on three consecutive days, at three different times, in the DSA and in the non-smoking area of the café simultaneously during normal opening times. These samples were analysed for the specific environmental tobacco smoke (ETS) markers nicotine, 3-EP and solanesol; and for the semi-specific particle markers PM2.5, PM5 and PM10. Measurements were also taken outdoors and in a local sports centre as references. The number of smokers, CO levels, temperature and humidity were also recorded in all locations.

Concentrations of the specific ETS markers were low in all environments (generally at or below the analytical limit of quantification) demonstrating the efficacy of the ventilation system in removing tobacco smoke from the DSA. Higher concentrations of semi-specific markers were found in both the DSA and non-smoking areas, probably reflecting sources additional to tobacco smoke such as combustion products from the cooking area. A comparison of the current results with published data on both specific and non-specific ETS markers shows that the levels in this study are amongst the lowest recorded in smoking areas.

JT International S.A., 1 rue de la Gabelle, CH-1211 Geneva 26, Switzerland

COVERLY S.(1); SHI Tingchuan(1); ZHANG Wei(2); WANG Ying(2); TANG Gangling(2)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST32

Safer nicotine analysis: proposed changes to CRM 35, total alkaloids in tobacco leaf

The most commonly used automated method for leaf nicotine analysis is CORESTA Recommended Method N° 35. This is based on the principle of continuous-flow analysis, using dialysis to remove interference from coloured leaf components followed by a colorimetric determination of alkaloids. The colour-producing reaction requires cyanogen chloride, CNCl, which is generated *in situ* from Chloramine T and KCN. As a result, considerable safety precautions are needed when preparing reagents and running the method.

In 2009 the Routine Analytical Chemistry Sub-Group considered a proposal for a safer nicotine method which generates the CNCl from NaOCl and KSCN. No special precautions are needed when handling the potassium thiocyanate. An inter-laboratory test was performed in 2010, with satisfactory results.

In 2010 Mehta *et al.*[1] proposed an improved procedure that replaces NaOCl with dichloroisocyanurate ("DCIC"), which has the advantage of providing a more stable chlorine source than NaOCl. We have compared this method with CRM 35 and the NaOCl / KSCN procedure in one internal and two international inter-laboratory tests, and here present the results.

[1] Mehta S.K.; Rajesh B.J.; Dhalewadikar S.V. – CORESTA Congress, Edinburgh, 2010, Smoke Science / Product Technology Groups, abstr. SSPT19

1. *SEAL Analytical GmbH, Werkstr. 5, 22844 Norderstedt, Germany*
2. *China National Tobacco Quality Supervision & Test Center, Zhengzhou, P.R. China*

CUNNINGHAM A.; SOMMARSTROM J.; ASHLEY M.; CAMACHO O.M.; McEWAN M.; PRASAD K.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT20

A longitudinal study to track changes in long-term smoking behaviour of smokers of 10 mg ISO tar cigarette in Germany

Many studies have reported the effects of short-term switching on smoking behaviour. However, few studies have been conducted recently involving the long-term monitoring of cigarette consumption, mouth level exposure to nicotine and tar, biomarkers of exposure for nicotine and other smoke constituents and in particular spontaneous cigarette brand switching.

Therefore, an ambulatory longitudinal study (clinical trial number ISRCTN95019245) comprising over 1000 smokers of the same commercial 10 mg ISO tar cigarette is being conducted in Germany to monitor smoking behaviour for up to five years, with six-monthly follow-ups.

Baseline data were presented at the 2010 CORESTA Congress (Edinburgh). The data presented here includes up to the third time point.

Results for over 580 subjects who have continued to smoke the original product will be presented for:

- Daily total urinary nicotine equivalents
- Daily mouth level exposure to nicotine
- Salivary cotinine
- Daily cigarette consumption

These data show that smokers who remained smoking their original cigarettes at all three time points showed consistent data for cigarette consumption, daily mouth level exposure to nicotine and salivary cotinine, with variation observed in daily total urinary nicotine equivalents.

Additional demographic overview data will be presented for those subjects who have switched to other cigarettes. Over 50 smokers had switched sideways to other 8-10 mg ISO tar cigarettes and over 60 smokers had switched to other cigarettes with lower tar yields (ISO tar 3-7 mg/cigarette) at time point three.

The main effects of product switching on the study variables will be presented analysed with a mixed model regression to account for repeated measures.

British American Tobacco, Group R&D, Regents Park Road, Southampton SO15 8TL, UK

DAVID F.(1); DUMONT E.(1); TIENPONT B.(1); MITSUI K.(2); OCHIAI N.(3); KANDA H.(3); HIGASHI N.(2); SANDRA P.(1)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. PT08

Heart-cutting two-dimensional GC in combination with isotope ratio mass spectrometry for the characterisation of lipids in tobacco leaves and smoke

During the last few years, interest in isotope ratio mass spectrometry (IRMS) has increased tremendously. IRMS is used for the determination of the origin/source of products and for adulteration detection. Application areas include natural product research, forensic and doping analysis (detection of endogenous *versus* exogenous origin of solutes).

Although IRMS is still often used for bulk analysis, the combination of IRMS with chromatographic separation is gaining a lot of attention, resulting in compound-specific isotope ratio determinations. Resolved solutes are directed towards a combustion oven for carbon or nitrogen isotope ratio determination or to a high temperature conversion oven for hydrogen or oxygen analysis.

GC-IRMS allows the analysis of more complex mixtures of solutes, but in order to successfully measure isotope ratios, isolation of target solutes is necessary. For this reason, the use of two-dimensional GC offers an important advantage compared to one-dimensional GC.

In this presentation, we will demonstrate a new configuration for GC-IRMS applying capillary flow technology and low thermal mass GC. As tobacco leaves and smoke are highly complex samples, these samples are very interesting for the evaluation of the two-dimensional GC-IRMS set-up. At first, the focus will be on the lipid fraction of these samples. Some data on isotope ratios obtained for tobacco leaves and their smoke analogues will be discussed.

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

DAVID F.(1); TIENPONT B.(1); MITSUI K.(2); OCHIAI N.(3); SASAMOTO K.(3); HIGASHI N.(2); SANDRA P.(1)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SS14

Applying gas chromatography with soft ionisation mass spectroscopy for the characterisation of tobacco leaves and tobacco smoke

In this presentation, the applicability of GC in combination with atmospheric pressure chemical ionisation mass spectroscopy (APCI-MS) and with supersonic molecular beam ionisation mass spectroscopy (SMB-MS) will be demonstrated for the characterisation of tobacco leaves and tobacco smoke. Both ionisation techniques are compatible with high temperature GC, extending the GC application range to high molecular weight solutes.

These soft ionisation techniques are especially useful for the identification of important apolar compounds, such as waxes, sterols, sterol esters and lipids. Using classical electron impact ionisation, strong fragmentation is obtained for these solutes, making unequivocal identification difficult. Soft ionisation in combination with accurate mass MS and/or software tools allowing molecular formula generation (*e.g.* Mass Works, Tel Aviv IAA) offers complementary information that helps to unravel the complexity of tobacco leaf and smoke composition.

Also in the field of metabolomics interesting results are obtained. For the analysis of polar fractions in biological materials (containing amino acids, sugars, etc.), silylation is often applied prior to GC-MS analysis. However, fragmentation in EI-MS mostly leads to non-characteristic fragment ions, making identification of possible biomarkers difficult. GC-APCI-MS and GC-SMB-MS can be used alternatively, resulting in easier feature extraction and identification.

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

DEGER A.; JACCARD G.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. PT07

Improved methods for the determination of crop protection agent residues in tobaccos and tobacco products by LC-MS/MS using the QuEChERS extraction

We will present two improved methods to determine Crop Protection Agent (CPA) residues in tobaccos and tobacco products and discuss their development and validation. The methods are applicable to 53 of the 118 CPA recommended in the CORESTA Guide N° 1[1]. The previous methods used a solid phase extraction followed by GC-MS/MS and LC-MS/MS. The new methods use a modified QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe)[2] extraction followed by LC-MS/MS. The validation was performed with spiked tobaccos (mixture of Burley and Virginia) with increasing levels of analytes. Results indicate that the methods are in line with the recommendations of the CORESTA Guide N° 5[3] in terms of recovery and relative standard deviation. The limits of quantification were below the respective ACAC Guidance Residue Limit for all substances. The main advantages of the new methods over the older methods are: reduced workload, lower consumption of solvents and supplies, good recovery rates obtained for a wide range of CPAs.

Method 1: Identification and measurement of 50 CPAs. Samples are prepared for analysis by 1) extraction of aqueous sample with acetonitrile; 2) a buffer salt induced phase separation; 3) a dispersive solid phase extraction in the acetonitrile phase. Quantitative analysis is performed by LC-MS/MS.

Method 2: Identification and measurement of three CPAs (2,4-D; Dicamba and 2,4,5-T). Due to their acidic nature the QuEChERS extraction is modified. Samples are prepared for analysis by 1) pH adjustment; 2) extraction of aqueous sample with acetonitrile; 3) a buffer salt induced phase separation. Quantitative analysis is performed by LC-MS/MS.

[1] CORESTA Guide N° 1: The Concept and Implementation of Agrochemical Guidance Residue Levels
[2] Anastassiades *et al.*, *Journal of AOAC International* 2003 86(2): 412-431
[3] CORESTA Guide N° 5, Technical Guideline for Pesticide Residues Analysis on Tobacco and Tobacco Products

Philip Morris International Management SA, Product and Process Quality, Quai Jeanrenaud 3, 2000 Neuchâtel, Switzerland

DRAKE L.; COCKAYNE L.; McADAM K.G.; McAUGHEY J.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT09

Is the 44 mm Cambridge filter pad suitable for trapping total particulate matter under ISO and Health Canada Intensive regimes?

Cambridge filter pads (CFP) are used for the collection of total particulate matter (TPM) in cigarette smoke and ISO standard 4387:2000 suggests a TPM limit of 150 mg per 44 mm CFP.
Machine smoke yields of current cigarettes are much lower than those when methods were originally developed with the consequence that more cigarettes are needed to produce an ISO TPM yield of 150 mg. The effectiveness of the CFP in these circumstances does not appear to have been assessed.

To characterise the trapping efficiency of the CFP with contemporary cigarettes and smoking regimes, the capability of 44 mm CFPs to trap smoke up to and beyond the current ISO standard limit has been examined.

Incremental numbers of 1R5F and 3R4F University of Kentucky reference cigarettes and three commercial brands were smoked onto separate 44 mm CFPs to obtain a range of TPM from approximately 50 to 400 mg/pad under ISO and Health Canada Intense (HCI) conditions. Puff number ranged from 40 to 1300 per pad.

Initial findings under the ISO smoking regime smoking showed that the trapping efficiency of the pad was constant up to 150 mg/pad. Above the ISO limit, the per-cigarette yields of TPM and water were found to reduce. In addition, the deviation from the standard ISO yield was greater for the lower yielding 1R5F cigarette than that of the 3R4F product. Under the HCI regime this trend was not observed for TPM, and was inconclusive for water.

In conclusion, this work has shown that, the capacity of the CFP is different when products are smoked under ISO and HCI smoke regimes. The losses, in particular water, that occur at the higher pad loadings under ISO smoking may be correlated to the total number of puffs, and the puff volume, taken during the smoking process.

British American Tobacco, Group R&D, Regents Park Road, Southampton SO15 8TL, UK

DUROT N.; ROUILLARD S.; RAVERDY-LAMBERT D.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT12

Reconstituted tobacco composition: fractionation of components between soluble and fibre fractions

The two step papermaking reconstitution is a well-known process to reshape raw materials that are unusable directly in the cigarette, generated during tobacco harvesting and cigarette manufacturing. An essential step of this process is the temporary fractionation of tobacco materials into a water soluble fraction and a fibrous fraction, which potentially allows the application of treatments to reduce certain smoke harmful constituent precursors.

The purpose of this presentation is to give an overview of the reconstituted tobacco composition variation, particularly the chemical components distribution between the fibre and soluble fractions.

Main components of reconstituted tobacco are: cellulose, hemicelluloses, lignin, proteins, pectins, organic acids such as malic acid and citric acid, sugars as glucose, fructose, saccharose, maltose, minerals such as potassium, calcium, nitrate, chloride, free amino acids, alkaloids, polyphenols such as chlorogenic acid and rutin, with a variable distribution depending on raw materials blending. Low amounts of minor alkaloids, terpenes, metals, carotenoids are also present.

Cellulose, hemicelluloses, lignin are recovered in the fibre fraction whereas proteins, pectins, and terpenes are split between both fractions with different ratios. Highly methylated pectins are mainly recovered in the soluble fraction whereas low methylated pectins and protopectins are observed in the fibrous one.

Sugars, are higher in Chinese flue-cured tobacco-solubles, lower in Oriental blends. Minerals in soluble fraction vary between 15% and 29% with a higher amount of potassium, nitrate in blended tobacco, and lower amounts of potassium, nitrate and chloride in Oriental blends.

Polyphenols, mainly recovered in tobacco solubles, are higher in Chinese flue-cured and Oriental tobacco blends. Alkaloids are mostly found in the soluble fraction. Total amount of free amino acids are similar in the various tobacco blends, but lower aspartic and glutamic acids, higher tryptophan contents are observed in flue-cured soluble fraction. High recovery of organic acids in solubles is observed, particularly malic acid. Metals (cadmium, mercury, selenium) are distributed between the soluble and fibrous fractions, except chromium which is mainly observed in fibres, and arsenic in the soluble fraction.

SWM International, LTR Industries, Usine le Mans, 72702 Allonnes Cedex, France

ECKER J.; STERZ K.; SCHERER G.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT27

Lipids and mediators as smoking related biomarkers of effect

Eicosanoids are key mediators and regulators of inflammation and oxidative stress involved in numerous physiological and pathophysiological processes. Precursors are polyunsaturated fatty acids liberated from membrane phospholipids. Many eicosanoid species are frequently used as biomarkers for diseases like cardiovascular disease (CVD), diabetes, cystic fibrosis (CF), multiple sclerosis, neurodegenerative diseases, rheumatoid arthritis, various forms of cancer and also for smoking.

Analytically, comprehensive and robust quantification of different eicosanoid species in a multi-method approach is problematic because most of these compounds are relatively unstable and may differ in their chemical properties. Here we describe a novel ultra-performance liquid chromatography-selected reaction monitoring mass spectroscopy (UPLC-SRM/MS) method for simultaneous quantification of key urinary eicosanoids, including the prostaglandins (PG) tetranor PGE-M, 8-iso-, and 2,3-dinor-8-iso-PGF_2; the thromboxanes (TXs) 11-dehydro- and 2,3-dinor-TXB_2; leukotriene E_4; and 12-hydroxyeicosatetraenoic acid (HETE). The method was validated and applied to human urine samples showing excellent precision, accuracy, detection limits, and robustness (Sterz *et al.*, J Lipid Res, 2012).

In a further application of the novel method we demonstrate that smoking affects urinary levels of tetranor PGE-M; 2,3-dinor- and 8-iso-PGF_2; 2,3-dinor-TXB_2. Importantly, urinary concentrations of these metabolites have also been found elevated in patients with cancer (*e.g.* lung cancer), diabetes or neurodegenerative diseases. Finally, we present data proposing that a combined profiling of eicosanoids and precursors/related molecules might be the most promising approach to evaluate inflammation related effects of smoking on human health.

ABF Analytisches Biologisches Forschungslabor GmbH, Goethestrasse 20, 80336 Munich, Germany

EITZINGER B.(1); COBURN S.(2); CASE P.(3)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. PT15

Comparison of two maximum-likelihood estimators of the rate of self-extinguishment in the ISO 12863 test

To check the compliance of cigarettes with low ignition propensity regulations, it is necessary to estimate the rate of self-extinguishment a cigarette will have, when tested according to ISO 12863. One way to do this is to actually perform the test according to ISO 12863, but the test suffers from a high variance. As a consequence high limits for the rate of self-extinguishment are set internally to safeguard against accidental non-compliance due to experimental variation. This leaves less room for adequate free-burn performance.

As an alternative a maximum likelihood estimator based on residual length data is proposed and compared theoretically and experimentally with a maximum likelihood estimator based on the test results of ISO12863. The theoretical results show that both estimators are unbiased, but the estimator based on residual length data has less than 25% of the standard deviation of an estimator based on ISO12863 data. For small sample sizes simulations show that the estimator based on residual length data provides an estimate closer to the true rate of self-extinguishment at a variance comparable to that of an estimator based on ISO 12863 data. This agrees with experimental data based on 38 non-banded cigarette designs with three to five replicates of the ISO 12863 test done on 40 cigarettes on 10 layers of filter paper. The results are primarily applicable to non-banded cigarettes, but it is discussed how the concept can be extended to banded cigarettes.

The results provide a precise estimate of the rate of self-extinguishment with less experimental effort and may help to get adequate free-burn performance without compromising legal compliance.

1. *delfortgroup AG, Fabrikstrasse 20, 4050 Traun, Austria*
2. *British American Tobacco, Group R&D, Regents Park Road, Southampton SO15 8TL, UK*
3. *Consultant to delfortgroup AG, Fabrikstrasse 20, 4050 Traun, Austria*

FARMEN R.H.(1); NEWLAND K.E.(2)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SS08

Fit for purpose bioanalytical validation and sample processing

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

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

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

1. Celerion, Lincoln, NE 68502, USA
2. Celerion, Zürich, Switzerland

FIELDS W.R.(1,2); LIEDTKE W.(2); LI J.(2); BOMBICK B.R.(1)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SS05

Evaluation of a panel of potential gene biomarkers in two *in vitro* lung models following exposure to tobacco smoke and extracts

Lung cells are frequently used in *in vitro* studies to assess the impact of cigarette smoke on defense and repair mechanisms of the lung. Previously, we identified a panel of fifteen genes representing inflammation, oxidative stress, metabolism and cellular growth regulation that were common *in vitro* to human *ex-vivo* lung comparisons.

The objective of this study was to evaluate the acute response of the panel of genes following exposure to tobacco smoke and tobacco extracts in two *in vitro* lung cell models – normal human bronchial epithelial (NHBE) and nuclear related factor 2 (Nrf2) luciferase/oxidative stress reporter cells. NHBE cells were initially evaluated following exposure to whole smoke from Kentucky Reference 3R4F (K3R4F) cigarettes *via* quantitative reverse transcriptase/polymerase chain reaction (QRT/PCR). Inflammatory responses were further assessed by multiplex cytokine analyses of the conditioned media. Subsequently, both models were exposed to total particulate matter (TPM) from K3R4F cigarettes and three smokeless tobacco extracts (STEs) from moist snuff, dry snuff and a smokeless tobacco blend. Gene expression and Nrf2 promoter regulation were then assessed *via* QRT/PCR and luciferase reporter assays.

Compared to air controls, whole smoke exposure induced gene expression levels that reached 5-fold (metabolism), 9-fold (inflammatory) and 20- to 100-fold (stress response). Elevated cytokine release confirmed the inflammatory indicators suggested by the mRNA changes. TPM exposure elicited gene expression responses $\geq$ 2-fold in the panel of genes in both NHBE (9 genes) and Nrf2 parental (BEAS2b) cells (7 genes). However, the targets were minimally impacted by the STEs as shown by $\leq$ 2-fold modifications of 14 of 15 of the targets. K3R4F TPM induced ~ 20-fold increase in Nrf2-regulated luciferase activity compared to the vehicle control while the STEs modulated the Nrf2 promoter $\leq$ 2-fold.

Collectively, the data indicate that the gene panel represents putative biomarkers of effect. The genes were responsive to tobacco exposures from multiple formats, were differentially regulated by combustible and non-combustible tobacco exposures and may further our understanding of the biological relevance of responses in *in vitro* models.

1. *R.J. Reynolds Tobacco Co., R&D Department, PO Box 1487, Winston-Salem, NC 27102, USA*
2. *Integrated Toxicology and Environmental Health Program, Duke University, Durham, NC 27710, USA*

FUJIMOTO H.(1); OKUBO C.(1); OZAKI H.(1); TSUJI H.(1); NISHINO T.(1); LEE K.M.(2); YOSHIMURA H.(1)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST01

Report on biological researches using a heated cigarette.
Part 1: *In vivo* inhalation and skin painting study

The heated cigarette (HC) generates mainstream cigarette smoke (MCS) primarily by vaporising compositions in the tobacco rod using a carbon heat source at the cigarette tip. Consequently, MCS of HC contains markedly less chemical constituents compared to combusted cigarettes. In this study, MCS from a non-ventilated HC (nvHC) was generated under a modified Canadian Intense Regimen and its biological activities were compared to those of Kentucky reference cigarette (3R4F), using a series of nose-only inhalation and skin painting testing. In a 5-week inhalation study, female SD rats were exposed to MCS of either cigarette at 600 or 1000 wet total particulate matter (WTPM) µg/L for 1 hr, 2 times/day, 7 days/week, 5 weeks. Pulmonary inflammation was significantly weaker in nvHC groups compared to 3R4F groups, based on the neutrophil counts and deviation enzyme levels in bronchoalveolar lavage fluid (BALF). In a 13-week inhalation study, male and female SD rats were exposed to MCS from each cigarette at 200, 600, or 1000 WTPM µg/L for 1 hr/day, 7 days/week, 13 weeks. Histopathological evaluation of the respiratory tract showed significantly lower incidence/severity in nvHC groups, especially on respiratory epithelium hyperplasia and accumulated pigmented macrophage in alveoli. In a 30-week skin painting study with application on the back skin of female SENCAR mice, first a single application of 7,12-dimethylbenz[a]anthracene (an initiator) was made, followed by MCS condensate prepared from either cigarette, up to 3 times/week for 29 weeks: the doses were 3.75, 7.5, 15, 22.5, and 30 mg "tar"/application. Tumour latency was prolonged and dermal tumour incidence and multiplicity were significantly lower in nvHC groups. In conclusion, nvHC demonstrated clear and significantly lower biological activities compared to 3R4F, based on the BALF parameters, histopathological findings of respiratory organs, and dermal tumorigenicity.

1. *Japan Tobacco Inc., R&D Group, Product Science Division, 6-2 Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan*
2. *JT International S.A., Scientific and Regulatory Affairs, 1 rue de la Gabelle, CH-1211 Geneva 26, Switzerland*

FUKUSHIMA T.; TSUJIMOTO T.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST05

Chemical and biological characterisation of mainstream smoke generated from commercial cigarettes available on the Japanese market

Mainstream cigarette smoke (MCS) is a complex mixture of more than 5000 compounds including toxicologically relevant constituents such as tobacco specific nitrosamines, polycyclic aromatic hydrocarbons, aromatic amines, carbonyls and phenolic compounds. Various *in vitro* and *in vivo* studies have indicated that MCS may also contribute to a wide variety of potentially toxic and biologically relevant responses. Meanwhile, it has also been reported that the chemical composition and *in vitro* biological activity of MCS varies with smoking regime and cigarette specification. Cigarette products marketed in Japan have a variety of characteristics including diverse blend types, charcoal or non-charcoal filter and a wide range of tar yields.

The aim of this study is to investigate the chemical and biological characteristics of commercial cigarettes available on the Japanese market. MCS from three reference cigarettes (Kentucky 1R5F and 3R4F, and CORESTA Monitor 6) and 15 commercially available cigarettes (tar range from 1 to 21 mg), which included a heated cigarette, were generated according to ISO and Canadian Intense smoking regimes. Generated MCS were subject to quantitative constituent analysis in accordance with those specified in the Canadian Regulations and analysed according to the *in vitro* assays recommended by CORESTA In Vitro Toxicity Task Force. (Ames, Micronucleus and Neutral red uptake assay).

Results revealed that the magnitude of mutagenic activity associated with total particulate matter (TPM) in the Ames assay as well as the quantity of aromatic amines in MCS (per TPM weight unit) had a wide distribution. This was in contrast to what was observed in the other assays. Blend type and tar yield with filter ventilation also had an impact on the magnitude of mutagenic response.

Notably, a drastic reduction in MCS chemical composition and associated *in vitro* biological activity was observed for the heated cigarette in comparison with all combusted cigarettes.

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

FURUKAWA C.; YOSHII H.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT05

Transfer of stable cesium isotope from cigarette into smoke

It is well known that tobacco contains various kinds of trace metals such as Ca, K and Mg and that some of these metals transfer to smoke when tobacco is smoked. On the other hand, only few data is publicly available with regard to transfer of Cs, one of the trace metals in tobacco, into tobacco smoke. The purpose of this study was to investigate the fate of ^{133}Cs, which is naturally contained in tobacco, while smoking. In order to calculate the transfer rate, mainstream and sidestream smoke from four brand styles of cigarette, which are commercially available in Japan, were collected using modified standardised methods. Ash and butts from the smoked cigarettes of two of the four brand styles were also collected for the estimation of mass balance. After digestion, the collected samples were analysed with ICP-MS to determine the amount of ^{133}Cs, and each amount was compared with original concentration in the cigarettes examined. The transfer rates of ^{133}Cs into mainstream smoke were in proportion to each tar (ISO) level, and those into sidestream smoke were not, but were similar among the brand styles investigated. The maximum transfer rates into mainstream and sidestream smoke were 0.35% and 0.57%, respectively, and almost all ^{133}Cs in tobacco was retained in ash and cigarette butts.

Japan Tobacco Inc., Product Quality Research Division, 6-2, Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan

GU Wen-bo; WU Da; LU Yi-feng; ZHOU Wan-hong; LIN Hua-qing; LIU Bai-zhan

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SS12(Poster)

Selective determination of tobacco-specific nitrosamines in mainstream cigarette smoke by gas chromatography-triple quadrupole mass spectrometry with ammonia positive CI source

A simple, sensitive and robust GC/MS-MS method for routine analysis of four tobacco-specific nitrosamines, N'-nitrosonornicotine (NNN), N'-nitrosoanatabine (NAT), N'-nitrosoanabasine (NAB) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), in mainstream cigarette smoke was developed. Under standard smoking regime, the particulate matter of mainstream cigarette smoke collected on a Cambridge filter pad was extracted with pH 1.0 HCl aqueous solution, cleaned up by a PCX solid-phase extraction (SPE) cartridge, and analysed by GC/MS-MS with positive CI source by using isotopically labelled analogues as internal standards. Four TSNAs were completely separated by an Agilent DB-35UI column, their contents were determined by gas chromatography-triple quadrupole mass spectrometry with ammonia positive CI source. This method is an effective, environmentally friendly approach for routine TSNA analysis with good repeatability and recovery, it also can significantly improve the detection capability for trace level of TSNAs in complex matrices. Four TSNAs in 1R5F and 2R4F reference cigarettes and some domestic Virginia type cigarette samples of commercial available brands were determined with the developed method, and the results well agreed with the data obtained by GC/TEA method.

Technology Center of Shanghai Tobacco Group Co., Ltd., No. 717 Changyang Road, Shanghai 200082, P.R. China

GUYARD A.; MEIER D.; CECCHETTO A.; HOFER R.; LI P.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. PT05

Impact of cigarette paper properties on smoke constituents' delivery under Health Canada Intense smoking regime

This investigation was undertaken to assess to which extent the changes of cigarette paper characteristics may impact the delivery of nine cigarette smoke constituents when measured under Health Canada Intense (HCI) smoking regime and normalised to smoke nicotine (SN). The characteristics of cigarette papers (CP) which have an influence on cigarette combustibility were considered. The impact on smoke constituents' delivery of paper permeability, burning agents' content and type, amount of filler and fibre, and implementation of Reduced Cigarette Ignition Propensity (RCIP) standard, were assessed under HCI smoking regime using the design of experiment (DOE) methodology. According to the interpretation of the DOE, only the variation of permeability and burning agent content have a significant impact on CO delivery. Up to 15% reduction of the CO/SN ratio was observed with the highest values of both permeability and burning agent content. All the other smoke constituents analysed were not affected by the variation of the cigarette paper characteristics and combinations thereof.

Philip Morris Products SA, Product Development – Applied Cigarette Research, Quai Jeanrenaud 3, 2000 Neuchâtel, Switzerland

HAO Ju-fang; GUO Ji-zhao; XIE Fu-wei; XIA Qiao-ling; XIE Jian-ping

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST14

Study on mechanisms of hydrocyanic acid formation during glycine pyrolysis

To find out the HCN formation process during glycine pyrolysis, the small molecule gaseous pyrolysates, H_2O, NH_3, CO_2, CO, HNCO and HCN, were analysed in real-time with TG-FTIR. The volatile pyrolysis products and solid residues at certain temperature were identified by online Py-two-dimensional GC-MS with heart-cutting and LC-MS/MS, respectively. The pyrolysis of 2,5-diketopiperazine (DKP), which was probably the intermediates of HCN forming from glycine, was studied. The results showed that: 1) The pyrolysis process of glycine could be divided into three temperature ranges: 200-300 °C, 300-440 °C and 440-900 °C, HCN was formed at each range with three peaks appearing at 273 °C, 422 °C and 763 °C; 2) The pathways of HCN formation from glycine at low- and high-temperatures were different. Below 273 °C, glycine underwent a decarboxylation reaction to produce methylamine, which subsequently formed HCN by means of dehydrogenation. Over 300 °C, glycine was dehydrated to form DKP, and then subsequently formed HCN.

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

HERTZ R.(1); BUSCH C.(1); STREIBEL T.(1,3); LIU C.(2); McADAM K.G.(2); ZIMMERMANN R.(1,3)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT32

Hyphenation of a cigarette smoking simulator and a micro-probe between a time-of-flight mass spectrometer for the analysis of pyrolysis and combustion products from a burning cigarette

The first part of the work addresses a coupling between a cigarette smoking simulator and a time-of-flight mass spectrometer which was constructed to allow the sampling and analysis of fresh tobacco smoke under simulated burning conditions. With flexible control of parameters such as smouldering and puff temperatures as well as combustion rate and puffing volume, which can be different from normal burning conditions, it is possible to investigate the compounds' formation mechanisms and pathways under simulated cigarette burning conditions. The first measurement series included the "smoking" of 3R4F reference cigarettes under nitrogen atmosphere in order to separate pyrolysis from combustion processes. The second part addresses the usage of a purposely constructed micro-probe which is inserted into a 2R4F reference cigarette to enable the direct analysis of the formed smoke compounds inside the cigarette during smoking. The smoke samples were taken directly from inside in the burning zone of the cigarette. This approach allows the monitoring of several smoke compounds during the puffing and smouldering phase as well as the change from pyrolysis to combustion conditions in the cigarettes' coal. Both approaches enable the direct sampling and analysis of fresh smoke produced under 35 mL puff volume, 2 s puff duration and one puff every 60 s, which was highly complex and dynamic. Therefore, time-of-flight mass spectrometry together with photo ionisation (SPI = single photon ionisation; REMPI = resonance enhanced multi photon ionisation) was applied to analyse smoke compounds on-line with a high time resolution. Both photo ionisation techniques do not ionise prominent bulk compounds of combustion such as nitrogen, carbon dioxide or water. In addition, REMPI is highly selective and sensitive for the detection of phenols and PAHs. The results from the first system demonstrate clear distinctions between the different experimental conditions based on their corresponding mass spectra and further statistical evaluations such as principal component analysis. The yield of nearly all compounds decreased while changing the burning atmosphere from nitrogen to air. Other compounds such as benzene and phenol were not significantly influenced by the type of burning atmosphere. The second setup revealed some formation profiles of several substances, such as isoprene, benzene or nicotine, connected with the chemical reactions occurring inside the coal during combustion and smouldering.

1. *Joint Mass Spectrometry Centre, Chair of Analytical Chemistry, Institute of Chemistry, University of Rostock, D-18057 Rostock, Germany*
2. *British American Tobacco, Group R&D, Regents Park Road, Southampton SO15 8TL, UK*
3. *Joint Mass Spectrometry Centre, Cooperation Group "Analysis of Complex Molecular Systems", Institute of Ecological Chemistry, Helmholtz Zentrum München – German Research Centre for Environmental Health, D-85764 Neuherberg, Germany*

HESFORD M.J.(1); VOLGGER D.(2); CASE P.(2); VANHALA A.(3)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. PT20

A further experimental design to investigate the influence of the LIP test substrate parameters on LIP pass rates and residual length measurements

Lower or Reduced Ignition Propensity (LIP or RIP) are terms used to describe modified cigarettes that demonstrate an increased propensity to self-extinguish when tested according to the standard test methods (*e.g.* ISO 12863:2010). The proportion of cigarettes that must self-extinguish during testing are specified in the performance criteria set by regulators.

LIP testing as conducted by the standard method has an inherent variability; the sources of which include the natural variability of cigarettes, the operator(s) or equipment used, etc. In addition, the manufacturing variability of the substrate upon which the LIP test is conducted may also have an influence on LIP test variability.

The current work complements an earlier study, the results of which were presented at the 2011 CORESTA SSPT Meeting (Graz). The aim was to investigate whether changes in three substrate physical parameters influence LIP test results, in order to determine the possible implications of substrate manufacturing tolerances on LIP test variability. A matrix of 15 substrate papers was manufactured by delfortgroup with basis weight (89-105 gsm), air permeability (99-351 CU) and roughness (659-2341 ml min^{-1}) target specifications. The LIP pass rates and residual lengths of two LIP cigarette designs were measured by Arista Laboratories UK, with the substrate papers being placed with their rougher sides facing upwards.

Statistical analysis of the data was performed in Minitab 16. The results demonstrated that roughness and basis weight were both found to be significant factors for one cigarette type, whereby increasing the roughness significantly decreased LIP pass rates and residual lengths and increasing the substrate basis weight increased LIP pass rates. Substrate air permeability was also found to have a significant impact on LIP pass rates and residual lengths for the second cigarette design and possible explanations for this will be given.

1. *British American Tobacco, Group R&D, Regents Park Road, Southampton SO15 8TL, UK*
2. *Papierfabrik Wattens GmbH & Co KG (delfortgroup), Ludwig-Lassl-Straße 15, A-6112 Wattens, Austria*
3. *Tervakoski Oy (delfortgroup), Vähikkäläntie 1, 12400 Tervakoski, Finland*

HOU Hong-wei; XIONG Wei; ZHANG Xiao-tao; TANG Gang-ling; HU Qing-yuan

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT26(Poster)

Determination of CEMA and HEMA in urine of restrictive smokers

Acrylonitrile is a reactive volatile compound, which is cytotoxic, mutagenic and possibly carcinogenic to humans. A major source for the non-occupational exposure to acrylonitrile is tobacco smoke. The major metabolites of acrylonitrile in human urine are 2-cyanoethylmercapturic acid (CEMA) and 2-hydroxyethylmercapturic acid (HEMA). In order to evaluate the possibility of using CEMA and HEMA as a tobacco specific related biomarkers for biological monitoring of smokers and non-smokers, a field study with 82 smokers and 58 non-smokers was performed. The creatinine, cotinine, CEMA and HEMA in the same urine sample were determined. The contents of CEMA and HEMA in urine samples of smokers were about ten and two times higher than those of non-smokers, respectively. Between smokers and non-smokers, the differences of two mercapturic acid levels in urine were statistically significant ($P < 0.001$), however, they did not reach a statistically significant level ($P > 0.001$) among the smokers smoking cigarettes with different tar deliveries. Urinary cotinine was detected by the proposed LC-MS/MS method. There was a significant correlation (Pearson, $R = 0.719$, $P < 0.001$) between CEMA and the nicotine metabolite, cotinine, in urine, which indicated that tobacco smoke exposure as well as cigarette smoking is the main source of acrylonitrile exposure for the general population. Due to the minimal environmental impact on the excretion of CEMA, it could be a potential biomarker for tobacco consumption.

China National Tobacco Quality Supervision & Test Center, No. 2 Fengyang Street, Zhengzhou High-Tech Industrial Development Zone, Zhengzhou 450001, Henan, P.R. China

HUGI S.(1); MEGER M.(1); MILLER J.(1); PANI J.(2); JONES I.(1)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST30

Nicotine, B[a]P and TSNA levels in commercial snus and moist snuff products, compared to CORESTA reference products

In 2009, the CORESTA Smokeless Tobacco Sub-Group oversaw the development of four new CORESTA Smokeless Tobacco Reference Products (CRPs), namely pouched snus (CRP1), moist snuff (CRP2), dry snuff (CRP3) and loose leaf (CRP4). These new CRPs are currently available from the North Carolina State University Tobacco Analytical Services Lab for research purposes.

The objective of this study is to determine how representative, in terms of chemical composition, are CRPs 1 and 2 in comparison to commercially-available snus and moist snuff products.

To facilitate these comparisons, analytical data on CRPs 1 and 2 was compared with equivalent data from a three-year market monitoring program (MMP), conducted between 2008 and 2010, covering both snus and moist snuff products on the Swedish, American, Canadian and Taiwanese markets.

In this presentation, data on benzo[a]pyrene (B[a]P), tobacco-specific nitrosamines (TSNAs) and nicotine are presented.

Results expressed on a wet weight (as used) basis indicate that nicotine levels in both CRP1 (0.94%) and CRP2 (1.26%) were comparable to the mean levels measured in commercial products (snus = 0.8% and moist snuff = 1.3%). Similarly, B[a]P levels in CRP1 (0.55 ng/g) and CRP2 (36.5 ng/g) were within the ranges measured in commercial products (mean levels for snus = 0.75 ng/g and moist snuff = 32 ng/g). The TSNA levels in CRP1 was found to be at the higher end of the ranges measured in commercial snus products, whereas the TSNA levels in CRP2 level were in the lower end of the range for NNN, NAT and NNK.

In conclusion, the results showed that, for six specific analytes addressed, both CRP1 and CRP2 were generally representative of commercially available snus and moist snuff products.

1. *JT International S.A., 1 rue de la Gabelle, CH-1211 Geneva 26, Switzerland*
2. *Ökolab Gesellschaft für Umweltanalytik Ges.m.b.H., Vienna, Austria*

HUMPHRIES K.E.; GILLMAN I.G.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SS19

Determination of volatile organic compounds from US FDA list of Harmful or Potentially Harmful Compounds in mainstream cigarette smoke by GC-MS

The US FDA's Tobacco Products Advisory Committee (TPSAC) list of harmful and potentially harmful compounds in mainstream smoke includes eleven compounds that may be considered as volatile organic compounds (VOCs). To analyze this wide range of compounds we wanted to apply CORESTA Recommended Method N° 70 "Determination of selected volatile organic compounds in the mainstream smoke of cigarettes" to the analysis of 1,3-butadiene, vinyl chloride, ethylene oxide, propylene oxide, furan, acrylonitrile, isoprene, nitromethane, vinyl acetate, benzene, and toluene in mainstream cigarette smoke. We will present the results of our method validation study including the trapping efficiency for each compound under ISO and Canadian Intense smoking conditions. We will also present data on the stability of ethylene and propylene oxide using several trapping solvents. Finally we will present data from our laboratory control charts and discuss the practical limitations of using liquid impingers to trap low level VOCs in mainstream cigarette smoke.

Enthalpy Analytical, Durham, NC 27713, USA

INOUE Y.; NAGAO A.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT10

Estimation of net heat generated from tobacco under puffing condition

Heat from the combustion of cut tobacco affected the mass burning rates of cigarettes, expressed in units of cal/g. Several investigations have been conducted to identify the heat of combustion of cut tobacco under static burning conditions. However, few studies have yet been conducted to identify the heat of combustion under burning conditions during puffing, because of the complexity caused by the effect of airflow and gas species resulting from the reaction of tobacco combustion. The objective of this study is to estimate the heat of combustion under puffing conditions.

The net heat generated from tobacco ($H_{puffing}$) was estimated from the following experiments and calculation. The cut tobacco was heated to 800 degrees with infrared furnace under conditions close to the cigarette puffing burn, and by-products from heated tobacco, such as carbon monoxide, hydrocarbons, tar, char and ash were determined using GC-TCD, GC-FID and CHN analyser, respectively. The heat of uncompleted combustion of by-products (H_{bp}) was calculated by summarising heats of combustion generated from complete combustion of by-products, (*e.g.* $CO + O_2 \rightarrow CO_2$). $H_{puffing}$ was calculated by subtracting H_{bp} from the heat of complete combustion of tobacco (H_{comp}), which was reported as 4000 cal/g in previous studies.

$H_{puffing}$ was estimated to be 1440 cal/g. Previous studies showed that the net heat generated from tobacco under static burn (H_{static}) was determined to be approximately 1900 cal/g. $H_{puffing}$ was 450 cal/g smaller than H_{static}. This result was mainly caused by the difference in H_{bp}. Most of H_{bp} under static burning condition consists of the heat of combustion of hydrocarbons, which were generated by reheating of Tar component. On the other hand, most of H_{bp} during puffing was constituted of the heat of combustion of Tar.

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

JI H.(1); FANNIN F.F.(1); JACK A.M.(2); BUSH L.P.(1)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST31

The use of nicotine to improve the stability of tobacco specific N-nitrosamines (TSNAs) standards

Analysis of tobacco specific N-nitrosamines (TSNAs) by gas chromatography (GC) using a thermal energy analyzer (TEA) detector has been the preferred method until quite recently. Although liquid chromatogram/mass spectrometer/mass spectrometer (LC/MS/MS) analysis is becoming more popular because of its high sensitivity and specificity, the equipment is expensive to purchase and maintain. GC-TEA analysis is still useful for routine analysis of TSNAs in a research setting as it involves less cleaning of extracts and does not require expensive deuterated internal standard. Stability of the pure TSNA standards necessary for the GC analysis has been a concern in our research. Even at -20 °C, the TSNA standards, particularly N-nitrosoanatabine (NAT) and N-nitrosoanabasine (NAB), have poor stability. Standard solutions have therefore been prepared immediately before analysis. However, it appears that TSNAs in methylene chloride extracts of tobacco are relatively stable, and can be stored for at least 24 hours in the freezer before use. As nicotine is present in all methylene chloride extracts of tobacco, we decided to test whether it might be useful in stabilising the TSNA standard solutions. Our preliminary tests indicate that nicotine can increase the stability of N-nitrosonornicotine (NNN), NAT, NAB and 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) as well as N-Nitroso-di-n-hexylamine (NDHA), our internal standard, in methylene chloride. Pure TSNA standards are expensive and preparation of standard curves is laborious, so the addition of nicotine to prolong the life of the standards would be very useful. Determining optimal conditions for storage of the standard solutions and understanding the reasons for their stability (or instability) may also be useful for reducing the final levels of TSNAs in tobacco products.

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

JONES B.A.; CHEN P.; SCHMIDT E.; PRASAD G.L.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT22

Development of biomarkers of effect from chronic tobacco usage.
Part 1: Study design and biomarkers of exposure

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

R.J. Reynolds Tobacco Co., R&D Department, PO Box 1487, Winston-Salem, NC 27102, USA

JOZA P.; BAO M.; WU J.; MASTERS A.; WAGSTAFF W.; RICKERT W.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SS16

Analysis of hazardous and potentially hazardous tobacco smoke constituents: methodology and yields of benzo[b]furan, acetamide, acrylamide and nitrobenzene from commercially available tobacco products

The recent FDA publication of a list of harmful and potentially harmful constituents has resulted in the development of a number of new or revised analytical methods for tobacco smoke chemistry. Analysis of the title compounds was achieved by extending an existing method used for the analysis of some semi-volatiles (Health Canada T-112).

Mainstream smoke was passed through a collection pad followed by two cryogenic traps (impingers) containing methanol. After addition of internal standard, the pad was extracted with the combined trapping solutions. The determination of benzo[b]furan and nitrobenzene was achieved by extending the existing GC/MS method analysis parameters, utilizing styrene-d8 and quinoline-d7 as internal standards. The analysis of acetamide and acrylamide required an independent GC/MS run of the sample extract, using selective ion monitoring with acetamide-d3 and acrylamide-d3 as internal standards.

This extended method was used to investigate the emissions from a series of twelve products that included a small cigar, a cigarillo and the Kentucky Reference 3R4F cigarette. These products were smoked under three smoking regimens defined by puff volume (mL), duration (seconds), frequency (seconds) and vent blocking (%); 35/2/60/0% (ISO), 55/2/30/100% (Canadian Intense (CI)), 60/2/30/50% (ISO/TC 126 WG 9 Option B (WG9B)). In all cases, nitrobenzene was determined to be below the method detection limit (LOD) of 0.013 µg/cig (ISO) and 0.027 µg/cig (CI and WG9B). Benzo[b]furan yields ranged from 0.048 to 2.23 µg/cig (LOD = 0.015 (ISO), 0.030 (CI and WG9B)). Acetamide and acrylamide yields ranged from 0.100 to 78.4 µg/cig (LOD = 0.049 (ISO), 0.099 (CI and WG9B)) and 0.060 to 14.2 µg/cig (LOD = 0.031 (ISO), 0.062 (CI and WG9B)) respectively.

The relationship between constituent yield and tar was basically linear for the emissions of cigarettes used in the study. However yields for the ‘little’ cigar and cigarillo were obviously different from those of the cigarettes in the study.

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

KASHIMA T.; ISHIDA N.; GOHARA Y.; YOKOI M.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. PT09

Quantitative analysis of labdanoids in tobacco leaves by gas chromatography

Labdanoids are one of the main diterpenoids in the leaf surface resin of Oriental tobacco. They are thought to give a unique taste and aroma to Oriental tobacco leaf. However, there have been no reports referring to the comprehensive quantitative results of the various labdanoids. Identification of the main labdanoids was therefore conducted, and the identified substances were subsequently quantified to compare their compositions among tobacco leaves. The procedure of identification was based on the following: The *n*-hexane extract from the Oriental tobacco was subjected to silica gel column chromatography for separation, and then each eluate was analysed using a GC/MS. Identification was carried out by comparing the mass spectrum with the previously reported one. This procedure enabled us to identify 15 labdanoids such as *cis*-abienol, sclareolide, epoxylabdans and levantenolides. Since several authentic standard components for calibration were not available, a GC/FID was selected to use its versatile sensitivity dependent on the molar quantity. The quantities were calculated by comparing them with the peak area of authentic sclareolide. Although the quantitative analysis was based on the procedure of identification, some modifications shown below were introduced; an internal standard (*n*-heptadecanol), solid phase extraction and appropriate column (DB-35MS) for separation were additionally given to the process. The validation study using sclareolide gave the desirable values as follows: linearity with a correlation coefficient greater than 0.999 (0.5 to 32.0 µg/mL), recovery ratio of 105.6, 101.9 or 103.5% (spiked with 1.02, 4.07 and 16.30 µg/mL of sclareolide, respectively), relative standard deviation of 2.18%, limit of detection of 0.071 µg/mL, and limit of quantification of 0.237 µg/mL. The quantification results showed that major variety of Oriental tobacco leaves had labdanoids except for Izmir (Turkish Oriental tobacco). Although the amount of total labdanoids differed by varieties, the composition ratio of labdanoids showed a similar pattern through varieties.

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

KATSUOKA T.; OSAKI E.; SAKAMOTO K.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT13

Kinetic analysis of changes in concentrations of tobacco constituents during drying

Reaction kinetics was applied to the estimation of changes in the concentrations of some constituents in tobacco cut-filler during drying. Nicotine, α-CBT and sugars related to the smell and taste of tobacco were chosen as the prime chemical species on the basis of our previous studies. The changes were simplified by regarding them as pseudo-reactions dependent on the following two prime factors: each initial concentration of the constituents; and each overall rate constant of the reactions. This was because the actual chemical reactions were too complex to determine them exactly.

A sample vessel packed with the filler of flue-cured tobacco was put into a through-flow dryer in which air with a humidity of 10 g of water/kg of dry air had been adjusted beforehand to a prescribed temperature in a range of 373 to 423 K with an air flow of 3 m/s. The temperature of the filler during drying was measured with a thermocouple 0.5 mm in diameter. After the temperature of the filler attained thermal equilibrium, all of the filler was taken out of the dryer. The constituents of the filler were extracted with several organic solvents and then the concentrations were determined with gas chromatography and liquid chromatography.

The concentration of the constituents decreased with increasing the temperature of the air, and the process could be approximated to pseudo first-order reactions. Moreover, the temperature dependence of the rate constant could be expressed by the Arrhenius equation, which allowed us to obtain activation energy and frequency factor as the parameters of the equation. The curves of the concentrations calculated with the parameters were in agreement with each experimental value under various drying conditions. It was therefore concluded that the simplification and the parameters of the reaction are valid.

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

LE BEC L.; LE MOIGNE C.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. PT06

Influence of cigarette paper permeability, basis weight and citrate level on the smoke yields in a flue-cured Chinese design

Cigarette paper is one of the tools that can be used to reduce certain smoke constituents of cigarettes. The reduction of the CO/tar ratio is an important objective for Chinese flue-cured cigarettes, most of them not being filter ventilated. In order to predict the influence of cigarette paper for a Chinese style cigarette, a face centred central composite design has been built. The effects of cigarette paper permeability, basis weight, citrate level and their interactions on the smoke yields have been assessed. The level of permeability was varied from 50 to 80 CU, the level of basis weight from 26 to 32 g/m^2 and the level of mixed citrate (2Na:1K) from 0.8 to 2.0%. The filler content was maintained constant at 32%.

Non-ventilated machine made cigarettes were manufactured using a Chinese flue-cured tobacco, the same mono acetate filter and at constant tobacco density. Smoking analysis was performed in standard ISO conditions and tar, nicotine, carbon monoxide deliveries and puff number were obtained.

Within the experimental region studied, increasing permeability and citrate level significantly reduces tar yields whereas the basis weight of the cigarette paper has no effect on tar yields. Citrate level, permeability and basis weight have significant effect on CO yields. Cigarette paper basis weight has a greater effect on the CO/tar ratio than permeability and citrate level. Increasing cigarette paper citrate level and basis weight have a significant effect in decreasing the puff number.

The quality of the models obtained is quite good with r^2 in the range of 80.6-98.4% and allows reliable predictions to be made.

SWM International, Le Mans, France

LAUTERBACH J.H.(1); GRIMM D.A.(2)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT11

Using tobacco chemistry to help explain toxicity data for mainstream smoke from cigarillos and filtered cigars

In 2011, Rickert *et al.* [*Regul. Toxicol. Pharmacol.* 2011 Nov;61(2):199-209] reported mutagenicity, cytotoxicity, and clastogenicity data for mainstream smoke (MSS) from cigarillos and similar products (sometimes called filtered cigars) that have dimensions similar to cigarettes. These products are often wrapped with a reconstituted tobacco wrapper without use of an underlying binder and have cellulose acetate filters similar to filters used on filtered cigarettes. The tobacco blends were suspected to be air-cured as is the case with most cigars, but the toxicity data reported by Rickert did not fully support that conclusion. Initial DS scan GC-MS analyses (SSPT 16, CORESTA Congress Edinburgh, 2010) revealed that products were fabricated from pipe tobacco, blended cigarette tobacco, or what appeared to be light air-cured tobacco blends that appeared to contain glycerine and sugars. However, the mainstream smoke from many of the filtered cigars had hedonic characteristics unlike larger cigars and unlike experimental cigarettes fabricated only with the grades of Burley tobacco used for US-blend cigarettes. Several Burley grades likely to be used in filtered cigars were obtained and routine tobacco analytes determined along with the detailed tobacco chemistries previously reported for filler from filtered cigars. Typical blend chemistries for the tobacco from filtered cigars were alkaloids, 1.3 to 1.5%; total sugars, 2.5 to 3.2%; reducing sugars, 2.5 to 3.2%; nitrate, 1.5 to 2.2%; and chloride, 1.5 to 1.9%. DS scan GC-MS data showed evidence for glycerine, fructose, glucose, caffeic acid (trace), sucrose, and chlorogenic acid. GC-MS data on Burley grades likely to be used for the blends used for the filtered cigars also showed evidence for the same set of compounds. Such blend chemistries may explain the toxicological findings as well as smoke sensory properties of these products.

1. *Lauterbach & Associates, LLC, Macon, GA 31210, USA*
2. *Tulane University, New Orleans, LA 70118, USA*

LAUTERBACH J.H.(1); RAHMAN A.(2)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT15

Use of terahertz spectrometry to perform analytical chemistry tasks likely to be required to meet tobacco products GMP

Section 906(e) of the US Family Smoking Prevention and Tobacco Control Act (P.Law. 111-31, "TCA") requires the US Food and Drug Administration ("FDA") to establish good manufacturing practice ("GMP") requirements. The FDA is likely to propose the initial GMP requirements in 2012 or 2013. The GMP requirements will likely call for analyses verifying that the tobaccos have been manufactured correctly and that the right tobacco blends are being used to manufacture a given cigarette brand-style. Currently such verifications are carried out through batch records and/or offline analyses. Terahertz spectrometry offers the possibility of real-time analyses without the need for complex sample preparation, a feature that is especially attractive to small business tobacco product manufacturers that do not have extensive quality assurance ("QA") laboratories. While reflectance infrared and near-infrared techniques have been used by QA laboratories to distinguish among samples with major differences in blend and casings, smaller differences are likely to go undetected. Terahertz ("THz") spectrometry has the power to detect smaller differences among similar samples. An initial evaluation of terahertz spectrometry was conducted to show its ability to distinguish among unprocessed tobacco, manufactured tobaccos, and tobaccos taken from finished cigarettes. The tobacco samples (at ambient moisture) were ground and a known weight of each tobacco sample was placed in rectangular glass cuvette. THz spectra were obtained with an ARP TeraSpectra spectrometer over the range of 0 to 3.5 E+13 Hz. Numerous differences were found between the spectra of tobacco taken from the KY3R4F reference cigarette and the tobaccos taken from commercial products of similar composition. The spectral differences between the reference tobacco and commercial product are due to additives not used to formulate the reference product. Thus, indistinguishable or near indistinguishable spectra from a known good batch of tobacco and test batches of tobacco would indicate whether the test batches of tobacco are acceptable for use.

1. *Lauterbach & Associates, LLC, Macon, GA 31210, USA*
2. *Applied Research & Photonics, Inc., Harrisburg, PA 17111, USA*

LAUTERBACH J.H.(1); FORBES M.F.(1); GRIMM D.A.(2)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST09

Additive-free processed cigarette leaf (PCL): a potential new use for an old material

Processed cigarette leaf (PCL) refers to several types of band-cast reconstituted tobaccos developed by Brown and Williamson Tobacco Corporation (B&W) and other British American Tobacco (BAT) units during the 1950s and 1960s (http://legacy.library.ucsf.edu/tid/wsa51f00/pdf). In the B&W implementation of PCL, there were no chemical additives used in the PCL process except for addition of glycerine as a humectant. PCL was used until the late 1970s, when it was replaced by a paper-type reconstituted tobacco that had much reduced costs associated with its use. However, the PCL process may offer advantages today if it could be used to produce a truly additive-free reconstituted tobacco. Consequently, we have produced PCL on a laboratory-scale using a recipe and process conditions from a 1967 B&W report (http://legacy.library.ucsf.edu/tid/hao00f00/pdf), and we have profiled the starting furnish and finished product with classical chemical analyses and two GC-MS techniques. Aqueous slurries of ground Burley stem and ground flue-cured stem were cooked under pressure for about 30 minutes at 143 °C and 132 °C, respectively. The cooked slurries were mixed and processed in a high-speed blender. After additional dilution with water, mixed 50/50 flue-cured/Burley lamina fines were added (lamina/stem 1.4/1), and the mixture was cooked for 4 h at about 90 °C with further water added to dilute the slurry to about 9% solids. The slurry was applied to drying sheets and dried at 100 °C until most of the water had evaporated. The dried tobacco films were equilibrated overnight at around 65% RH. They were flexible without addition of humectants, and gave off a pleasant aroma during smoulder. Chemical changes shown by the GC-MS analyses were those expected from treating tobacco with hot water.

1. *Lauterbach & Associates, LLC, Macon, GA 31210, USA*
2. *Tulane University, New Orleans, LA 70118, USA*

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

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. PT04

Effect and influence of perforation methods for tipping paper on the control of basic smoke yields

Perforation of tipping paper is the method of choice for adjusting the ventilation of a cigarette to achieve specific smoke deliveries. Hereby, the investigation of the air flow through the perforation zone of the tipping paper and the dynamic smoke stream within the filter plug towards its mouthend is the first step to gain profound knowledge about the dilution impact on cigarette smoke. Physical and geometrical parameters of individual tipping perforation types serve as basis for the second step in studying ventilation processes – to determine the influence of possible diffusion effects. The reduction of carbon monoxide (CO) is without doubt mainly governed by diffusion through the inherently porous cigarette paper, whereas the concentration of nicotine-free dry particulate matter ("tar") and nicotine is predominantly controlled by dilution. However, the idea is to reveal the contribution of different tipping perforation methods to the diffusive regulation of the CO quantity. The experimental section comprises the analysis of variously ventilated cigarettes in terms of the three basic smoke yields and the comparison of the individual CO / tar ratios. A mathematical combination of the volumetric air flow through capillary tubes, the flow of a fluid through porous medium and one-dimensional gas diffusion establishes the theoretical approach to confirm the observed measurement results. The findings open the potential to consider the most suitable and efficient perforation configuration in respect of the fundamental smoke composition which finally affects the sensory quality of cigarette smoke.

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

MARINER D.C.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT33

A comparison of potential FCTC and US FSPTCA tobacco product data reporting requirements

The World Health Organisation (WHO) Framework Convention on Tobacco Control (FCTC) is the major driver of tobacco product regulation in most countries. Since its entry into force in February 2005 Guidelines on several of the Articles to the FCTC have been adopted by the Conference of the Parties (COP). Partial Guidelines for Articles 9 and 10 were adopted at COP4 in 2010 and are likely to be expanded at COP5 in November 2012. The current Partial Guidelines require the disclosure of ingredients and product characteristics and will be expanded to include constituents and emissions, some details of which can be anticipated, based on available documents from the Working Group on Articles 9 and 10 and the WHO Study Group on Tobacco Product Regulation.

In addition to the international developments as a result of the FCTC, a number of countries have proceeded to develop regional or national tobacco product regulations. The most detailed of these is the United States Family Smoking Prevention and Tobacco Control Act (FSPTCA) which, in 2009, gave authority to the Food and Drug Administration (FDA) to develop the relevant regulatory infrastructure. The FSPTCA requires the reporting of ingredients and selected tobacco and smoke constituents, details of which have recently been set out as draft guidance.

This paper will compare the anticipated data reporting requirements of the FCTC Partial Guidelines and those of the FDA, the processes by which the reporting requirements have been developed, and the methods and standards under which the data should be generated.

British American Tobacco, Group R&D, Regents Park Road, Southampton SO15 8TL, UK

MARTIN A.; WILLIAMSON F.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. PT13

Extraction efficiency of selected alkaloids from tobacco products

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

The objective of this work was to develop a simple, robust and efficient method to analyze nicotine and the minor alkaloids in a wide range of tobacco products/matrices without requiring standard addition. A variety of published methods were evaluated with the focus on extraction efficiency and quantitation. It was observed that the most difficult alkaloid to extract was nornicotine. The methods examined required up to three hours to reach an extraction plateau, which is often (erroneously) equated with complete extraction.

Due to the poor extraction efficiency of the methods evaluated, a new method was developed that extracts the alkaloids within 30 minutes using 5N NaOH (aqueous) to pre-treat the matrix and diluting with methanol to make the extract suitable for GC/MS. By using GC/MS as the analytical system, the alkaloid levels in Quest 3 tobacco and a variety of flavored STPs were determined with excellent precision and no observed interferences. The levels of nornicotine from this method ranged from 15 to 80% higher than the other methods tested, while the nicotine was not significantly different.

Arista Laboratories, 1941 Reymet Road, Richmond, VA 23237, USA

MARTIN A.; KALATA S.; SODEN C.; WILLIAMSON F.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST29

An improved method for the determination of selected humectants in tobacco products

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

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

The objectives of this study were to improve methodology by reducing sample extraction time and expand the scope to include a variety of other tobacco products (*e.g.* moist snuff and Kreteks).

During development, it was observed that moist snuff products reached an extraction plateau after 30 minutes, while dry products had not. It was postulated that the elevated moisture content of certain tobacco products proved beneficial to the extraction efficiency of humectants. Water was incorporated into the extraction process resulting in a more efficient extraction of the dry products.

Using the improved method, humectants were extracted from tobacco, first with water to hydrate the tobacco cell structure; then with methanol to allow the extracts to remain amenable to GC-FID analysis. Quantitation was achieved using 1,3-butanediol as an internal standard.

The improved method was fully validated with a reduced extraction time and the scope extended to include various tobacco products.

Arista Laboratories, 1941 Reymet Road, Richmond, VA 23237, USA

MASTERS A.; LIU X.; BAO M.; JOZA P.; WAGSTAFF W.; RICKERT W.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SS17

Analysis of hazardous and potentially hazardous tobacco smoke constituents: methodology and yields of ethyl benzene, ruran, vinyl acetate, nitromethane and related volatile organic compounds

The recent FDA publication of a list of harmful and potentially harmful constituents has resulted in the development of a number of new or revised analytical methods for smoke chemistry. This paper describes the extension of Health Canada method, T-116, to include the determination of ethyl benzene, furan, vinyl acetate, nitromethane from a single smoking.

Independent aliquots of the sample extract are analyzed using two analytical runs in the existing method. The challenge was to incorporate the new compounds into these analytical runs, while maintaining comparable limits of detection. Ethyl benzene was directly incorporated into an existing analytical run. However, vinyl acetate, furan and nitromethane required an additional run due the diverse nature (polarity and functional groups) of the compounds analyzed.

This extended method was used to investigate the emissions from a set of 12 brands that included typical Canadian cigarettes, a 'little' cigar, a cigarillo and the Kentucky Reference 3R4F cigarette. Products were smoked under three smoking regimens defined by puff volume (mL), duration (seconds), frequency (seconds) and vent blocking (%); 35/2/60/0% (ISO), 55/2/30/100% (Canadian Intense (CI)), 60/2/30/50% (ISO/TC 126 WG 9 Option B (WG9B)).

Ethyl benzene yields ranged from 1.9 to 40.1 µg/cig (LOD = 0.160 (ISO), 0.321 (CI and WG9B)); Furan from 3.5 to 70.3 µg/cig (LOD = 0.090 (ISO), 0.181 (CI and WG9B)); Nitromethane 27 to 1390 ng/cig (LOD = 5.21 (ISO), 10.4 (CI and WG9B)); and vinyl acetate 52.6 to 955 ng/cig (LOD = 8.25 (ISO), 16.5 (CI and WG9B)).

The relationship between constituent yield and tar was basically linear for the emissions and cigarettes in the study. However yields for the 'little' cigar and cigarillo were obviously different from those of the cigarettes in the study.

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

MASUGI E.(1); DUNKLE M.(2); MITSUI K.(1); OCHIAI N.(3); KANDA H.(3); HIGASHI N.(1); DAVID F.(2); SANDRA P.(2)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST22

Characterisation of tobacco leaf types using GC-MS analysis and statistical approach

Tobacco leaf is highly complex, containing hundreds of different components that range in molecular weight and polarity, and these components can be used to characterise and differentiate leaf types using advanced analytical techniques and data processing.

Metabolomics is a powerful tool used for the characterisation of biological samples in life science research and has become a 'Hot Topic' for the analysis of complex samples, making it an ideal process to be used with tobacco leaf. In this study, a metabolomics approach was applied to the characterisation and comparison of tobacco leaf type (flue-cured, Burley and Oriental).

Tobacco leaves were extracted using different solvents (hexane, water / MeOH / MeCN), and the extracts were analysed by GC-MS. The GC-MS data was processed using deconvolution software (AMDIS, Agilent Technologies) prior to analysis in multivariate software (MPP, Agilent Technologies). Using MPP, various manipulations were performed, such as filtering by frequency, T-test, Volcano and PCA plot construction along with the identification of unique entities.

By comparing the PCA plots from the different extraction conditions, it was observed that the results were complementary to one another. The PCA plot obtained from the hexane extracts showed good discrimination between the three leaf types. On the other hand, the polar extracts (water / MeOH / MeCN extracts) were only able to distinguish Burley from the flue-cured and Oriental. Lastly, using the information obtained from the Volcano Plots, feature identification of the hexane extracts was performed and 18 potential marker components were identified.

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, 8500 Kortrijk, Belgium*
3. *Gerstel K.K., 1-3-1 Nakane, Meguro-ku, 152-0031 Tokyo, Japan*

MAYR M.; VOLGGER D.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. PT19

Influence of band width and band material coverage rate (total band area / total paper area) on smoke yields, SE test and free burn

The objective of this analysis was to evaluate the difference in smoke yields, self-extinguishment performance and free burn rate depending on the different amount of band coverage rate on the FSC cigarette papers. The cigarette base sheet parameters were kept constant, for substance at a level of 24 gsm, chalk content at a level of 29% and an amount of burning additive (tri-potassium-citrate) of 2.0%. The aim was to vary the coverage rate of the band material with a constant diffusivity level (RT) of 0.05 cm/s on the bands. The defined levels were:

- 5/19 mm with 20.8% coverage rate
- 6/21 mm with 22.2% coverage rate
- 6/18 mm with 25.0% coverage rate
- 7/20 mm with 25.9% coverage rate

All cigarettes were produced maintaining one specification and the same tobacco blend (American Blend). At least two identical bands were present on each of the cigarettes. All cigarettes were smoked according ISO 4387 with the ISO Regime (35/2/60) and the Canadian Regime (55/2/30). The results showed a clear increase of T-N-C with increasing band coverage rate on the FSC cigarette paper. All cigarettes were tested on self-extinguishment performance according ASTM E.2187-09. They have also been tested on three layers of Whatman #2 and LIPCan filter papers to achieve a better characterisation and discrimination of the self-extinguishment performance.

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

MEHTA S.K.; RAGHU H.S.; SANGLI N.D.; GOURISANKAR S.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST11

Simultaneous quantification of 4-hydroxy coumarin, coumarin, 6-methyl coumarin and eugenol in cigarette filler and in mainstream smoke using HPLC-PDA

Eugenol is the main volatile compound of extracted oil from clove bud (*S. aromaticum* L.) that is used in traditional medicine as a bactericide, fungicide and anaesthetic.

Coumarin has long been used by the tobacco industry as an additive in cigarettes. Despite its flavour enhancing properties, numerous studies, beginning in 1855, have indicated that coumarin has toxic effects on the nervous system, heart, blood vessels, and liver of animals as well as inducing cancerous tumours and toxic conditions in humans.

Current literature indicates the GC-MS technique as being suited only for eugenol and coumarin in mainstream smoke and involves tedious headspace solid phase micro-extraction.

Based on these considerations, this study proposes a far simpler analytical process using HPLC with a Photo diode array (PDA) detector. It involves extraction of cigarette or mainstream smoke particulate matter previously collected on a Cambridge filter pad with ethanol. The analytical conditions enable separation of all four compounds eugenol, coumarin, 4-hydroxy coumarin and 6-methyl coumarin using C18 Column [25 cm × 4.6 µm × 5 µm] with water:methanol [50:50] mobile phase and column flow of 0.7 ml/min.

The method has been validated and it exhibits excellent linearity (R^2 larger than 0.9998) over a wide range of concentrations [10-1000 ng/ml] and recovery varies from 96 to 102%. The limit of detection is 0.0004 mg/Cig and quantification is 0.0012 mg/Cig. The method is robust in a commercial laboratory setting and can be applied to the above flavoured compounds analysis in different cigarette filler / smoking tobacco products.

This method was recently adopted for the analysis of nine international brand Kretek style samples for the above compounds in cigarette filler and in mainstream smoke.

ITC R&D Centre, ITC Ltd., Bengalaru 560058, India

MEHTA S.K.; RAJESHA B.J.; GOURISANKAR S.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST17

Determination of hydrazine in tobacco smoke by HPLC

Hydrazine is known to be a human carcinogen with a dangerous level of exposure being 50 ppb (Occupational Safety & Health Administration (OSHA) 2008; EPA 2008). Hydrazine is part of a family of highly volatile liquids and is present in tobacco and cigarette smoke, consequently the risk of exposure through inhalation is of great concern.

The development of a simple method for the qualitative and quantitative determination of hydrazine in tobacco smoke is presented as follows.

This method uses HPLC with photo diode array (PDA) detector, wherein cigarette smoke is trapped into an impinger containing a mixture of methanol and 0.1 N sulphuric acid in the ratio (70:30). The smoke extract is derivatised using benzaldehyde solution wherein the hydrazine is converted to benzalazine and subsequent chromatographic separation on Lichrospher RP – 18e (250 mm × 5 micron × 4.0 mm) (Merck), under isocratic conditions using a mobile phase containing acetonitrile (70%) methanol (5%) and distilled water (25%).

The separations are monitored by PDA detector at 313 nm. Retention time for hydrazine derivative is found to be 8.5 ±0.5 min and retention time for benzaldehyde is found to be 3.0 ±0.5 min at a flow rate of 1.0 ml/min. Quantitation is based on the external standard technique. The method has been validated by standard validation protocols *i.e.* limit of detection, limit of quantification, recovery, repeatability and reproducibility. Minimum recovery of 70.8% was obtained with a linear regression coefficient of 0.9998 for the range of 20 to 4000 ppb hydrazine and limit of detection was 20 ppb.

The method presented here provides several advantages over the current ones in terms of factors such as speed, simplicity and ease of sample preparation. The method was found suitable for rapid determination of hydrazine in cigarette smoke.

ITC R&D Centre, ITC Ltd., Bengalaru 560058, India

MIN H.J.; KIM I.J.; LEE J.M.; JANG G.C.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT30

Comparative study of solvent extraction and thermal desorption method to determine volatile organic compounds in cigarette sidestream smoke

The determination of volatile organic compounds (VOCs) in sidestream smoke is a field of increasing interest, because they have a significant impact on finished tobacco products. The flavour of tobacco typically comprises contributions from hundreds of VOCs and those at lowest concentration have often the most important effect. Conventional sample preparation methods, such as solvent extraction, do not offer the sensitivity required and may also distort the vapour profile so that it is no longer representative of the flavour perceived by consumers. Therefore, it is necessary to study a new method for VOCs determination in cigarette sidestream smoke.

This paper investigates a new analytical method to determine VOCs in cigarette sidestream smoke by thermal desorption coupled to gas chromatography-mass spectrometry, and compares VOCs profiles of two methods. The first method involves solvent extraction and uses an impinger. After sampling, the extract is analysed in GC-MS. The second method involves thermal desorption and uses Tenax TA and Carbograph 1TD as sorbents, which allows the whole sample to be analysed. The solvent extraction method is compatible with high molecular mass and thermally unstable compounds. However, the sample is diluted with solvent, which increases the detection limit. The thermal desorption method provides enhanced sensitivity and is compatible with thermally stable polar and non-polar compounds. Also this method enables the reuse of the adsorbent tubes and the recollection of samples. Thermal desorption also allows peak interferences such as methanol to be selectively purged to vent prior to analysis. The performance of both methods was tested in commercial tobacco products, both methods found differences in the VOCs profiles. In addition, LSS (Less Smell Smoke) products were tested, and VOCs profiles identified.

KT&G R&D Headquarters, Daejeon 305-805, South Korea

MINAGAWA K.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT36

The long-term variability of selected smoke constituents of commercial brands in the Japanese market

To control or evaluate cigarette smoke constituents, it is important to consider their long-term variability. The aim of this study is to understand how the variability of smoke constituents changes over the long term and what is the key factor of the variability. We selected 20 brands from the Japanese market for analysing nine smoke constituents under ISO and CIR conditions and physical measurements. We sampled these brands from the market five times a year and obtained three sets of data of smoke constituents for each sampling. To compare the long-term and short-term variability of smoke constituents, a selection of five brands were additionally analysed once again within two weeks after obtaining the data in each sampling. All data were examined using one-way ANOVA in each sampling. Almost all yields of NNN and NNK were roughly the same in each brand through all tests. It was observed that there were statistically significant differences ($p<0.05$) between different samplings in only a few brands whose tobacco TSNAs changed substantially. As for B[a]P, it was observed that there were statistically significant differences in only the long-term test in all brands, which suggested that the measurement variability was larger than product variability. In other constituents, there were neither statistically significant differences nor trends through all tests, which suggested that the measurement variability was the same as product variability. Finally we concluded that the long-term variability of nine smoke constituents in 20 brands on the Japanese market was almost the same as measurement variability.

Japan Tobacco Inc., R&D Group, Product Science Division, 6-2 Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan

MITSUI K.(1); DAVID F.(2); TIENPONT B.(2); OCHIAI N.(3); SASAMOTO K.(3); HIGASHI N.(1); SANDRA P.(2)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST13

Comparison of two pyrolysis systems in combination with gas or liquid chromatography for tobacco material

For the tobacco industry, the clarification of the relationship between tobacco leaf and cigarette smoke is one of the most interesting topics that has been studied for many years and various approaches and techniques have been adopted. Among these, analytical pyrolysis is a powerful tool to simulate the smoking process and to study the breakdown products formed.

Pyrolysis of natural products produces a complex mixture of solutes, dependent on the pyrolysis conditions and the instrument. Various pyrolysis systems have been developed in recent years. It is important to understand the specific advantages and limitations of available instrumentation.

In this study, two resistive heating pyrolysis devices, namely a modified thermal desorption system (TDU-P, Gerstel GmbH) and a recently introduced PyroVial system (RIC), were compared. Tobacco samples and mixtures of amino acids and sugars were pyrolysed under different conditions and the pyrolysates were analysed by gas chromatography-mass spectrometry (GC-MS). Both systems resulted in complex chromatograms. Several volatile and semi-volatile solutes were identified.

The PyroVial system also allowed the collection of the pyrolysis products in a solvent, followed by liquid injection in liquid chromatography-time of flight-mass spectrometry (LC-TOF-MS). In this way complementary information can be obtained.

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, 8500 Kortrijk, Belgium*
3. *Gerstel K.K., 1-3-1 Nakane, Meguro-ku, 152-0031 Tokyo, Japan*

MONTELEONE J.(1); GUNAWAN R.(1); LEE K.M.(2)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST08

Assessment of mathematical models in literature that estimate the tobacco-related population effect

There are increasing literature examples where mathematical models are used to forecast the public health effect of various tobacco products. This computational approach, which links diverse data on health risk and tobacco use behaviors and quantifies the net population effect, has been suggested by the US Food and Drug Administration as part of pre-market evidence for supporting the application of new tobacco products, including modified-risk tobacco products. Here, relevant modelling literature was reviewed to assess the scientific basis and readiness for use in tobacco product assessment. Public sources (*e.g.*, PubMed, Google, government reports) were searched to identify tobacco-related documents using mathematical models, from which representative models were identified and evaluated for their strengths, weaknesses, and validity for assessing the population effect (*e.g.*, mortality or prevalence). The modelling purpose, assumptions, structure, data sources, software, and the degree of validation were also reviewed. Overall, current models share a basic structure (*i.e.*, population dynamics model) and a common endpoint prediction (*i.e.*, change in population). The prediction can change based upon introduction of different tobacco control policies or tobacco products. To the basic structure, specific components for populations of interest and tobacco use patterns were incorporated and the projected difference between the baseline or 'status quo' and the 'what-if' scenarios were reported as the anticipated public health impact. However, most models used point estimates as inputs and often no statistics were reported on the uncertainty and quality of the prediction. In summary, simulation models have the ability to conceptualize and link complex and potentially confounding components to provide a prediction of net population effect attributable to tobacco products. Robust, transparent, and standard quality measures should be defined and put in place, especially for parameter estimation and model validation, if results from simulation models are objectively used for new tobacco product assessments.

1. *Pharsight Consulting Services, 5520 Dillard Dr., Suite 260, Cary, NC 27518, USA*
2. *JT International S.A., 1 rue de la Gabelle, CH-1211 Geneva 26, Switzerland*

MOSTOVOJUS V.; TUCINSKAS G.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST25

Comparative analysis of paper and cellulose acetate filters in low tar cigarettes

Low tar cigarettes are designed in ways supposed to reduce the amount of harmful compounds from mainstream cigarette smoke. There are many different filter models for achieving this including the use of dual or other combined filters containing charcoal or other filtration materials.

In this study, cellulose acetate (CA) tow and filter paper (FP) were used as filtration media. Indeed, one of the additional advantages of filters containing a paper part is environment friendliness due to faster disintegration in the environment compared with cellulose acetate tow filters.

Monoacetate, monopaper as well as acetate-acetate and acetate-paper filters, both in dual and triple options, were compared while keeping pressure drop and other key physical parameters constant. Cigarettes with the above described filters were made using CM6 tobacco columns with and without ventilation. The smoke was collected on Cambridge filter pads using the ISO smoking regime.

Our research shows that filters combining CA and paper segments have better total particulate matter removal properties from tobacco smoke compared to monoacetate and acetate-acetate filters. However, the paper filter adsorbs more moisture condensate from cigarette smoke than acetate and this could be one of the disadvantages for paper filter due to supposed dry smoke taste.

We believe that a filter combining paper and acetate tow parts have a relatively good potential for achieving low tar and environmentally friendly cigarettes with balanced taste properties.

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

NARA H.; ERAMI K.; ISHIKAWA S.; NAGATA Y.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST03

Report on biological researches using a heated cigarette.
Part 3: *In vitro* toxicological study by the direct exposure method

The objective of this *in vitro* study was to evaluate the toxicity of the mainstream cigarette smoke (MCS) of a heated cigarette (HC) by the direct exposure method in comparison with combusted cigarettes such as the Kentucky reference cigarettes, *i.e.*, 3R4F and 1R5F, and a commercial cigarette (cigarette C).

Prior to the *in vitro* toxicological evaluation, we determined the method of toxicological comparison among the test cigarettes in accordance with the characteristics of the direct exposure method. After the direct exposure of the MCS generated from the test cigarettes using a whole smoke exposure system (CULTEX® system) at the air-liquid interface, the mutagenicity, cytotoxicity and genotoxicity of the test cigarettes were evaluated by Ames assay with *Salmonella Typhimurium* strains (TA98 and TA100), neutral red uptake assay (NRU assay) with a Chinese hamster ovary cell line (CHO-K1), and micronucleus assay (MN assay) with a Chinese hamster lung cell line (CHL/IU), respectively.

As a result, the MCS of HC was proven to be generally less toxic than those of all the combusted cigarettes tested in terms of mutagenicity, cytotoxicity and genotoxicity.

Japan Tobacco Inc., R&D Group, Product Science Division, 6-2 Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan

NELSON P.R.; CHEN P.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT19

Analysis of results from four U.S. mouth level exposure studies

R.J. Reynolds completed four large studies of mouth level exposure (MLE) to cigarette smoke in over 3700 U.S. subjects between 2007 and 2010. Data collected included subject demographics, self-reported and actual product usage, and MLE "tar" and nicotine on a per-cigarette and per-day basis. Analysis of the data across studies allows a broad look at cigarette usage and the relationship between smoking behaviors and MLE.

There is considerable variability in MLE both within and across cigarette brand-styles. The overall average MLE "tar" exposure was 17.7 mg/cig and 250 mg/day. For nicotine, the average MLE exposure was 1.53 mg per cigarette and 21.5 mg per day.

In the first MLE exposure study, smokers received their study cigarettes at no cost. On average, those smokers consumed approximately eight more cigarettes per day than smokers in subsequent studies who provided their own study product. Due to an atypical number of cigarettes being smoked in the first study, the evaluations of smoking behavior reported below were conducted using data from subjects who provided their own study product.

Daily exposure to "tar" was examined as a function of T:N ratio determined from MLE results. The results obtained suggest that as T:N ratios decrease, daily exposure to "tar" is correspondingly reduced. Also, there was no significant relationship between the number of cigarettes smoked per day and MLE "tar" or nicotine.

Males were found to have a statistically significantly higher per cigarette and daily exposure to both "tar" and nicotine than females. There was no difference in per cigarette MLE "tar" exposure among the different ethnic/racial groups evaluated. Whites had a statistically significantly higher per cigarette nicotine and daily "tar" and nicotine exposure than either blacks or Hispanics.

R.J. Reynolds Tobacco Co., PO Box 1487, Winston-Salem, NC 27106-1487, USA

NIE Cong(1); SUN Xue-hui(1); WANG Ming-feng(2); ZHAO Le(1); JIANG Ju-xing(2); PENG Bin(1); WANG Yi-peng(1); YANG Song(1)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST27

Preparation of dual functional mesoporous MCM-41 containing alkaline and transition metal groups for selectively removing hydrogen cyanide from cigarette smoke

In this study, inorganic mesoporous MCM-4l was synthesised by hydrothermal synthesis and functionalised by both alkaline and transition metal groups. The structures of the obtained dual functional MCM-41 were characterised by XRD, elemental analysis, thermographimetry (TG) and nitrogen adsorption at -196 °C. The results showed that the aminopropyltriethoxysilane (APS) and transition metal ion modified MCM-41 had been successfully synthesised, meanwhile, the modified MCM-41 retained its hexagonal structure and high specific surface area with good thermal stability at 180 °C. A model reactor was used to evaluate the HCN removing ability from cigarette smoke and optimise the preparation conditions of the material. When alkaline functional reagent dosage was 4 mL APS/(g MCM-41) following modification by transition metal ion Zn^{2+}, the corresponding material Zn^{2+}/*c*-APS/ MCM-41 was found to be the most effective for reducing HCN in cigarette smoke. The material was applied in cigarettes by means of dual filter. Compared with the control cigarette, the HCN delivery of the testing cigarettes was selectively reduced by 31.2%, while the deliveries of tar, nicotine, CO, B[a]P, NNK, NH_3, crotonaldehyde and phenol changed little. The hazard index was reduced by 0.4.

$$\text{The hazard index} = \left(\frac{X_{\text{co}}}{14.2} + \frac{X_{HCN}}{146.3} + \frac{X_{NNK}}{5.5} + \frac{X_{NH_3}}{8.1} + \frac{X_{B[a]P}}{10.9} + \frac{X_{\text{Phenol}}}{17.4} + \frac{X_{\text{Crotonaldehyde}}}{18.6}\right) \times \frac{10}{7}$$

The overall sensory quality and aroma style of the test cigarettes were almost similar to those of the control. The results indicated that the dual functional mesoporous MCM-41 containing both alkaline and transition metal groups was a promising filter additive for removing HCN from cigarette smoke.

1. *Zhengzhou Tobacco Research Institute of CNTC, No. 2 Fengyang Street, Zhengzhou High-Tech Industrial Development Zone, Zhengzhou 450001, Henan, P.R. China*
2. *Hongyun Honghe Tobacco (Group) Co., Ltd, No. 181 Hongjin Road, Wuhua District, Kunming 650202, P.R. China*

PEITSCH M.C.; MATHIS C.; WAGNER S.; KOGEL U.; POUSSIN C.; SCHLAGE W.; STINN W.; MEURRENS K.; WEISENSEE D.; GEBEL S.; BELCASTRO V.; XIANG Y.; MARTIN F.; SEWER A.; HENGSTERMANN A.; ANSARI A.; HOENG J.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SS02

Development of a systems toxicology approach and its application to quantify the biological impact of tobacco smoke *in vitro* and *in vivo*

The potential health risks of long-term exposure to biologically active substances such as therapeutic drugs or environmental toxins are frequently determined a posteriori through clinical epidemiology studies. However, disease may take decades to manifest, and changes in therapeutic regime, lifestyle or exposures may impact the risk of disease onset. Moreover, correlative disease risk assessment in epidemiology is not intended to elucidate the mechanisms linking perturbations in molecular signalling to disease and thus provides fewer options for intervention. We propose to quantify the biological network perturbations caused by active substances and thereby identify mechanisms and biomarkers that are modulated in response to exposure and are related to disease onset.

Two distinct metrics were developed: NPA (network perturbation amplitude), which assesses the amplitude of signalling in a network, and BIF (biological impact factor), which aggregates the amplitude scores of multiple networks. These methods rely on the development of causal models of biological networks that include measurable downstream quantities affected by pathway perturbations. Here we present a five-step biological impact evaluation and sample applications to quantify the biological perturbations induced by whole cigarette smoke (CS).

Firstly, we studied transcriptomics in organotypic 3-dimensional cultures of human bronchial epithelium and modelled the biological network perturbations induced by CS exposure. When compared to human bronchial epithelium *in vivo*, a single exposure to CS in this *in vitro* system induced biological perturbations similar to those observed in the airway epithelium of human smokers *in vivo*.

Secondly, we augmented the OECD TG412 28-day inhalation study with transcriptomics and phosphoproteomics to evaluate biological perturbations by CS *in vivo* and to derive a quantitative BIF of various exposures. The observed histopathological end points correlated with the degree of perturbation of their associated biological networks *in vivo*, thus providing a powerful approach to investigate disease mechanisms *in vivo*.

Philip Morris International R&D, Philip Morris Products SA, Neuchâtel, Switzerland

PIKE L-J.(1); HURLE P.(1); ROBERTS P.(2); POWLEY S.(1); ASHMORE L.(1); BETSON T.R.(1)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SS20

Smoke analysis made easier: two case studies

There is increasing focus globally on the measurement and reporting of tobacco product chemistries, such as smoke emissions and tobacco contents, from both scientific and regulatory perspectives. This increases the workload of laboratories which then need to deploy strategies to deliver a higher number of results within the same time frame. Using case studies, two strategies are illustrated. One aims to reduce instrument run time and maintenance while the other focusses on automation of sample preparation and injection.

The first example examines a GC-MS method to analyse for Pyridine, Quinoline and Styrene in mainstream smoke. Each run was 39 minutes long despite the retention time of the last compound of interest being 17 minutes. This was due to a temperature ramp optimised to elute high-boiling-point compounds at the end of the run but frequent column trimming was still necessary to remove residual compounds leading to instrument down-time. A backflush system was installed to flush high boiling point compounds facilitating a 23 minutes run and a significant decrease in instrument maintenance. A comparative study showed that the results for all analytes at ISO and Canadian Intense regime with or without the backflush were not significantly different.

The second example details the extraction method for the analysis of Phenol, *ortho*-, *meta*- and *para*-Cresol, Resorcinol, Hydroquinone and Catechol in mainstream smoke. The method calls for a solvent extraction of a Cambridge filter pad, followed by derivatisation before injection onto a GC-MS. This multi-step method was transferred to a prep-station (Gerstel MPS 2 XL xt) which can add the solvent and internal standard, shake, derivatise and inject the sample automatically. Preliminary results showed that the prep-station can prepare and inject the samples to produce acceptable results for a reference. The use of such a system significantly increases an analysts' capacity.

1. *British American Tobacco, Group R&D, Regents Park Road, Southampton SO15 8TL, UK*
2. *Anatune Ltd, Unit 4, Wellbrook Court, Girton Road, Cambridge, CB3 0NA, UK*

PRASAD G.L.(1); CHEN P.(1); JONES B.A.(1); KENNEDY A.D.(2)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT24

Development of biomarkers of effect from chronic tobacco usage.
Part 3: Potential metabolomics biomarkers of tobacco effect

Epidemiological data indicate that consumption of moist snuff is associated with reduced harm relative to cigarette smoking. However, a need exists for interim biomarkers of effect (BioEff) that would be useful to assess the health effects of tobacco. To identify potential BioEff in smokers and moist snuff consumers (MSC), we performed global untargeted metabolomic profiling of plasma and urine collected from smokers and MSC, using mass spectrometry. Analyses revealed a general concordance between the data obtained from the two matrices among biological pathways influenced by tobacco exposure (details presented in an accompanying poster). For example, smokers experienced exacerbated oxidative stress and inflammatory pathways relative to MSC. Based on the differential levels of metabolites detected, random forest analyses separated non-tobacco consumers (NTC), smokers, and MSC with a high (96%) accuracy when all metabolites were included. Overall, smokers showed more pronounced biochemical changes compared to MSC, which facilitated the separation of smokers from non-smokers (MSC and NTC). On the other hand, MSC showed more subtle changes in metabolite profiles, and were more difficult to separate from NTC but could still be separated. The smokers and MSC cohorts could also be segregated with a high (95%) accuracy. In addition, panels of a few metabolites that may be useful for segregating the two tobacco consumer cohorts were identified from these metabolomic profiling data. These metabolites could be used as potential BioEff, pending further validation. In summary, global metabolomic profiles and panels of selected metabolites may be used to assess the effect of tobacco consumption on biological pathways in plasma and urine.

1. *R.J. Reynolds Tobacco Co., R&D Department, PO Box 1487, Winston-Salem, NC 27102, USA*
2. *Metabolon, PO Box 110407, Research Triangle Park, Durham, NC 27709, USA*

PRASAD G.L.; JONES B.A.; CHEN P.; SCHMIDT E.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT23

Development of biomarkers of effect from chronic tobacco usage.
Part 2: Inflammation and oxidative stress

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

R.J. Reynolds Tobacco Co., R&D Department, PO Box 1487, Winston-Salem, NC 27102, USA

PRASAD G.L.(1); CHEN P.(1); JONES B.A.(1); KENNEDY A.D.(2)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST36

Development of biomarkers of effect from chronic tobacco usage.
Part 4: Metabolomic profiles from cigarette smokers and moist snuff 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 effects of tobacco exposure, we evaluated the biochemical profiles of 40 smokers, 40 moist snuff consumers (MSC), and 40 non-tobacco consumers (NTC) using UHPLC-mass-spectrometry based global metabolomics. Twenty-four hour urine samples and matching plasma samples were collected from adult male subjects who abstained overnight from both food and tobacco. Metabolomic profiling and data analyses were performed at Metabolon Inc., (Durham NC). In this global profiling study, a total of 511 biochemicals (290 known and 221 unknown metabolites) were detected in the plasma, whereas 972 biochemicals (396 known and 596 unknown) were found in urine. For example, biochemicals from amino acid, carbohydrate, fatty acid, lipid, nucleotide and xenobiotic metabolism were identified. These biochemicals fit into distinct metabolic pathways such as oxidative stress and inflammation, and cholesterol, glucose and amino acid metabolism. In addition, a large number of structurally unknown biochemicals were detected. Cigarette smoking, relative to moist snuff consumption, appears to lead to the most changes in biochemical profiles observed in this study. Biochemical changes which point to a hyperglycemic state and hyperlipidemia are among the key perturbations noted in the smoker cohort, compared to non-smoking cohorts. In summary, these data show changes in global biochemical profiles in generally healthy cigarette smokers and moist snuff consumers. These differences in the metabolite profiles may be useful in understanding the higher risks associated with smoking relative to consumption of smokeless tobacco products.

1. *R.J. Reynolds Tobacco Co., R&D Department, PO Box 1487, Winston-Salem, NC 27102, USA*
2. *Metabolon, PO Box 110407, Research Triangle Park, Durham, NC 27709, USA*

RAJNOCH C.; GAFNER J.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST07

Nicotine and cotinine in hair as biomarkers of tobacco smoke exposure

Tobacco smoke exposure is often assessed using self-report questionnaires which, because of recall bias, can be unreliable. Better results can be obtained when biomarkers are measured in combination with self-reports. Nicotine and cotinine have emerged as the preferred biomarkers to assess tobacco exposure, since both metabolites can be quantified in body fluids. However, nicotine and cotinine in these matrices are subject to daily-variability and only representative of the exposure from the past few days. Their use in large epidemiologic studies can also be debated, since nicotine pharmacokinetics may vary between individuals.

Because of these restrictions, detection of nicotine metabolites in hair has emerged as an alternative way to quantify tobacco smoke exposure, since it is not subject to daily-variability and offers the possibility to define exposure long after it has occurred. Nonetheless, biomarker measurements in hair also suffer from drawbacks including issues such as hair melanin concentration, hair treatment and hair nicotine uptake mechanisms, which can all impede hair nicotine metabolite levels. Furthermore, factors influencing nicotine metabolism such as age, ethnicity, diet and genetic variability may also affect hair nicotine and cotinine concentrations.

This review discusses and evaluates the use of hair nicotine and cotinine to assess tobacco smoke exposure. Representative data from studies found in the scientific literature are presented and the many experimental designs and analytical methods used by different research groups are discussed.

JT International S.A., 1 rue de la Gabelle, CH-1211 Geneva 26, Switzerland

ROEPER W.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SS01

Ten years and more of 'biosciences' in CORESTA – What have we learnt so far?

In June 2001, the CORESTA Board formally decided to broaden the scope of CORESTA by engaging into biosciences (other than tobacco agronomy and phytopathology), starting with *in vitro* toxicity testing and biomarkers of tobacco (smoke) exposure. Until then, since 1996, work addressing biological aspects of smoking had only been done by a special committee within CORESTA, reporting directly to the Scientific Commission, the Smoking Behaviour Committee. Membership of that committee was – similar to ACAC – by invitation only. The broadened scope consequently led to the re-shaping of the then Study Groups "Smoke" and "Technology" into "Smoke Science (SS)" and "Product Technology (PT)".

Subsequently, three Sub-Groups (SG) and Task Forces (TF) were set up, reflecting this change: i) SG Smoking Behaviour (name change of former committee 2001), ii) TF 'Nicotine Intake' (2001, later on 'Nicotine Uptake', disbanded 2009) and iii) TF '*In vitro* Toxicity Testing of Tobacco Smoke' (2002). Finally, a new SG 'Biomarkers' was launched in 2009 with a wider scope than its predecessor TF 'Nicotine Uptake'. The work of these groups has had and still has significant impact on the scientific work within CORESTA, leading to numerous presentations at CORESTA meetings and publications in peer-reviewed journals.

This paper provides a brief analysis of some 270 presentations and posters addressing tobacco smoke toxicity, human smoking behaviour or biomarkers, delivered at CORESTA Congresses and SSPT Joint Meetings between 1993 and 2011. More than 50% of these papers covered different aspects of toxicology, mainly *in vitro* toxicity testing methodologies, smoke exposure systems and other equipments. Other papers described the influence of cigarette design parameters on smoke toxicity. Approaches to human risk assessment were presented, including the search for suitable *in vitro* models of the major smoking related human diseases.

CORESTA began discussing smoking behaviour topics at its Vienna meeting in 1995 and received five respective presentations there; indeed, the issue has various aspects, from smoking topography and human smoke yield to smoke uptake, deposition and retention, and ... why do people smoke at all?

As early as 1996, a presentation was given on measurement of urinary mutagenicity in volunteers exposed to ETS, apparently indicating a need for CORESTA to engage in this field and to face new challenges. Indeed, our knowledge of biomarkers and how to measure them has increased considerably over the years, and there is a clear trend to use this knowledge for conducting clinical studies into the assessment of 'modified risk tobacco products'.

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

SAKAGUCHI C.; KAKEHI A.; MINAMI N.; KIKUCHI A.; ONOZAWA M.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST02

Report on biological researches using a heated cigarette.
Part 2: Clinical exposure study

The objective of this clinical study was to investigate changes in levels of biomarkers of cigarette smoke exposure (BoExp) in healthy adult male smokers who switched to heated cigarettes (HC).

This was a controlled, randomised, open-label, residential study conducted in Japan in accordance with the principles of GCP. A total of 70 healthy Japanese male smokers were enrolled (one subject from the 10 mg tar combusted cigarette (CC) group withdrew from the study). Most subjects smoked a 10-15 mg tar CC with a daily consumption of at least 20 cigarettes. Following enrolment, subjects smoked their usual brand of cigarette for two days (baseline period) and were subsequently randomised either to a HC group (46 smokers smoking approximately 20 HCs per day, 8 puffs per cigarette) or a CC10 group (23 smokers smoking approximately 20 assigned 10 mg tar CCs per day, 8 puffs per cigarette) for four consecutive weeks (investigation period). Levels of BoExp for eight selected cigarette smoke constituents (nicotine, CO, benzene, 1,3-butadiene, acrolein, hydrogen cyanide, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone [NNK], pyrene) and urine mutagenicity were measured at several time points during the study period.

At the end of the investigation period, except for the CO BoExp, BoExp levels for the other seven constituents and urine mutagenicity were significantly lower in the HC group compared to the CC10 group. The value of the CO BoExp was slightly higher in the HC group compared to the CC10 group but was without statistical significance.

Japan Tobacco Inc., R&D Group, Product Science Division, 6-2 Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan

SAKAKI T.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT35

The twenty years of activity of the Asia Collaborative Study

The Asia Collaborative Study (ACS) involves proficiency testing of NFDPM and nicotine in mainstream smoke. It was started in 1992 with nine laboratories from six countries when the Asian laboratories numbered only three. This report will explain the history and the latest activities of ACS.

The establishment of ACS was triggered by the ISO Standards for NFDPM and nicotine analyses which were published in 1991. The new ISO Standards were based on the CORESTA Task Force activities such as "the Review of Smoking Methods". Several Asian government and industry laboratories felt the necessity of interlaboratory testing prior to the adoption of new ISO Standards and planned the first ACS which was reported in 1992.

Meanwhile, the Tobacco Institute of Japan (TIOJ) Collaborative Study had been started in 1989 for harmonising the TIOJ smoking method with the ISO Standards. It was performed by TIOJ members' laboratories and the TIOJ Testing Laboratory. The technical experts of TIOJ members' laboratories were also involved with the ACS activity. The TIOJ Collaborative Study was merged into the ACS in 1994. It was the 3rd ACS and the 8th TIOJ Collaborative Study. The TIOJ Testing Laboratory was assigned as the distributor of the ACS sample cigarettes which have been provided by TIOJ members and voluntary companies since then.

The participants of the 19th and latest ACS in 2011 included 57 laboratories from 26 countries. The Asian region laboratories were 36 and represented 63% of the total participants of the 19th ACS. The others were mainly from Europe and the Americas. They submitted 80 data sets of five sample cigarettes including the CORESTA Monitor CM6. The 19th ACS report was discussed at the 2012 ACS meeting at Bali, Indonesia on 17 May 2012. The ACS is now a global proficiency testing group organised in Asia.

Tobacco Institute of Japan, Testing Laboratory, 3-1-39 Wakamiya, Ishioka, 315-0017, Japan

SAWAGURI J.(1); HORI K.(2); SAKIMURA M.(3); SOFUNI T.(1)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST19

The *in vitro* micronucleus assay for cigarette smoke condensate samples: Photomicrographs for micronucleus scoring and analysis of historical data obtained from Kentucky reference cigarette 3R4F

The *in vitro* micronucleus (MNvit) assay is a genotoxicity test that was developed as an alternative to the chromosomal aberration assay with the advantage of detecting both structural aberrations and numerical aberrations (aneuploidy). We conducted the MNvit assay for the evaluation of cigarette smoke condensate (CSC) samples to detect the genotoxicity of tobacco materials as one of the *in vitro* toxicity assays. In order to maintain the uniformity of the evaluation criteria, we prepared photomicrographs of micronucleated cells and defined detailed micronucleus classifications. The accumulated historical data of the micronuclei (MN) in the CSC samples derived from 3R4F was analysed from several aspects.

In the CSC sample treated groups, a decrease in the cell survival with increasing the MN frequency was observed both with and without metabolic activation (+S9 and -S9), and the maximum MN frequency (average of all studies) was found to be 450 µg/mL (-S9) and 600 µg/mL (+S9). The MN frequency of the solvent control ranged from 0.5 to ~1.5% and the maximum frequency of MN in the CSC sample treated groups fluctuated from ~3 to ~8% both with and without S9. Regarding the size of the MN, CSC sample treatment mainly induced small MN rather than large ones, suggesting that the CSC sample induces structural aberrations rather than aneuploids.

The OECD Test Guideline 487 (MNvit assay) states that when cytochalasin B is not used, evaluation of cytotoxicity based on RICC or on RPD is recommended, and the highest concentration should aim to produce 55 ± 5% cytotoxicity. However, in the evaluation of CSC samples, data with more than 60% cytotoxicity should be included in the evaluation because CSC samples are sporadically judged to be "negative" when they are eliminated, and in these cases we cannot compare the genotoxicity of two or more CSC samples.

1. *Japan Tobacco Inc., Scientific and Regulatory Affairs Division, 2-1-1, Toranomon, Minato-ku, Tokyo 105-8422, Japan*
2. *Mitsubishi Chemical Medience Corp., Drug Development Service Segment, 14-1, Sunayama, Kamisu, Ibaraki 314-0255, Japan*
3. *Japan Tobacco Inc., Product Science Division, 6-2 Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan*

SCHERER G.; ECKER J.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT16

Smoking behaviour and compensation: an update of the literature since 1999

An update of the literature in relation to a review published in 1999 on compensational smoking (Scherer, 1999, *Psychopharmacology* 145: 1-20) has been performed. A number of events in the field of smoking and health undoubtedly influenced the general opinion on compensation. These include the IOM Report on tobacco harm reduction in 2001, the proposed normalisation of yields and properties of smoke to 1 mg nicotine per cigarette by the WHO Study Group on Tobacco Product Regulation (TobReg) in 2007, and the FDA (draft) Guidelines for testing of Modified Risk Tobacco Products (MRTPs). These events consider compensation in their strategies in any way.

In the categories of cross-sectional field studies and brand-switching studies, 12 and 10 studies, respectively, published since 1999 were found to be suitable for evaluation in this literature update. The additional studies support the conclusions from the first review to the effect that compensation widely occurs and is predominantly partial and that the mechanism of compensation is mainly by change in puffing volume rather than number of cigarettes smoked.

As in the previous review, there were investigations addressing the question of compensation for nicotine, belonging to the categories (a) switching between cigarettes which differ predominantly in the nicotine yields, (b) nicotine supplementation and simultaneous smoking, (c) application of nicotine agonists/antagonists and simultaneous smoking. Two additional categories were included in this literature update: (d) Smoking behaviour in rapid and slow nicotine metabolisers and (e) influence of polymorphic nicotine receptors on smoking behaviour. The information available gives further support to the compensation for nicotine hypothesis. However, compensation for nicotine is by far not precise. Also as previously, there is some limited evidence that other factors such as amount of smoke per cigarette (irrespective of the nicotine yield), sensory effects (rather than pharmacological effects) of nicotine, brain receptor activating factors in smoke of denicotinised cigarette smoke and charcoal filter tips determine the smoking pattern beyond the nicotine yield of the product.

In summary, the update of the literature confirms the conclusions of the previous review stating that compensation for changed yields in cigarettes widely occurs and is in most cases partial. Nicotine is probably the most important, but not the only factor which drives compensational smoking.

ABF Analytisches Biologisches Forschungslabor GmbH, Goethestrasse 20, 80336 Munich, Germany

SHIN H.J.; JANG M.; HYUN H.C.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SS06

Relationship between leaf/smoke components and *in vitro* biological activity using several kinds of leaf tobaccos

This study was conducted to get the information about relationship among leaf and smoke components and *in vitro* toxicity according to several kinds of leaf tobaccos. Twelve Burley and ten flue-cured leaf tobaccos which were of different grades, three reconstituted and two expended tobaccos were used for this study. Leaf components (sugar, total nitrogen compound, polyphenols, alkaloid, organic acid and inorganic acid) and smoke components (phenols, carbonyls, volatile compounds, aromatic amines) were analysed. The cytotoxic potencies of both the total particulate matter (TPM) and gas/vapour phase (GVP) were assessed using neutral red uptake assay and GSH consumption assay, respectively. The assessment for genotoxicity of smoke from leaf tobacco was determined using *Salmonella* mutagenicity. On the basis of 3R4F reference cigarette, flue-cured tobaccos showed higher cytotoxicity and lower mutagenicity than that of 3R4F's TPM. But Burley tobaccos showed high mutagenicity and low cytotoxicity. All of the chemical components changed with different grade leaf tobaccos. As the grade ascended, mutagenicity increased and cytotoxicity decreased. This result can be used to estimate the biological characteristics of a cigarette blend using different grades of leaf tobacco.

KT&G Research Institute, 22 Gajeong-ro Shinseong-dong, Yuseong-gu, Daejeon, South Korea

SHINKAWA T.; YAJIMA M.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST26

Evaluation of chemical compound sorption into packaging materials

Packaging film materials (polymers) require a low sorption property to preserve product quality. Generally, it is difficult to estimate the sorption of chemical compounds into polymers, and a practical evaluation method needs to be established.

In this study, solubility parameters (SP) were applied to estimate the sorption amount of chemical compounds into polymers. The polymers and compounds were selected to have a wide range of SP values. The polymers were polypropylene (PP, SP=16 $MPa^{1/2}$) and ethylene vinyl alcohol copolymer (EVOH, SP=39 $MPa^{1/2}$), while the compounds were 16 substances with SPs between 16 and 32 $MPa^{1/2}$. The SP values of the polymers were taken from published data, and those of the compounds were calculated using Molecular Modeling Pro software published by Norgwyn Montgomery Software Inc. Tobacco leaf with the mixture of 16 compounds was stored with each polymer in a sealed pouch at 42 °C for a week. The decreased amount of the compounds in the tobacco after storage was measured by GC/MS to evaluate the sorption.

The data obtained showed that the sorption amount was related to the differences between the SP values of the polymers and compounds. Compounds with SPs close to the polymers' SPs tended to be sorbed more significantly than those with distant SPs. It was suggested that SP was a useful parameter for estimating the sorption amount of chemical compounds into polymers.

Japan Tobacco Inc., Product Technology Development Div., 1-17-7, Yokokawa, Sumida-ku, Tokyo 130-8603, Japan

SLAYFORD S.; NOTHER K.; PRASAD K.; MARINER D.C.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT17

The SA7 Smoking Analyser – A puff topography device with a difference

The Smoking Analyser Number 7 (SA7) is a topography device developed by British American Tobacco in conjunction with C-Matic Systems Ltd. The instrument is a portable mains powered device. All data on the SA7 are collected on a puff by puff basis and total yields are determined by adding the individual puff values together.

Puffs are triggered by flow and pressure values rising above and falling below pre-determined thresholds for a set time. The equipment can be taken to a central location, where large numbers of consumers can smoke in a controlled environment. A calibration procedure based on known flows and pressures is used to ensure accurate flow and pressure measurements.

However, unlike other devices, the SA7 additionally measures the optical density of smoke, referred to as Optical Tar (OT), this real time measurement is an estimation of the Tar generated by the consumer from the cigarette. Calibration of the relationship between the extinction of the light signal and the Optical Tar concentration is determined by smoking cigarettes under a range of smoking regimes, with simultaneous measurements of OT and NFDPM yields.

Results will be presented for a study in which 50 UK smokers smoked their regular brand (a commercial 5 mg ISO tar cigarette) through the SA7 holder in a central location on three separate occasions. It was determined that for this commercial product Optical Tar = (0.94 × NFDPM + 0.89) with an R^2 value of 0.99. Using the BAT part filter methodology to determine the consumers Mouth Level Exposure (MLE) for each cigarette, the relationship between MLE and OT will be shown.

We propose that this is a robust system which provides real time estimation of MLE, providing a potential tool to supplement, and on occasions, replacement for filter analysis.

British American Tobacco, Group R&D, Regents Park Road, Southampton SO15 8TL, UK

STEACH J.K.; FISHER JONES D.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. PT01

Photodegradability testing of cellulose acetate filters – outdoor weathering vs. accelerated weathering

Degradation testing in the laboratory is based on understanding a material's exposure to specific environmental forces, which can play a key role in the mechanisms of degradation. Cellulose acetate filters can be degraded by various environmental forces, which include physical forces, biological processes, heat, light, and water. These various forces can lead to degradation mechanisms, such as photodegradation, biodegradation, chemical degradation, dispersion, and disintegration.

For this study, the testing was focused on the photodegradation mechanism of cellulose acetate filters by two different tests. An accelerated test method, based on the 1995 CORESTA Task Force utilising bench top weatherometers and a roof top weathering test method was used. A comparison of the results for accelerated bench top testing and roof top testing showed that one week of accelerated testing is equal to one month of roof top testing. Also, the impact of paper on the filter *versus* removed from the filter was studied. It was determined that the impact of paper on the rate of photodegradation was larger for samples which had photodegradation enhancing additives incorporated into the cigarette filters. These findings indicate that care should be taken in understanding the test method for the specific degradation mechanism being investigated.

Eastman Chemical Company, PO Box 511, Kingsport, TN 37662, USA

SUZUKI H.; NISHIJIMA Y.; FUKANO Y.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST04

Report on biological researches using a heated cigarette.
Part 4: *In vitro* oxidative stress assays

Mainstream cigarette smoke (MCS) is a mixture of several thousand constituents and is reported to include many oxidants, such as reactive oxygen species (ROS). ROS induced oxidative damage at molecular and cellular levels. At the 2010 CORESTA Congress (Edinburgh), we presented investigations regarding the cigarette smoke constituent associated with 8-hydroxydeoxyguanosine (8-OHdG) induced in cultured cells and oxidised low-density lipoprotein (ox-LDL) by aqueous extracts of MCS. Our results showed that the gas vapour phase (GVP) was more involved in the induction of both 8-OHdG and ox-LDL than the particulate phase in MCS, and the acrolein in MCS was a major constituent in the induction of 8-OHdG and ox-LDL.

In this study, both oxidative stress assays were applied for estimating the *in vitro* biological activity of a Heated cigarette (HC) that we developed. The Kentucky reference cigarette, 3R4F, and a commercial cigarette (cigarette C) were used for comparing the activity of a HC. The whole smoke-bubbled PBS (WS-PBS) of a HC indicated less 8-OHdG induction than these combusted cigarettes, while the GVP-PBS of a HC showed the same tendency for 8-OHdG induction. Additionally, both the WS-PBS and GVP-PBS of a HC showed the lowest ox-LDL induction in this experiment.

Japan Tobacco Inc., R&D Group, Product Science Division, 6-2 Umegaoka, Aoba-ku, Yokohama, Kanagawa 227-8512, Japan

TAKANAMI Y.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT02

Mechanisms generating reactive oxygen species in cigarette smoke

Cigarette smoke generates reactive oxygen species such as hydrogen peroxide, superoxide anion radicals and hydroxyl radicals. Although cigarette smoke has been studied intensively, the mechanisms generating these reactive oxygen species have not yet been clarified. The major reason is the difficulty of analysing these species in the presence of matrices such as phenolic constituents. In order to clarify the mechanisms, firstly a novel method for analysing hydrogen peroxide was developed which uses an HPLC-ECD (electrochemical detector). Hydrogen peroxide in the matrices of the smoke constituents was successfully analysed by this method. Secondly, the generation of superoxide in the aqueous extract of the smoke was demonstrated by ESR analysis using a spin-trapping agent, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). In addition, while some phenolic compounds were determined to contribute to the generation of hydrogen peroxide, the amount of hydrogen peroxide observed in a solution containing these phenolic compounds was lower than that in the smoke extract. This indicated that other smoke constituents were contributing to the generation of hydrogen peroxide. These results clarified the consecutive mechanisms generating the reactive oxygen species; initially the phenolic compounds and other smoke constituents generate superoxide, then superoxide is converted to hydrogen peroxide.

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

TAYLOR M.J.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT37

The effect of puff duration and volume on the yields of e-cigarettes

In recent years there has been a large increase in the number of e-cigarettes on the market, the majority of which are designed to resemble the appearance of a standard cigarette. These devices are claimed to deliver nicotine to the smoker without any of the pyrolysis products associated with lit cigarettes. The e-cigarettes normally operate by heating an element containing a solution containing flavours and nicotine so that some of these chemicals are released to the air stream and thus delivered to the smoker. At the beginning of a puff a flow sensor initiates the heating process to allow the temperature of the element to be raised and improve the transfer efficiency to the air stream.

Due to the nature of the operation of e-cigarettes it may be expected that the parameters of principally puff duration and to some extent puff volume would have an effect on the deliveries to a smoker. At this moment in time no standard method or smoking regime exists for the machine testing of e-cigarettes. The effect of puff duration and volume on the yields of total particulate matter, nicotine and water will be presented with data from standard cigarettes for comparison. Puff volumes in the range 35 to 55 ml and puff durations in the range 2 to 4 seconds have been studied.

Filtrona Technology Centre, Shaftesbury Avenue, Jarrow, Tyne and Wear NE32 3UP, UK

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

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT04

Investigation of tobacco precursors on the formation of smoke acetaldehyde: sugars, sucrose esters and sugars from cellulose

The generation of smoke acetaldehyde has been cited as being linked to the combustion of carbohydrates, particularly sugars and cellulose on the basis of pyrolysis experiments or cellulose made cigarettes[1].

In this study, the effects of tobacco free sugars (glucose, fructose, sucrose), sucrose esters and sugars from cellulose on smoke acetaldehyde yields are investigated.

These potential tobacco precursors of acetaldehyde were determined in 30 different single tobacco grades (10 Burley, 10 Virginia, and 10 Oriental origins). Free sugars was analysed as their sugar monomers by ion chromatography with amperometric detection after aqueous extraction. Sucrose esters and cellulose were analysed as their sugar monomers by the same method after hydrolysis by aqueous sodium hydroxide and sulphuric acid solutions, respectively. Their levels in tobacco were expressed on a per cigarette basis. The expected ranges of contents have been observed across single tobacco grades.

For each grade, experimental cigarettes were made with a single design similar to commercial cigarettes. The cigarettes were made to the same pressure drop by tobacco weight adjustment.

Measurements of mainstream smoke acetaldehyde yields (ISO smoking regime) were performed by high performance liquid chromatography with UV detection after 2,4-dinitrophenylhydrazone derivatisation. Smoke acetaldehyde yields ranged from 432.3 to 771.0 µg per cigarette.

The relationship between tobaccos and smoke levels has been investigated by hierarchical ANOVA and linear correlation models. The observed effects of these tobacco precursors on smoke acetaldehyde yields will be presented and discussed.

[1] Seeman, J.I., M.Dixon and H.J. Haussmann. Acetaldehyde in mainstream tobacco smoke: formation and occurrence in smoke and bioavailability in the smoker. *Chem. Res. Toxicol.* 15 (2002) 1331-1349.

1. *SEITA, Imperial Tobacco Group, 48 rue Danton, 45404 Fleury-les-Aubrais, France*
2. *Imperial Tobacco Limited, PO Box 525, Winterstoke Road, Bristol BS99 1LQ, UK*

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

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SS07

Analytical method validation – application of the accuracy profile to the method "determination of total alkaloids in tobacco"

In the context of methodology for cigarette product characterisation, it is important to subject the method to its intended-use (*e.g.* regulatory requirement) in order to confirm its "fitness for purpose" as described in ISO17025. The objective is to determine if the method can provide results with the required accuracy, a combination of precision and trueness, for all samples coming in for testing. This paper describes how to build a graphical representation, called the Accuracy Profile, based on measurements generated under intermediate precision conditions. This representation helps decision making regarding performance (namely the expected tolerance interval) and intended use (namely the acceptability interval).

As an example, validation of the determination of total alkaloids in tobacco by continuous flow analysis illustrates how this approach can be carried out.

The end-user defines first the limit of acceptability (*e.g.* ±15%) on the difference between the measurement and the true value, and the confidence level of the tolerance interval (*e.g.* 95%).

As no reference products with assigned values currently exist, five different samples with different alkaloid levels from 0.27 to 4.10% (dry weight basis) have been selected and values were assigned by spike calibration.

The experimental design consisted of preparing two independent replicates of the calibration standards and tobacco samples. This was repeated for five days involving three different operators preparing new reagents each day.

From this experimental design, intermediate precision standard deviation, bias, acceptability interval and expected tolerance interval were calculated. The accuracy profile representation allowed the conclusion that the total alkaloid method is valid from 0.73 to 4%, with 0.73% as the limit of quantification (LOQ).

Unlike classical validation methods that do not manage simultaneously trueness and precision, the Accuracy Profile approach allows a clear and easy comparison between method performance and the intended use.

1. *SEITA, Imperial Tobacco Group, 48 rue Danton, 45404 Fleury-les-Aubrais, France*
2. *Imperial Tobacco Limited, PO Box 525, Winterstoke Road, Bristol BS99 1LQ, UK*

TERAUCHI D.; MIKITA A.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST24

Identification of pigment in yellowish spots on cigarette papers of menthol products

The formation of spots on cigarette papers of products is a well-known phenomenon, and it is one of the issues affecting the appearance quality in the cigarette industry. There are a few studies on the countermeasures for spots, but no report about the direct detection of "key pigment" in spots. This research focused on identification of the key pigment in yellowish spots on cigarette papers from menthol products. This study was done because the market share of menthol products has been growing in Japan.

Generally, it is considered that the key pigment in spots comes from substances in tobacco. However, there are a variety of pigment families in tobacco such as carotenoids, flavonoids, chlorophylls, and quinoids. Thus, it is important to reveal the chemical characteristics of the yellowish pigment to clarify the target pigment family.

At first, the chemical characteristics (polarity, stability in air and stability to light) were investigated. As a result, the yellowish "pigment" has the characteristics of non-polarity, instability to air and light. This result and the yellowish colour of the spots suggests that the key pigment is in carotenoids.

A substantial number of spots were cut out from approximately 800 cigarettes and pigment was extracted with hexane. Liquid chromatography with a photo diode array detector (LC-PDA; 190-800 nm) was selected in order to identify the key pigment. Beta-carotene, lutein, and beta-damascenone were identified by comparison of absorption spectrum on LC-PDA and retention time to corresponding authentic samples.

Moreover, each of the absorption coefficients of the three pigments was investigated to estimate their contribution to colour intensity. As a result, the absorption coefficient of beta-carotene and lutein were higher than that of beta-damascenone. This suggests that beta-carotene and lutein are the key pigments of the yellowish spots in the three pigments identified.

Japan Tobacco Inc., R&D Group, Product Quality Research Div., 1-17-7, Yokokawa, Sumida-ku, Tokyo 130-8603, Japan

THOMPSON K.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST23

Calculation of the annual effective radiation dose arising from four radionuclides present in tobacco

The current scientific literature documents the presence of at least 25 radionuclides in tobacco. Several authors have attempted to calculate the annual effective radiation dose (AERD) originating from one or more of these radionuclides and have suggested that the AERD to the respiratory tract from smoking twenty cigarettes per day over the course of a year may reach several hundred microsieverts (µSv). This is important when regulatory bodies generally report that an individual's radiation exposure beyond background levels should not exceed 1 mSv (1,000 µSv) per year, from all sources. However the estimates in these studies may be challenged due to their use of questionable tobacco-to-smoke transfer rates. Calculations using more appropriate transfer values and recent data for caesium-137, lead-210, polonium-210 and potassium-40 (the only four radionuclides for which these calculations can currently be performed) indicate that the combined AERD in tobacco is 57 µSv. Since potassium-40 is by far the most abundant radionuclide present in tobacco, with lead-210 and polonium-210 regarded as being the most volatile, this value represents a reasonable surrogate for the total AERD from tobacco. By way of comparison, an AERD of this magnitude represents only around 5% of the mean worldwide background radiation dose obtained from the inhalation of radon gas (1.26 mSv, UNSCEAR 2008*).

* United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR). Sources of Ionizing Radiation. Volume I: Report to the General Assembly, Scientific Annexes A and B (2008).

JT International S.A., 1 rue de la Gabelle, CH-1211 Geneva 26, Switzerland

TICHA J.(1); FROSTINA J.(1); DONARSKI J.(2); BROKL M.(3); VAN HEEMST J.(1); BISHOP L.(1); WRIGHT C.(1)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SS15

Hyphenated and spectroscopic techniques for the screening of multiple analyte groups in tobacco smoke

In 2009, the Family Smoking Prevention and Tobacco Control Act granted to the US Food and Drug Administration (FDA) the authority to regulate the manufacture, marketing and distribution of tobacco products to protect public health. A list of harmful and potentially harmful constituents (HPHCs) in tobacco products and tobacco smoke was published in April 2012, comprising of more than 90 individual substances.

Methods have been established by different laboratories for the targeted analysis of tobacco and/or tobacco smoke constituents (predominantly Hoffmann analytes), usually for the determination of a single chemical class of substances by each method, but in many cases the methods have not been validated between laboratories. Such approaches require thorough optimisation of analytical procedures and frequently include clean-up steps to remove un-wanted matrix artefacts in order to determine sometimes challengingly low levels of target analytes.

As well as methods for the determination of HPHCs, fast screening methods for multiple classes of HPHCs are desirable for research purposes and to guide the development of confirmatory methods.

The requirement to measure increasingly large numbers of analytes with varying physico-chemical properties has encouraged the evaluation of novel screening analytical techniques that may enable not only non-targeted screening and "fingerprinting" of samples, but also rapid identification and semi-quantification of extended suites of target analytes.

We are exploring the potential capability of selected spectroscopic and hyphenated chromatographic techniques such as GC×GC/TOF-MS and GC/HRTOF-MS to identify substances in tobacco smoke. The techniques will be described and examples of the data obtained will be discussed.

1. *British American Tobacco, Group R&D, Regents Park Road, Southampton SO15 8TL, UK*
2. *The Food and Environment Research Agency, Sand Hutton, York YO41 1LZ, UK*
3. *University of Liège, 7 Place du 20-Août, 4000 Liège, Belgium*

TINDALL I.F.(1); CRUMPLER L.P.(2); MASON T.J.P.(1)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT06

Smoking machine design and yield errors under intense smoke regimes.
Part 1: The influence of dead volume on yield

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

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

The relationship between puff volume and vapour desorption is identified as a secondary mechanism for lowering yields and is the subject of a separate paper.

The hazards inherent in using a remote capture pad and the consequences for CI smoking are made clear. Recommendations for design changes that minimise the apparent lowering of yields are presented.

1. *Cerulean, Rockingham Drive, Milton Keynes, UK*
2. *Cerulean, Richmond, VA, USA*

TINDALL I.F.(1); CRUMPLER L.P.(2); MASON T.J.P.(1)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT07

Smoking machine design and yield errors under intense smoke regimes.
Part 2: The influence of puff volume on desorption of volatile smoke components

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

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

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

A model was developed using a synthetic TPM mixture that allowed the relationship between measured yield, puff intensity and machine type to be defined empirically.

The problems inherent in using a capture pad with high total air flow and the consequences for CI smoking are made clear. Predictions are made on the basis of an empirical model developed using the synthetic TPM mixture.

1. *Cerulean, Rockingham Drive, Milton Keynes, UK*
2. *Cerulean, Richmond, VA, USA*

TINDALL I.F.(1); CRUMPLER L.P.(2); MASON T.J.P.(1)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST12

A methodology for calculating the effect of puffing on desorption of condensate in HCI and ISO smoking

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

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

It is shown that this has particular relevance when comparing smoking machine types under HCI and ISO smoking and how clearing puffs can introduce unwanted variability if not consistently specified.

The applicability of this model is tested by comparing with experimental data gathered from smoking of cigars and cigarettes where high levels of TPM have been generated.

1. *Cerulean, Rockingham Drive, Milton Keynes, UK*
2. *Cerulean, Richmond, VA, USA*

TROFIMOV A.V.; MENSHOV V.A.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SS04

Redox processes in a smoker's organism: Between the imbalance and the stress

Estimating a smoker's health may utilise approaches based on the role of oxidative stress in pathogenic developments. In this context, oxidative stress is often considered as an imbalance in redox processes. Nevertheless, our analysis of the literature showed that although this imbalance may indeed furnish the chief cause of oxidative stress, it does not always provide sufficient condition on redox processes themselves. Cigarette smoke is a source of reactive oxygen species (ROS), which may cause oxidative modifications of biomolecules and intervene as signalling agents in adaptational and regulatory mechanisms. The protracted exposure of the smoker to smoke-borne and secondary (cell-derived) ROS is considered as one of the reasons for aging and weakening of the cellular antioxidant defense systems. However, to date little account has been taken of the fact that cigarette smoke is also a supplier of inhibitory species that retard free-radical processes, and whose role in triggering oxidative stress has barely been studied. Based on literature data (> 5000 publications), we have carried out a comprehensive analysis of potential biomarkers and regulators of oxidative stress. We conclude that at the current stage of knowledge it is untimely to consider smoking as the necessary immediate prerequisite of oxidative stress. Instead, we believe that to proceed from redox imbalance to oxidative stress, and in doing so triggering cellular dysfunctions, one should consider the coupling regulatory entity. The latter may be considered as the phase of accumulation of disturbances in the functioning of biological structures.

Emanuel Institute of Biochemical Physics, Russian Academy of Sciences, 119334 Moscow, Russian Federation

TROFIMOV A.V.; FEDOROVA G.F.; MENSHOV V.A.; TSAPLEV Yu.B.; VASIL'EV R.F.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT31

Smoke-derived chemiluminescence as phenomenon and experimental tool

Chemiluminescence emission can be detected from cigarette smoke. This is thought to link to smoke free radicals, many of which are reactive oxygen and nitrogen species of excited states. Smoke-derived chemiluminescence was first observed in the mid-1970s, however, to date its mechanistic nature remained unclear. A comprehensive knowledge on the nature of the excited state generation in smoke is required if facile chemiluminescence assays are to be developed into an analytical tool for studying the free radical content of cigarette smoke and its oxidative potential. The present study used commercial cigarettes of different tar levels available in Russia. The mainstream whole smoke was generated using a syringe simulating the machine-smoking condition using 35 mL puff volume, 2-s puff duration and one puff every 60 s. The experimental results provided insight into the general mechanism of the mainstream cigarette smoke derived chemiluminescence, which involved excited state generation in unimolecular transformation of smoke borne free radical species. However, the concentration of these radicals was found to obey a bimolecular (second-order) kinetics and depended on the amount of total particulate matter (TPM). Surprisingly, no energy transfer took place from the primary excited light-emitting species to luminophoric molecules in the smoke. The implications of the results are discussed in relation to the free radical activities in smoke.

Emanuel Institute of Biochemical Physics, Russian Academy of Sciences, 119334 Moscow, Russian Federation

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

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. PT17

LIP cigarettes: effect of band positioning

LIP cigarette paper is usually a standard cigarette paper on which zones with starch or another material with reduced air permeability have been printed equidistantly. The porosity of the LIP zones (reduced porosity zones) is designed so that the cigarettes will "stop" (minimum 75% test) smouldering when both conditions, that is, not puffed and in contact with a substrate, are satisfied. Currently, for technical reasons, the manufacturing of LIP products generates bands on the cigarettes that fall completely at random positions. We decided to study the impact of the band positioning (random and controlled) taking into account the moment when the smoker drops his cigarette. In order to assess the impact of the band position, we have developed an approach based on simulations using specific laws of probability to take into account the moment when the smoker drops his cigarette, the position of the LIP band, and the capacity of the LIP band in stopping the combustion. The burning time after dropping was used to determine if the control of the band position could be beneficial in improving the safety from burning cigarettes.

1. *SEITA, Imperial Tobacco Group, 48 rue Danton, 45404 Fleury-les-Aubrais, France*
2. *Imperial Tobacco Limited, PO Box 525, Winterstoke Road, Bristol BS99 1LQ, UK*

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

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SS11

Trend analysis: a relevant tool to assess post-regulation impacts

After the implementation of a new regulation, which imposes some changes on products or consumer behaviours, it is important to know if the impact is in line with the expectations or not. To assess this impact the best method is to compare the figures before and after the implementation and to show the significance of the change. Sometimes, conclusions are rapidly drawn up from few data in order to show a potential positive impact and to justify the regulatory development. Often, these conclusions are based on a short period of time consisting in the comparison of the two consecutive years before and after the implementation. However, this comparison can only show a difference between two years but cannot in any circumstance lead to the conclusion on an effect of the new regulation. Indeed, a solid conclusion can only be claimed if a thorough study of data over a long period of time is performed taking into account the controlled (*e.g.* trend, seasonality) and random variations. In this paper, we show the important factors that should be taken into account and the techniques for extracting an underlying pattern of behaviour in a time series (trend analysis). Examples on the impact on new regulations will also be discussed (*e.g.* LIP regulation).

1. *SEITA, Imperial Tobacco Group, 48 rue Danton, 45404 Fleury-les-Aubrais, France*
2. *Imperial Tobacco Limited, PO Box 525, Winterstoke Road, Bristol BS99 1LQ, UK*

WANNA J.; LE MOIGNE C.; LE BEC L.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. PT18

Tobacco column influence on cigarette paper

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

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

SWM International, 100 Northpoint Center East, Suite 600, Alpharetta, GA 30022, USA

WATANABE N.; KATSUYAMA N.; YUASA H.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST28

Measurement of the filling capacity of tobacco using near-infrared spectroscopy analysis

The filling capacity of leaf tobacco is an important physical characteristic which should be taken into consideration in the product design of a cigarette. The conventional method of measuring the filling capacity requires sample conditioning for several days in accordance with ISO 3402, and then the measurement has to be made using special instruments and skilled techniques, taking much time.

In recent years, near-infrared (NIR) spectroscopy analysis has become very easy following the remarkable development of the processing capabilities of computers, and it is now being widely used in the field of constituent measurement of chemical, food, pharmaceutical and agricultural products.

A simple method of measuring the filling capacity of tobacco using NIR spectroscopy was developed with acceptable accuracy. This measuring method detects the penetration absorption spectrum or the diffuse reflectance spectrum obtained by irradiating the ground tobacco with near-infrared rays, and then quantifying the filling capacity of the tobacco using a calibration curve which was prepared beforehand. Using many samples, the calibration curve for the quantitation was generated by multiple linear regression analysis or PLS regression analysis and it showed high correlation and an acceptable standard error.

Because this measuring method does not require time-consuming sample conditioning beforehand and takes only a little time (about one minute) for measuring each sample compared with the conventional method, the efficiency of the filling capacity measurement is improved dramatically. Moreover, since any type of tobacco (flue-cured, Burley, and Oriental) can be measured using the same analysis method, applications to the measurement of tobacco types other than leaf tobacco (*e.g.* expanded tobacco and reconstituted tobacco) are expected.

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

WEAVER S.A.; INTORP M.; PURKIS S.; ROEPER W.; STOTESBURY S.; WILLIAMS J.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST21

Use of an experimental smoking room to investigate claims that ETS residue (third-hand smoke) reacts with air pollutants to produce TSNAs

It has been reported that constituents of environmental tobacco smoke (ETS) may become sorbed onto household surfaces (such as furniture, walls and clothing) after a cigarette is smoked and subsequently desorbed over time. This type of smoke residue has been termed 'third hand smoke' and it has been suggested that it may be a potential health hazard. There have been recent claims made in the literature that reactions may occur between sorbed nicotine and atmospheric nitrous acid (HONO) to produce a novel tobacco-specific nitrosamine (TSNA), 4-(N-methyl-N-nitrosamino)-4-(3-pyridyl)-1-butanal (NNA), that is not routinely detected in tobacco smoke.

The objective of this study was to determine whether TSNAs are generated on the surface of three substrates (stainless steel, polyethylene and cellulose) after they have been exposed to sidestream cigarette smoke in an experimental chamber. The experimental chamber used in this study was an 18 m^3 airtight room with stainless steel interior walls. A temperature of 22 °C and a range of relative humidities between 30 and 70% were chosen for the experimental work. To determine the effect of differing concentrations of nicotine, NO/NO_x and TSNAs in the ambient air, two cigarette brands were chosen for analysis. Three 1 m^3 substrates (stainless steel, polyethylene and cellulose) were placed vertically on the walls of the experimental chamber, equidistant from the smoke source. In order to generate side stream smoke, ten cigarettes were smoked on a rotary smoking machine placed in the centre of the experimental room. Swabs were taken from the surface of each substrate on days one and four after smoking. LC-MS/MS analysis was performed to determine the concentrations of TSNAs in the sample material. We will discuss the results of this study and outline any further investigations required.

Imperial Tobacco Limited, PO Box 525, Winterstoke Road, Bristol BS99 1LQ, UK

WEN Jian-hui(1); DU Wen(1); PENG Bin(2); ZHANG Xiao-bing(2); LIU Xiao-qing(1); LI Yan-ping(1); XIE Fu-wei(2); WANG Zhi-guo(1); LIU Hui-min(2)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. PT02(Poster)

Filtration efficiencies and distribution patterns of nicotine in filters of different structure

The filtration efficiencies of filters of different structure to nicotine and the space distribution patterns of nicotine retention in filters were studied. By quantitatively analysing the nicotine deliveries in mainstream cigarette smoke and nicotine retention in filters, the filtration efficiencies, namely the ratio of nicotine retention amount by filter to total nicotine delivery, of four different filters were compared. With the same raw and auxiliary material, filter length and pressure drop, the filtration efficiencies of four different filters, regular CA (cellulose acetate) filter, external grooved CA filter, internal grooved CA filter and CA filter with cavity, were 39.06%, 45.45%, 39.63% and 31.75%, respectively. The filters were transversely and longitudinal concentric cut by a precision laser cutter, and then the nicotine retention in those divided filter parts was quantitatively analysed. The nicotine distribution data were calibrated firstly and then treated with polynomial fitting and interpolation analysis. The nicotine retention distribution patterns, including longitudinal, cross section radial and tridimensional space, in different filters were obtained. The practical contribution rates of individual longitudinal parts to nicotine retention were determined according to the longitudinal distribution patterns of nicotine retention in filters, and the space distribution pattern differences were discussed based on the changes of smoke flow in different filters.

1. *Research and Development Center of China Tobacco Hunan Industrial Co., Ltd., No. 426 Laodong Road, Changsha 410007, Hunan, P.R. China*
2. *Zhengzhou Tobacco Research Institute of CNTC, No. 2 Fengyang Street, Zhengzhou High-Tech Industrial Development Zone, Zhengzhou 450001, Henan, P.R. China*

WIECZOREK R.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SS03

The effect of charcoal filters on gas vapour phase *in vitro* toxicity tested in different air/liquid interface exposure systems

In addition to particulate matter, gas-vapour phase contributes significantly to smoke aerosol *in vitro* toxicity. Standard tests using cigarette condensate reflect cigarette toxicity to a limited extent only.

A number of methods in aerosol research were developed and applied for whole smoke testing. Some of them are hardly suitable for cigarette smoke since they are not free of artefacts. Trapping of gas phase in buffer or nutrient medium is limited due to different solubility of gaseous substances. Long term bubbling procedure or storage under cooling conditions lead to aging effects and changes in the biological activity. The dynamic nature of cigarette smoke has to be considered in the smoking test procedure in order to obtain reliable toxicological data. Rapid dilution and transport of whole smoke to exposure systems with cells at an air/liquid interface is essential for effective interaction of cells with the aerosol. Systems with transwell/inserts and cells in wetted surface area in 96 round well plate allow at least partially direct contact with smoke aerosol.

The fresh smoke aerosol cell exposure apparatus Bt020 can be used with both exposure systems. Cigarettes with standard cellulose acetate filter and highly loaded charcoal filter were tested. Toxicity (Neutral Red Uptake test) of gas vapour phase and whole smoke toxicity were determined for different exposure systems. Transwell systems as well as cells exposed in round bottom wells showed similar effects.

Charcoal filters decreased the contribution of gas vapour phase *in vitro* toxicity in whole smoke.

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

WILLIAMSON F.; MARTIN A.

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. PT12

***N*-Nitrosodiethanolamine in tobacco: method validation and levels present in US tobacco products**

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

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

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

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

Arista Laboratories, 1941 Reymet Road, Richmond, VA 23237, USA

YIN Dong-hong; CHEN Qian; ZHAO Li-hong; QIN Liang-sheng; GUO Xiao-yi; TAN Xin-liang; LIU Jian-fu

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. PT03(Poster)

Preparation of basic functionalised material and its application in simultaneously selectively reducing hydrocyanic acid and crotonaldehyde in cigarette smoke

For selectively reducing hydrocyanic acid (HCN) and crotonaldehyde in cigarette smoke, a series of novel basic functionalised materials were prepared by immobilising amides on different solids (such as silica gel, active carbon, alumina and tobacco stem) and then added into cigarette filter. In view of tobacco additive safety and the removal efficiency of HCN and crotonaldehyde, it was found that one of the amides immobilised on silica gel was the optimal filter additive, which was obtained by screening and optimising the varieties of amides and supports, as well as amide loading, impregnating duration, drying temperature and time, and the amount added in filter. The element analysis of the basic functionalised material indicated that there is no significant loss of amide during preparation process and the amount of surface basic sites (represented as $-NH_2$ groups) was 0.26 mmol/g. However, the BET surface area, pore volume, and pore size decreased slightly when amide was immobilised on silica gel. The FT-IR spectra suggested that there was hydrogen bond interaction between amide and the hydroxyl on silica gel surface. The cigarette with a dual filter containing 30 mg/cig of basic functionalised material was manufactured. It was found that compared with the control cigarette, the basic functionalised materials in filters could simultaneously selectively remove 20.7% of HCN, 32.2% of crotonaldehyde, and 50.9% of formaldehyde from cigarette smoke. The moisture content in smoke increased slightly, and the deliveries of tar, nicotine, and CO were almost unchanged. The smoothness and dryness of cigarette smoke was improved, its irritation was decreased, while the smoke style character of the cigarette was not changed by the basic functionalised material.

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

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

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT01

Study on the formation mechanisms of 4-aminobiphenyl in the combustion of cigarette including isotope-labelled phenylalanine

Tobacco smoke is a complex mixture of a wide variety of chemical compounds, including polycyclic aromatic amines (PAAs). In past studies, it was proposed that proteins in tobacco are major precursors of PAAs. At the 2007 CORESTA SSPT Meeting (Jeju), the authors reported that the addition of phenylalanine (Phe) to tobacco accelerated the formation of 4-aminobiphenyl (4-ABP) during inert pyrolysis. The aim of the current study is to identify the key intermediates of 4-ABP formation in the combustion of Phe-added tobacco. Incorporation of isotopic atom in Phe into 4-ABP was investigated using three kinds of stable isotope-labelled Phe, such as Phe-^{15}N, ^{13}C on the phenyl-1 position (Phe-(phenyl-1)-^{13}C), and ^{13}C on the benzyl position (Phe-3-^{13}C).

Each isotope-labelled Phe was added to flue-cured cut tobacco individually. Non-filter test cigarettes were made from the Phe-added tobacco. Total particulate matter (TPM) of the test cigarettes were collected on a glass filter under ISO standard smoking conditions. TPM was extracted with hydrochloric acid. The extract was applied to solid phase extraction followed by derivatisation. The derivatised PAAs were detected using GC/EI-TOF-MS. The obtained ion counts of isotope were corrected by using a natural isotope abundance ratio.

By the addition of Phe-^{15}N, the [M+1] ion of derivatised 4-ABP accounted for 60% of the total molecular ion counts of derivatised 4-ABP. In the cases where Phe-(phenyl-1)-^{13}C or Phe-3-^{13}C were added, it was determined that 80% of the total molecular ion counts of derivatised 4-ABP were observed as the [M+1] ion. These results indicate that both the phenyl group and the amino group in Phe would be important moieties to form the structure of 4-ABP in the combustion of Phe-added tobacco.

In this presentation, the results obtained using the equimolar mixture of two kinds of isotope-labelled Phe (*e.g.*, Phe-(phenyl-1)-^{13}C and Phe-^{15}N) will also be discussed.

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

YOSHIMURA Y.(1); OCHIAI N.(2); SASAMOTO K.(2); MITSUI K.(1); KANDA H.(2); HIGASHI N.(1); DAVID F.(3); SANDRA P.(3)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPT28

Comprehensive analysis of sulfur-containing compounds in tobacco smoke by using new separation and analytical techniques

Sulfur-containing compounds in food have been extensively studied because of their effect on aroma and taste. It is well known that sulfur compounds in complex matrices, such as tobacco smoke, are difficult to analyse. Sulfur compounds are typically present at very low concentrations and in GC-MS analysis they co-elute with other analytes from the tobacco smoke matrix. Therefore the required chromatographic resolution is much higher than conventional ^{1}D GC can provide.

In this presentation, a FEDHS-TD-GC-selectable ^{2}D/^{3}D GC-SCD/MS approach will be demonstrated for comprehensive analysis of sulfur compounds in tobacco smoke. For sample introduction, a specific dynamic headspace technique called Full Evaporation Dynamic Headspace (FEDHS) was applied in order to obtain high sensitivity, similar to large volume injection, while sample matrix effects are minimised. After conventional separation in a pre-column, fractions can be further analysed on a second and third dimension column. The selectable ^{2}D/^{3}D GC with selective sulfur detection using a sulfur chemoluminescence detector (SCD) is based on capillary flow technology and low thermal mass GC (LTM-GC). The main advantages of this system are simple and fast selection of ^{2}D GC-MS or ^{3}D GC-MS without any instrument set-up change.

After analysis of tobacco smoke, identification of each peak on SCD chromatograms was done by Aroma Office 2D software (GERSTEL) that provides highly accurate searches through the use of retention indices and database of over 70000 entities.

By using this system, we could isolate and detect over 450 sulfur-containing compounds on ^{2}D SCD chromatograms and identify 18 unique compounds.

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

YOSHIMURA Y.(1); T'KINDT R.(2); KAMIYA N.(1); MITSUI K.(1); OCHIAI N.(3); KANDA H.(3); HIGASHI N.(1); DAVID F.(2); SANDRA P.(2)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST15

Applying a lipidomics approach for the characterisation of tobacco smoke

Lipids are considered as an important fraction in biological samples. In lipidomics, a non-target analytical approach is applied to monitor different lipid classes and to obtain detailed information on the qualitative and quantitative composition of this fraction in a set of samples. This approach is typically used for biological samples, such as blood, tissue or skin. In this work, the applicability of lipidomics using LC-Q-TOF-MS will be demonstrated for the characterisation of the apolar fraction of tobacco smoke.

Four tobacco smoke extracts were compared. Tobacco smoke was collected and fractionated using a standardised protocol. The lipid fractions were subjected to a lipidomics approach to differentiate the tobacco types. The comparison of all extracts was performed using a defined lipidomics platform, based on JetStream electrospray ionisation (ESI) coupled to high-resolution quadrupole time-of-flight (Q-TOF) mass spectrometry (MS). Data analysis focused on identifying lipid signatures that can be linked to differences between the sample types. A large number of features could be unravelled as being statistically significant. After data processing, the focus was put on six significant features displaying the highest signal intensity, and representing the most abundant compounds that differ between the sample groups. Lastly, these six lipids were identified as unsaturated fatty acid esters of sterol-like compounds.

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, 8500 Kortrijk, Belgium*
3. *Gerstel K.K., 1-3-1 Nakane, Meguro-ku, 152-0031 Tokyo, Japan*

YU Jing-jing; WANG Sheng; XIE Fu-wei; XIA Qiao-ling; ZHAO Ge; ZHANG Xiao-bing

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST18

Simultaneous determination of ten aromatic amines in mainstream cigarette smoke by liquid chromatography/electrospray ionisation tandem mass spectrometry

For rapidly, sensitively and comprehensively analysing the aromatic amines in mainstream cigarette smoke, a liquid chromatography-electrospray ionisation tandem mass spectrometric (LC-MS/MS) method coupled with solid phase extraction (SPE) was developed. The particulate phase of mainstream smoke was collected on Cambridge filter pads, while the gas phase was trapped by 25 mL 5% HCl solution. The pads were extracted in an ultrasonic bath with HCl solution, the solution was purified with a HLB solid phase extraction column after being neutralised with NaOH, and then analysed with LC-MS/MS. The sample pre-treatment of the developed method was simple. Five groups of aromatic amine isomers were separated by means of HPLC column selecting and HPLC condition optimising. Ten aromatic amines including aniline, ortho-toluidine, meta-toluidine, para-toluidine, 1-naphthylamine, 2-naphthylamine, 3-aminobiphenyl, 4-aminobiphenyl, meta-phenylenediamine and meta-anisidine were quantitatively analysed. The limits of detection and recoveries of the method ranged from 0.04-1.03 ng/mL and 72.8%-133.5%, respectively; and the intra-day and inter-day precisions were less than 10% and 16%, respectively.

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

ZACHARIAS W.(1); GAO H.(1); PRASAD G.L.(2)

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. SSPTPOST06

Combusted, but not smokeless tobacco product preparations, cause DNA damage in human oral cavity cells

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

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

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

1. *Dept. of Medicine, J.G. Brown Cancer Center, University of Louisville, 505 S. Hancock St., CTRB 218, Louisville, KY 40202, USA*
2. *R.J. Reynolds Tobacco Co., R&D Department, PO Box 1487, Winston-Salem, NC 27102, USA*

ZHANG Wen-juan; LI Peng; HE Bao-jiang; ZHANG Jian-xun; ZONG Yong-li

CORESTA Congress, Sapporo, 2012, Smoke Science/Product Technology Groups, abstr. PT14(Poster)

Evaluation of release behaviour of nicotine from tobacco chewing gum

A simulated chewing machine capable of evaluating the *in vitro* release behaviour of nicotine from tobacco chewing gum (TCG) was developed. It was composed of a temperature control module, simulated chewing module, artificial saliva adding module, release solution collecting module and it could simulate buccal conditions from three respects of buccal temperature, salivary compositions and the rate of salivary secretion. The nicotine concentration in TCG was determined with a reversed-phase high-performance liquid chromatographic (RP-HPLC) method, and the release rate of nicotine was estimated by analysing the amounts of nicotine released from TCG and remaining in residues. The influences of flow rate of artificial saliva, temperature of release test and composition of artificial saliva on release rate were studied. The nicotine released from four samples of two commercially available TCG brands was evaluated with the simulated chewing machine and there was significant difference of nicotine release between TCG of different brands. The HPLC assay method developed for nicotine was validated in terms of linearity, limits of detection and quantitation (LOD and LOQ), inter-day and intra-day precisions, accuracy, and stability of solutions. The results of nicotine release from TCG obtained by the simulated chewing machine agreed with those of *in vivo* release experiments. The method was suitable for regulating the nicotine content in TCG and assessing the bioavailability of nicotine to TCG consumers.

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

ZIMMERMANN R.(1); EHLERT S.(1); SARAJI-BOZORGZAD M.(2); BENTE M.(2); WALTE A.(2)

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Detection of the molecular composition of pyrolysis gases of tobacco and cigarette materials in thermogravimetry with evolved gas analysis (TG-EGA) and on-line analysis of cigarette smoke by photo ionisation TOF mass spectrometry

It has been shown recently that mass spectrometry with photo ionisation (SPI-TOFMS) is well suited for on-line detection of volatile and semi-volatile compounds in tobacco smoke. Another interesting subject in tobacco science is the evolved gas analysis (EGA) in thermogravimetry of tobacco products (TG) by mass spectrometry (MS). TG-MS is a powerful technology for analysis of thermal degradation products from organic materials such as polymers, bio mass, paper or tobacco. TG-MS with electron ionisation (EI) gives predominately information on the evolved small molecules such as CO, CO_2, or H_2O. Complex organic molecules, however, often are not detectable due to the fragmentation associated with EI. Mass spectrometry using single photon ionisation (SPI-MS) now allows the analysis of the evolved pattern of intact organic molecules in conjunction with thermal analysis (TA) data. In this work the coupling of thermal analysis (TG and differential calorimetry, DSC) with SPI-TOFMS is described[1]. The SPI approach used for the TG-SPI-MS coupling is based on an innovative incoherent EBEL VUV-light source (Electron Beam pumped rare gas Excimer Light source). The VUV-light emitted by the EBEL is focused into the ion source of an ultra-compact orthogonal-acceleration-TOFMS (oaTOFMS). The new EBEL-SPI-oaTOFMS instrument achieves detection limits for organics in the ppb region. In addition to the technology, applications on different materials are presented. This includes polymers, tobacco, cigarette filter material as well as cigarette paper. Further applications in the field of tobacco science are motivated and discussed.

[1] R. Geißler, M.R. Saraji-Bozorgzad, T. Gröger, A. Fendt, T. Streibel, M. Sklorz, B.M. Krooß, K. Fuhrer, M. Gonin, E. Kaisersberger, T. Denner, R. Zimmermann, *Anal. Chem.* 81 (2009) 6038-6048

1. *Chair of Analytical Chemistry, University of Rostock, Rostock, Germany*
2. *Photonion GmbH, Schwerin, Germany*