Food and Chemical Toxicology 49 (2011) 1684–1696

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

The use of a novel tobacco-substitute sheet and smoke dilution to reduce toxicant yields in cigarette smoke

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ARTICLE INFO

Article history:
Received 1 October 2010
Accepted 4 April 2011
Available online 8 April 2011

Keywords:
Smoke toxicants
Tobacco-substitute
Filter studies
Biomarkers

ABSTRACT

The Institute of Medicine encouraged the pursuit and development of potential reduced-exposure products, tobacco products that substantially reduce exposure to one or more tobacco toxicants and can reasonably be expected to reduce the risk of one or more specific diseases or other adverse health effects. One approach to reducing smoke toxicant yields is to dilute the smoke with glycerol. We report chemical, biological and human exposure data related to experimental cigarettes containing up to 60% of a novel glycerol containing "tobacco-substitute" sheet. Analysis of mainstream smoke from experimental cigarettes showed reductions in yields of most measured constituents, other than some volatile species. *In vitro* toxicological tests showed reductions in the activity of smoke particulates in proportion to their glycerol content. Human exposure to nicotine was reduced by a mean of 18% as determined by filter studies and by 14% using 24 h urinary biomarker analysis. Smoke particulate exposures were reduced by a mean of 29% in filter studies and NNK exposure by similar amounts based on urinary 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol concentrations. These results show that reducing exposure to some smoke toxicants is possible using a tobacco-substitute sheet, although some smoke toxicants, and the sensory attributes of the smoke, remain as technical challenges.

1. Introduction

In 2001, the US Institute of Medicine reported that, since smoking related diseases were dose related, and because epidemiological studies show reduction in the risk of smoking related diseases following cessation, it might be possible to reduce smoking related risks by developing potential reduced-exposure products (PREPs). These they defined as products that (1) result in the substantial reduction in exposure to one or more tobacco toxicants and (2), if a risk reduction claim is made, products that can reasonably be expected to reduce the risk of one or more specific diseases or other adverse health effects (Stratton et al., 2001). Tobacco smoke is a complex, dynamic, mixture of more than 5000 constituents (Rodgman and Perfetti, 2009) of which approximately 150 have been documented as toxicants (Fowles and Dybing, 2003; Green et al., 2007). The toxicants are present in the mainstream smoke (MS) inhaled by a smoker and are also released between puffs as constituents of sidestream smoke (SS). While it is not known which of the many smoke toxicants are most related to tobacco-related diseases, and what level of reduction in exposure to these toxicants would be biologically relevant, the regulation of the yields of some toxicants in MS has been proposed (Burns et al., 2008).

There is a long history of attempts to modify tobacco products to make them less hazardous (Baker, 2006a; Waller and Froggatt, 1996; Hoffmann et al., 2001). For many years, governments and public health authorities in various parts of the world considered lower ISO tar yielding cigarettes as a way to reduce the health risks of smoking for those smokers who do not quit smoking (Froggatt, 1989). However, this product modification approach has more recently been highly criticised by various bodies, including the US National Cancer Institute (US National Cancer Institute, 2001). Modern filters are typically made from cellulose acetate (CA), sometimes with the dispersion of activated charcoal in the CA fibres of one section, giving a "dual carbon" (DC) or "Dalmatian"

Abbreviations: CA, cellulose acetate (filter); COHb, carboxyhaemoglobin; DC, dual carbon (filter); DMEM, Dulbecco's Modified Eagle's Medium; EU, European Union; FTC, (USA) Federal Trade Commission; IC_{50}, Concentration producing a 50% inhibition of growth of the responder cells; ICH, International Conference on Harmonisation; ISCSH, (UK) Independent Committee on Smoking and Health; ISO, International Organisation for Standardisation; MS, mainstream smoke; NFDPM, nicotine-free dry particulate matterl; NHFDPM, nicotine and humectants-free dry particulate matter; NIH, National Institutes of Health; NAB, *N*′-nitroso anabasine; NAT, *N*′-nitroso anatabine; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; PREP, a potential educed-exposure product; SS, sidestream smoke; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N*′-nitroso nornicotine; OECD, Organisation for Economic Cooperation and Development; TPM, total particulate matter.

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doi:10.1016/j.fct.2011.04.002

filter. Other additions may result in "triple filters". All filters reduce the amount of particulate matter in the MS and CA filters selectively reduce some phenolic compounds (Hoffmann and Wynder, 1963), while carbon removes additional volatile smoke constituents (Coggins and Gaworski, 2008).

Smoke dilution has also been used to reduce the overall smoke constituent yields of MS. Methods that have been used for smoke dilution include increasing the amount of air drawn through a filter during smoking and the use of glycerol as smoke diluents (Renne, 1992; Patskan and Reininghaus, 2003; Liu, 2004; Green et al., 2007). Air dilution is achieved by two main routes: filter ventilation and the use of cigarette paper with increased porosity.

The introduction of alternative materials into tobacco blends to reduce the overall amount of tobacco available for combustion has also been attempted, and in the UK several commercial cigarettes were launched during the 1970s containing 'Cytrel' or 'New Smoking Material'. These cigarettes were criticised by public health groups and did not prove popular with consumers and were withdrawn from the market (Waller and Froggatt, 1996).

Glycerol has been used as a carrier for semi-volatile tobacco constituents in a cigarette-like device that heats rather than burns tobacco (Gardner, 2000) and in an electrically heated cigarette (Patskan and Reininghaus, 2003) as well as by addition to tobacco blends (Liu, 2004).

This paper describes several experimental cigarettes that use a combination of approaches to make cigarettes with decreased yields of MS constituents. These experimental cigarettes were made with a novel tobacco-substitute sheet (TSS) that releases glycerol on heating. Thus, the TSS has a dual function: first, it decreases the amount of tobacco available in the overall blend, reducing its overall potential to generate smoke toxicants; and, second, it releases glycerol into MS to dilute the concentration of tobacco combustion sourced particulate constituents, including toxicants. Smoke chemistry, toxicological investigations and preliminary human studies on experimental cigarettes made with the TSS are described. They demonstrate that the generation of combustible experimental cigarettes using TSS can lead to reductions in overall exposure to some smoke toxicants. However, use of the TSS does reduce the sensory attributes of the smoke which might lead to smoker rejection of such products.

2. Materials and methods

2.1. Tobacco-substitute sheet specifications and construction

The TSS was made from calcium carbonate, bound with sodium alginate, loaded with glycerol and, in some iterations, coloured with caramel E150a, which makes the sheet look similar to the overall tobacco blend. Specifications for the materials used in the TSS construction are shown in Table 1. The TSS was manufactured in a band casting process, with all storage, piping and flow-control machinery meeting EU food hygiene standards. Briefly, an aqueous slurry of sodium alginate was prepared to which pre-determined amounts of calcium carbonate, glycerol and caramel were added. The mixture was thoroughly homogenised with a food-standard K blender and the viscosity adjusted by addition of potable water before pumping to a feed reservoir. The sheet was created by slowly laying down a liquid film onto a moving band, heated to 110 °C and the sheet was allowed to dry for 5–6 min before winding onto a bobbin for subsequent cutting to the desired dimensions for incorporation into a tobacco blend, during experimental cigarette manufacture.

The construction of experimental cigarettes and the physical characteristics required for optimal construction, and to provide burn and smoulder characteristics comparable to that of a conventional cigarette, was described by Dittrich et al. (2003a, 2003b). From these studies it was found that a mean calcium carbonate particle diameter of approximately 170 μm was the optimum for sheet formation because lower particle diameters gave a less pliable sheet that required more sodium alginate to hold together. Furthermore, in early experimental cigarette batches, ash formation was more uniform at this mean particle diameter. At greater calcium carbonate particle diameters, sheet formation became more difficult and the sheet itself was less uniform, often with a perforated appearance. Colouring of the substitute sheet was found to be important in some initial investigations in which the inclusion of non-coloured substitute sheet gave a white speckled appearance to the blend, leading to concerns over the unusual appearance of the experimental cigarettes.

2.2. Experimental cigarette specifications

Many experimental cigarettes were generated during the course of this work. However, most were minor design variations to address technical aspects of the production of TSS and its incorporation into experimental cigarettes or were minor adjustments to the paper permeability, filter ventilation and overall pressure drop, required to achieve a desired draw resistance and machine smoked nicotine-free dry particulate matter (NFDPM) yields. The physical characteristics of the key experimental cigarettes which showed major design variation are described in Table 2. The key variations were in the level of inclusion of TSS in the final blend, the addition of glycerol to the final blend and the type of filter used: either a cellulose acetate (CA) or a dual segment carbon (DC) filter. For all experimental cigarettes, appropriate 100% tobacco blend control cigarettes with matching filter types were also manufactured as controls for use in tests. The experimental cigarettes described were constructed using product design characteristics similar to brands of commercial cigarettes and in many tests they were compared directly with these commercial cigarettes as well as the control products.

2.3. Commercial comparator cigarettes

Silk Cut King Size (SCKS) filtered cigarettes, of the same nominal NFDPM yields as the experimental cigarettes, were purchased in the UK between 2001 and 2005.

2.4. Smoke chemistry testing

Prior to smoke chemistry analysis, cigarettes were conditioned according to the requirements specified in ISO 3402 (1999). Routine chemical analyses were performed according to the smoking conditions specified in ISO 3308 (2000) (i.e. a 35 ml puff of 2 s in duration taken every 60 s, abbreviated as 35/2/60) and ISO 4387 (2000) which was developed for NFDPM and nicotine analysis. A slight modification to the smoking parameters was required for other analytes, as described by Gregg et al. (2004). In addition to the standard 35/2/60 smoking parameters, in some studies smoke constituent yields were also determined under modified smoking machine puffing conditions such as 45/2/30, with half of the filter ventilation holes closed, as specified by the Massachusetts Department of Public Health (Commonwealth of Massachusetts, 1997). Sidestream smoke (SS) yields were also measured as described by Health Canada (1999), but only under ISO smoke generation parameters and for a wider range of smoke constituents.

2.4.1. Vapour phase analysis of MS

Vapour phase analysis of MS was based on the method described by Dong et al. (2000). Briefly, 5 experimental cigarettes were smoked using a Borgwaldt RM20/CS smoking machine, according to the parameters outlined in ISO 3308 (2000). The MS was passed through a 44 mm Cambridge filter pad, to remove particulates, before the remaining vapour phase was collected in a 3 L Tedlar bag. An internal standard of deuterated acetonitrile was added before injection into a GC/MS system for separation and analysis.

2.4.2. Particulate phase scans for MS

Particulate phase scans for MS matter trapped on the Cambridge filter pad during smoking to ISO 3308 (2000) conditions were carried out using two separate forms of analysis: headspace analysis (for released volatile or semi-volatile

Table 1
Components of tobacco-substitute sheet.

Ingredient	CAS no.	Use level (% by mass)	Other information
Calcium carbonate	471-34-1	78.5–80.0	Conforms to British & European Pharmacopoeia
Glycerol	56-81-5	12.5	British Pharmacopoeia, E422
Sodium alginate	9005-38-3	7.5	Food Grade, E401
Caramel	8028-89-5	0–1.5	Food Grade, E150a

Table 2
Characteristics of the experimental cigarettes.

	Cigarette code								
	S618	S619	S620	N324	R817	P462	T291	J473	U271
Nominal tobacco-substitute sheet inclusion (% dwb)[a]	0	60	30	0	60	60	50	60	50
Total blend weight (mg)	536	833	642	581	821	898	895	877	861
Tobacco weight in blend (mg)	536	312	446	581	313	363	456	351	431
Tobacco-substitute sheet weight in blend (mg)	0	521	196	0	508	535	439	526	431
Total blend moisture content (%)	13.6	10.4	12.4	10.0	11.4	12.5	11.7	13.5	10.3
Tobacco blend glycerol content (%)	0	0	0	0	0	0	2.5	0	2.5
Tobacco-substitute sheet glycerol content (%)	0	12.5	12.5	0	12.5	12.5	12.5	12.5	12.5
Total glycerol content (%)	0	7.8	3.8	0	7.7	7.5	7.4	7.5	7.5
Target NFDPM yield (I–SO) (mg/cig)	12[b]	12[b]	12[b]	5	5	5	5	5	5
Paper permeability (CORESTA units[c])	50	50	50	42	42	42	42	50	71
Rod length (mm)	62.5	62.5	62.5	56	56	56	56	56.5	57.8
Circumference (mm)	24.7	24.7	24.7	24.6	24.6	24.6	24.6	24.6	24.8
Filter type[d]	CA	CA	CA	CA	CA	DC	DC	CA	DC
Filter length (mm)	21	21	21	27	27	27	27	27	27
Filter ventilation level (%)	0	0	0	55	55	55	55	55	55

[a] These values were the target% inclusions, allowing for a manufacturing tolerance of ±5% – actual levels were within this tolerance.
[b] These experimental cigarettes were manufactured and tested before EU legislation banned the manufacture of cigarettes with an NFDPM yield of >10 mg, under ISO machine smoking conditions.
[c] Volume of air (cm^3) passing through 1 cm^2 min^{-1} at constant pressure difference of 1.0 kPa.
[d] DC filter contained 40 mg carbon in a Dalmatian format.

compounds) and solvent extraction of the filter pad residue (to analyse smoke constituents with a low vapour pressure). These are qualitative analyses and simple comparison of chromatographic traces was the most appropriate analysis.

2.5. In vitro toxicology testing

All tests were performed on smoke particulate matter trapped on a Cambridge filter pad after smoking experimental cigarettes using a Borgwaldt RM20/CSR smoking machine, according to the parameters outlined in ISO 3308 (2000). The Cambridge filter pads were cut into a three-quarter and a one quarter piece. The larger segment was immediately extracted into dimethyl-sulphoxide (DMSO) and the MS particulate solutions were stored on dry ice, protected from light. The DMSO extracts were diluted into culture medium for use within 24 h of generation, for all tests. The remaining quarter of the Cambridge filter was used to confirm NFDPM, water and, where applicable, glycerol yields.

The Neutral Red Cytotoxicity assay was performed as described by Baker et al. (2004). Briefly, V79 cells were maintained in tissue culture in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, now INVITROGEN, Paisley) supplemented with 10% (v/v) heat inactivated foetal calf serum (JRH Bioscience, Andover), and 0.52% Penicillin/Streptomycin. Cytotoxicity was expressed as a reduction in the uptake of Neutral Red dye into the lysosomes of cells after 48 h culture, measured by absorbance at 450 nm. Serial dilutions of MS particulate extracts were made to determine concentration-dependent inhibition of V79 cell growth. Four separate assays were performed for each test substance and IC_{50} values were derived by software analysis of the dose–response curves obtained. A higher IC_{50} value represents a lower cytotoxicity. This protocol conforms to the guidelines issued by the National Institutes of Health (USA) (2001).

The Ames' test was performed as described by Baker et al. (2004) using 5 *Salmonella typhimurium* responder strains: TA98, TA100, TA102, TA1535 and TA1537 in the presence and absence of an S9 metabolic activation system. The S9 phase consisted of the post-mitochondrial fraction of the livers from rats treated with Arochlor 1254 (MolTox, USA). All tests were performed in duplicate. A further two experiments using strains TA98, TA100 and TA1537 in the presence of S9 were carried out over the linear portion of the dose–response curve. All data were analysed using Dunnett's test for significant differences between solvent control plates and those treated with MS particulate extracts. The numbers of revertants per μg smoke particulate matter were calculated using the data from the linear portion of the dose–response curve. Subsequently, Tukey's statistic was used to compare specific activities of the test condensates. This protocol conforms to the OECD guideline 471 (OECD, 1997a) and the ICH guideline S2 (ICH, 2008).

The *in vitro* Mammalian Micronucleus Test was performed as described by Baker et al. (2004). Briefly, duplicate V79 cell cultures in DMEM (Gibco, now INVITROGEN, Paisley) supplemented with 10% foetal calf serum (JRH Bioscience, Andover) were pulsed with test or control samples for 3 h followed by a 17 h recovery both with and without S9 metabolic activation. Other cultures were exposed to test or control agent continuously for 20 h without S9 metabolic activation. At least 6 dose levels for each unknown were scored for cytoxicity and for micronucleus formation in bi-nucleate cells, on duplicate slides. Data were analysed by calculation of a mean value per unit dose and a test for normal distribution performed. Normally distributed data were analysed using a combined paired Student's *t*-test and data not normally distributed were analysed using Wilcoxon's signed rank test. This testing approach was based on the OECD draft guideline 473 now updated as draft guideline 487 (OECD, 2007).

The *in vitro* Mouse Lymphoma Assay was performed using L5178Y tk +/− cells, cultured in DMEM (Gibco, now INVITROGEN, Paisley) supplemented with 10% foetal calf serum (JRH Bioscience, Andover), as described by Clive et al. (1995) based on OECD guideline 476 (OECD, 1997b). Four experiments to encompass three treatment regimes were employed: two independent experiments using a 3 h treatment with metabolic activation; and two independent experiments to cover 3 and 24 h exposure without metabolic activation. Data are plotted as the means of duplicate cultures within each experiment.

2.6. Sensory evaluation of experimental cigarettes

Groups of 50 smokers of specific popular brands of commercial cigarettes were recruited. Smokers were of either sex, aged between 21 and 60 years, and the recruitment target was 50% male and 50% female subjects. A number of studies were performed: direct, single blind comparisons of experimental cigarettes or a tobacco blend control against smokers' usual brand at a central laboratory location; single blind analysis of experimental cigarettes compared to smokers' usual brands or tobacco blend controls at a central laboratory location; and open label, home use studies with limited numbers of experimental cigarettes. Detailed study plans were explained to all participants and informed consent documents were required to be signed as a condition of enrolment.

In the home use studies and during some laboratory tests, used cigarette filters were collected for chemical analysis to estimate mouth level exposure, as described below.

2.7. Used filter analysis

The analysis of used cigarette filters to determine the mouth-level exposure of smokers to nicotine and NFDPM has been described by Shepperd and Mariner (2001) and St Charles (2001). Minor modifications to these methods were made and the assays were performed as described by Shepperd et al. (2006).

2.8. Biomarker analysis

In a randomised, open label, double-crossover study conducted at Inveresk Research, Edinburgh UK, a group of 20 smokers of either sex, aged 21–60, who smoked Silk Cut King Size (SCKS) filtered cigarettes, attended a clinical centre for 15 consecutive days, including weekends, for a period of approximately 2 h between 10.00 and 12.00 am, each day. Although the subjects attended the clinical centre daily, the study was non-residential. Recruitment criteria included having been a smoker of more than 10 but fewer than 28 SCKS cigarettes per day for at least the previous 6 months. Exclusion criteria were: a positive pregnancy test, smoking outside the specified range of cigarettes per day, a COHb of <1.0%, plasma cotinine >50 ng/ml, recent blood or plasma donation, use of nicotine-replacement therapy in the previous 14 days, resting blood pressure or heart rate outside a predefined range, an adverse pulmonary function test, and a positive hepatitis A or B or human immunodeficiency virus test. The clinical study was conducted in accordance with the Declaration of Helsinki guidelines, and the principles of the ICH Good Clinical

Practice. The Inveresk Independent Research Ethics Committee approved the study and detailed study plans were explained to all participants who were then required to sign informed consent documents as a condition of enrolment.

On day 1 blood and saliva samples were taken and subjects were randomly assigned to usual commercial cigarette (SCKS) or experimental cigarette for the initial arm of the study. Subjects were provided with one day's supply of the appropriate product, a container for the collection of a 24 h urine sample and a containers for the collection of smoked cigarette filters. On each subsequent visit to the clinic, blood samples were taken and the previous 24 h urine sample collected. A smoking record for the previous 24 h and the container with the smoked cigarette filters were collected. Subjects were given another day's supply of cigarettes and containers for the 24 h urine and used filter collections. Each arm of the study (i.e. test product or usual brand cigarette smoking) lasted 7 days. On day 8 the switch to the other arm of the study occurred without any wash-out period or break in continuity. On day 15 samples and records were collected but further cigarettes and collection containers were not provided.

Subjects were asked to follow normal diet and exercise routines throughout the study and were advised that they could withdraw at any point.

Plasma and urine samples were split into aliquots and frozen before dispatch to independent analytical laboratories. The biomarkers measured were nicotine, cotinine, 3′-hydroxycotinine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) and their glucuronide conjugates. Urinary creatinine and 1-hydroxypyrene concentrations were also measured. The methods used for biomarker analysis were those described by Lowe et al. (2009). Statistical evaluation of biomarker concentrations was performed using a paired Student's *t*-test and data were examined for sequence and period effects by analysis of variance and Fisher's exact test. All statistical comparisons were made using Minitab software.

3. Results

3.1. Smoke chemistry studies on experimental cigarettes

The results of standard smoke chemistry analyses for the experimental cigarettes, with the additional analysis of glycerol yields, are shown in Table 3.

Experimental cigarettes S618, S619 and S620 were designed to give a machine NFDPM yield of 12 mg, prior to the imposition of the EU legislation limiting NFDPM yields to 10 mg, and N324, R817, P462, T291, J473 and U271 were designed to give a machine NFDPM yield of 5 mg, using the ISO puffing profile of 35/2/60. The NFDPM yields shown in the table include the glycerol content of MS. The glycerol yield is subtracted to give nicotine- and humectant-free dry particulate matter (NHFDPM) yield, as shown in the last column of Table 3. Under these machine smoking conditions inclusion of TSS in the tobacco blend, and additional glycerol in the blend for cigarettes T291 and U271, resulted in increased glycerol in the MS, decreasing the overall NHFDPM content. This decreased NHFDPM gives an effective glycerol dilution value which, for S619 (60% TSS) was 38.6%; for S620 (30% TSS) 15.1%; for R817 (60% TSS) 41.8%; for P462 (60% TSS) 45.1%; for J473 (60% TSS) 43.4%; for T291 (50% TSS + 2.5% tobacco blend glycerol) 39.3% and for U271 (50% TSS + 2.5% tobacco blend glycerol) 34.8%. Thus, the dilution of MS by glycerol was increased over the range 30–60% TSS inclusion, taking into account glycerol added to the tobacco blend in some experimental cigarettes. The CO yields of the experimental cigarettes were not decreased by the inclusion of TSS or by the addition of glycerol to the tobacco blends.

3.1.1. Extended chemical analyses

The analysis of a greater range of smoke constituents from the experimental cigarettes was performed under ISO smoking conditions. Representative results from three replicate analyses for yields of an extended range of smoke constituents from two experimental cigarettes with different levels of TSS inclusion and a 100% tobacco blend control are shown in Table 4. From these data it is apparent that, for most of the smoke constituents measured, there is a reduction in the MS yields from experimental cigarettes S619 and S620 compared to the tobacco blend control, S618. Also, for most constituents, there was a lower yield as the percentage inclusion of TSS increased and the reductions were approximately by the amount expected based on the glycerol yields from the experimental cigarettes. Ammonia, 4-aminobiphenyl, formaldehyde, NAB, quinoline, cadmium and mercury did not fit this general pattern as their yields were not statistically significantly different from the control cigarette for at least one of the two test samples.

3.1.2. Vapour phase analysis

The chemical analyses shown in Table 4 did not measure uniform reductions in MS constituents, particularly for the volatile smoke constituents. Therefore, the vapour phase constituents were examined using smoke captured in a Tedlar bag before analysis, which differs from the ISO standard smoking procedure. Results are shown in Supplementary Table 1 (available in the online version of the paper). For the majority of volatile smoke constituents, there was a greater reduction in yield as the percentage TSS inclusion in the experimental cigarettes increased; although, for some (crotonaldehyde, propionitrile and toluene) the pattern of change was not uniform. Furthermore, acrolein and carbon disulphide gave increased yields at both levels of TSS inclusion. These results, while consistent with an overall smoke dilution effect, highlight the variable nature of changes in volatile smoke constituents when only TSS is used as an approach to toxicant yield reduction.

3.1.3. Particulate and vapour phase scans

Particulate material extraction and qualitative scanning tests with a range of different experimental cigarettes showed some peaks that were not observed with matched control cigarettes made without TSS (data not shown). The most abundant peak was always glycerol and occasional other peaks were tentatively assigned identities of constituents that have previously been found in cigarette MS (Rodgman and Perfetti, 2009). Overall, no new peaks were seen in the vapour phase scans of experimental cigarettes (data not shown). Collectively, these scans suggest that, with the exception of the measurement of up to 40% glycerol, the overall spectrum of smoke constituents in experimental cigarettes is minimally affected by the inclusion of TSS in the tobacco blend.

3.1.4. Use of carbon in filters

The variable reductions for volatile smoke constituents suggested that other experimental cigarette design modification

Table 3
Mainstream smoke yields of experimental cigarettes under ISO 4387 machine smoking conditions.

Cigarette code	NFDPM (mg)	Nicotine (mg)	CO (mg)	Glycerol (mg)	NHFDPM (mg)
S618	12.8	1.10	11.3	0.0	12.8
S619	12.7	0.66	11.1	4.9	7.8
S620	12.6	0.97	10.9	1.9	10.7
N324	5.1	0.59	5.2	0.0	5.1
R817	5.5	0.34	5.5	2.3	3.2
P462	5.1	0.33	5.1	2.3	2.8
T291	5.6	0.40	5.5	2.2	3.3
J473	5.3	0.33	3.9	2.3	3.0
U271	4.6	0.32	4.8	1.6	3.2

Table 4
Smoke constituent yields in experimental and control cigarettes generated under ISO machine smoking conditions. All values are expressed to 1DP or 3 SF.

Cigarette code	Yield		Δ %[a]	*P* value	Yield	Δ %[a]	*P* value
% TSS inclusion	S618 0	S620 30			S619 60		
NFDPM (mg)	12.8	12.6	−2	12.7	−1		
Ammonia (ng)	16.3	17.3	6	NS	15.8	−3	NS
1-Aminonaphthalene (ng)	2.9	1.9	−35	<0.05	1.7	−40	<0.01
2-Aminonaphthalene (ng)	1.1	0.6	−43	<0.05	0.6	−50	<0.05
3-Aminobiphenyl (ng)	0.6	0.3	−43	<0.05	0.3	−52	<0.05
4-Aminobiphenyl (ng)	0.3	0.2	−21	NS	0.2	−21	NS
Benzo[a]pyrene (ng)	7.9	6.3	−20	<0.01	6.1	−23	<0.01
Formaldehyde (ng)	70.7	60.1	−15	NS	62.9	−11	NS
Acetaldehyde (μg)	540	421	−22	<0.05	335	−38	<0.05
Acetone (μg)	301	223	−26	<0.05	172	−43	<0.05
Acrolein (μg)	74.0	62.9	−15	<0.05	64.4	−13	<0.05
Propionaldehyde (μg)	47.5	36.6	−23	<0.05	25.7	−46	<0.05
Crotonaldehyde (μg)	20.3	16.0	−21	<0.05	9.3	−54	<0.05
Methyl ethyl ketone (μg)	50.9	34.1	−33	<0.05	37.7	−26	<0.05
Butyraldehyde (μg)	31.2	23.7	−24	<0.05	13.7	−56	<0.05
Hydrogen cyanide (μg)	122	91.5	−25	<0.05	58.6	−52	<0.05
NNN (ng)	19.0	14.1	−26	<0.01	12.9	−32	<0.01
NAT (ng)	38.0	27.0	−29	<0.01	22.8	−40	<0.01
NAB (ng)	5.0	5.0	0	NS	LOQ	>−25	–
NNK (ng)	32.0	22.1	−31	<0.01	19.8	−38	<0.01
Phenol (μg)	15.7	9.1	−42	<0.01	3.3	−79	<0.01
o-Cresol (μg)	2.8	1.8	−35	<0.01	0.8	−71	<0.01
m-Cresol (μg)	3.7	2.4	−36	<0.01	1.1	−71	<0.01
p-Cresol (μg)	7.6	4.7	−38	<0.01	1.9	−75	<0.01
Catechol (μg)	75.6	56.7	−25	<0.01	32.5	−57	<0.01
Resorcinol (μg)	1.6	1.2	−22	<0.05	0.7	−54	<0.01
Hydroquinone (μg)	65.3	48.3	−26	<0.01	27.4	−58	<0.01
Pyridine (μg)	9.0	6.9	−23	<0.05	5.9	−34	<0.05
Quinoline (μμg)	0.8	0.9	9	NS	0.3	−58	<0.01
Styrene (μg)	7.6	5.9	−23	<0.01	5.6	−26	<0.01
Chromium (ng)	126	125	−1	NS	97.0	−23	<0.05
Nickel (ng)	8.1	5.5	−32	<0.05	8.7	**7**	NS
Arsenic (ng)	78.6	59.8	−24	<0.05	44.5	−43	<0.01
Selenium (ng)	270	205	−24	<0.01	141	−48	<0.01
Cadmium (ng)	68.4	68.9	1	NS	66.1	−3	NS
Mercury (ng)	8.3	9.9	19	NS	5.2	−37	<0.01
Lead (ng)	54.6	45.3	−17	<0.05	44.8	−18	<0.05
Expected change (%)[b]	–	–	−15	–	–	−39	–

[a] The difference between the control and experimental cigarette yields expressed as a % (rounded to nearest whole number).
[b] Based on the glycerol yields of experimental cigarettes. LOQ, value less than the limit of quantification of the assay.

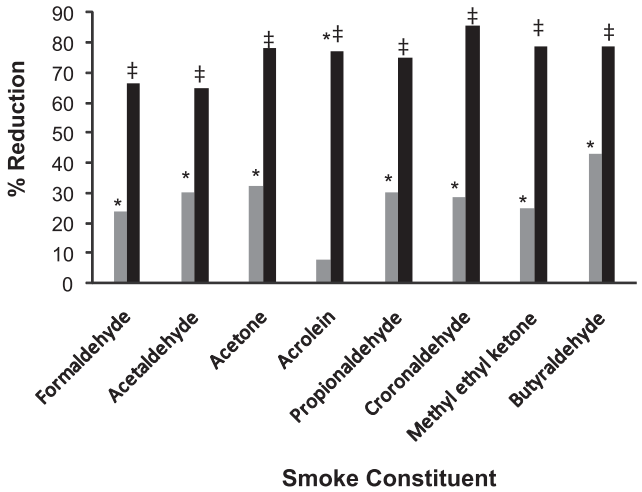

Fig. 1. Reductions in volatile carbonyls in experimental cigarettes with and without the addition of activated carbon to the filter. Reductions were calculated by differences in the mean values between the blend control with a CA filter (N324) and the experimental cigarettes: R817 (CA filter), P462 (DC filter). $^{*}p < 0.05$ compared with control cigarette. $^{‡}p < 0.05$ comparing experimental cigarettes.

should be used to reduce the overall yields of smoke toxicants. One approach taken was the inclusion of activated carbon in the filters of experimental cigarettes and a comparison of volatile carbonyl yields from two experimental cigarettes, one with a cellulose acetate filter (R817) and the other with 40 mg activated carbon in a DC filter (P462), compared to the blend control cigarette (N324) is shown in Fig. 1.

In Fig. 1 the volatile carbonyl yields measured in MS were reduced in both experimental cigarettes containing TSS compared to control cigarettes but the reduction was greater for P462 with the DC filter. The greatest change between experimental cigarettes was for acrolein yields, where a non-significant reduction of 8% was measured from R817 (TSS and a CA filter) compared to the control cigarette but a statistically significant 77% reduction was measured in P462, containing TSS and a DC filter.

3.1.5. Commercial comparators

Comparisons of experimental cigarette constituent yields with yields from commercial cigarettes under both ISO machine smoking conditions and more intense smoking conditions (the Massachusetts Department of Public Health smoking conditions, 45/2/30, with filter ventilation 50% blocked) were also performed. The comparison of yields from an experimental cigarette, U271 with a DC filter, and a commercial cigarette, SCKS, is shown in Table 5. Both cigarettes had a nominal NFDPM yield of 5 mg under ISO smoking conditions.

Table 5
Smoke constituent yields for experimental cigarette U271 and a commercial cigarette tested under ISO and Massachusetts machine smoking conditions. All values are expressed to 1DP or 3 SF.

Smoke constituent	MA smoking conditions				ISO smoking conditions			
	Yield		Δ %[a]	*P* value	Yield		Δ %[a]	*P* value
	SCKS	U271			SCKS	U271		
NFDPM (mg)	15.2	14.5	−4.6	NS	4.5	4.6	2	NS
Ammonia (μg)	12.7	15.7	24	NS	6.1	7.1	16	NS
1-Aminonaphthalene (ng)	10.2	6.5	−36	<0.05	5.8	LOQ	>−11[b]	–
2-Aminonaphthalene (ng)	12.3	7.0	−43	<0.05	4.6	LOQ	>−24	–
3-Aminobiphenyl (ng)	2.4	1.5	−37	<0.05	LOQ	LOQ	–	–
4-Aminobiphenyl (ng)	1.9	1.1	−40	<0.05	0.8	LOQ	>−20	–
Benzo[a]pyrene (ng)	13.2	11.1	−16	NS	3.8	4.1	8	0.05
Formaldehyde (μg)	38.0	27.0	−29	<0.05	10.0	9.0	−10	NS
Acetaldehyde (μg)	679	531	−22	<0.05	188.0	146.6	−22	<0.05
Acetone (μg)	395	251	−36	<0.05	111.0	53.3	−52	<0.05
Acrolein (μg)	95.0	84.0	−12	<0.05	24.0	14.9	−38	<0.05
Propionaldehyde (μg)	64.0	45.0	−30	<0.05	17.0	10.0	−41	<0.05
Crotonaldehyde (μg)	37.0	17.0	−54	<0.05	8.0	4.0	−50	<0.05
Methyl ethyl ketone (μg)	111	59.0	−47	<0.05	36.0	18.0	−50	<0.05
Butyraldehyde (μg)	52.0	28.0	−46	<0.05	17.0	10.0	−41	<0.05
Hydrogen cyanide (μg)	123	55.8	−54	<0.05	20.9	5.4	−74	<0.05
NNN (ng)	16.0	10.5	−34	<0.05	7.9	LOQ	>−37	–
NAT (ng)	33.4	15.3	−54	<0.05	15.9	6.3	−60	<0.05
NAB (ng)	5.3	3.6	−32	<0.05	LOQ	LOQ	–	–
NNK (ng)	19.9	10.1	−49	<0.05	8.0	LOQ	>−10	–
Phenol (μg)	31.2	4.2	−87	<0.05	7.9	LOQ	>−10	–
o-Cresol (μg)	8.6	1.6	−82	<0.05	2.6	0.1	−96	<0.05
m-Cresol (μg)	6.8	1.5	−78	<0.05	1.5	0.1	−95	<0.05
p-Cresol (μg)	13.9	2.7	−81	<0.05	3.5	0.3	−92	<0.05
Catechol (μg)	89.4	42.1	−53	<0.05	27.9	12.0	−57	<0.05
Resorcinol (μg)	1.8	0.9	−47	<0.05	0.4	0.1	−84	<0.05
Hydroquinone (μg)	83.5	36.0	−57	<0.05	25.5	9.4	−63	<0.05
Pyridine (μg)	10.5	2.2	−79	<0.05	2.1	0.4	−81	<0.05
Quinoline (μg)	0.5	0.1	−76	<0.05	0.2	LOQ	>−80	–
Styrene (μg)	15.4	4.3	−72	<0.05	2.3	0.7	−68	<0.05
Benzene (μg)	95.9	49.1	−49	<0.05	23.6	8.9	−62	<0.05
1,3-Butadiene (μg)	48.6	45.1	−7	NS	12.0	9.8	−18	NS
Isoprene (μg)	437	410	−6	NS	135.4	79.4	−41	<0.05
Toluene (μg)	151	50.0	−67	<0.05	31.1	LOQ	>−36	<0.05
Chromium (ng)	29.0	21.9	−25	<0.05	9.4	5.0	−47	<0.05
Arsenic (ng)	4.3	7.7	77	<0.05	1.5	1.9	24	NS
Selenium (ng)	14.9	9.3	−38	<0.05	5.7	2.5	−57	<0.05
Cadmium (ng)	18.8	13.2	−30	<0.05	5.6	2.4	−57	<0.05
Mercury (ng)	3.0	3.2	7	NS	3.5	1.9	−47	<0.05
Lead (ng)	17.4	22.2	27	<0.05	LOQ	LOQ	–	–

[a] The expected difference, based on glycerol yield under these machine smoking conditions, was −30% under ISO smoking conditions and −32% under Massachusetts smoking conditions, calculated using the assumption of identical blends.
[b] Where a difference is shown as >, the difference was based on the LOQ value. LOQ, value less than the limit of quantification of the assay.

Experimental cigarette U271 is an example of an experimental cigarette with high filter ventilation (55%) and, even under more intense machine smoking conditions with partial filter ventilation blocking most smoke constituents were lower by an amount comparable to the expected level based on the glycerol yield (Table 5). The volatile constituent yields from U217 were lower than those from SCKS, emphasising the role of the DC filter in this experimental cigarette.

3.1.6. Sidestream smoke (SS) analysis

Analysis of sidestream smoke constituents generated under ISO smoking machine conditions are shown in Table 6. These results are typical of many runs performed with experimental cigarettes manufactured with TSS. Most smoke constituents are present with a decreased yield in SS compared to control cigarettes made from 100% tobacco; however some constituents have consistently elevated yields. The greatest elevation was measured with formaldehyde, which had an almost 200% increased SS yield from experimental cigarette R817. Acetaldehyde and acrolein SS yield increases were also measured from R817 and this has been noted with other experimental cigarettes in these studies. The SS NFDPM also contains glycerol, which also contributes to an overall increased yield value.

3.2. Toxicological investigations on experimental cigarettes

3.2.1. In vitro cytotoxicity assays

For all experimental cigarettes, dose–response curves were constructed for Neutral Red uptake in V79 cells exposed to particulate matter, extracted with dimethylsulphoxide and diluted with culture medium. This allowed IC_{50} values to be calculated on a total particulate matter (TPM), NFDPM and NHFDPM basis. The results are shown in Table 7.

On a TPM and NFDPM basis, the IC_{50} values obtained for experimental cigarettes containing TSS were higher than those of matched control cigarettes although the values were not statistically significantly different for S620 which contained 30% TSS, the lowest level tested. These higher IC_{50} values show lower cytotoxic potency for the experimental cigarettes than control cigarettes. When the IC_{50} values were calculated on an NHFDPM basis, i.e. allowing for the glycerol content of the NFDPM phase, then no statistically significant differences between any of the samples were seen.

Table 6
Sidestream smoke yields in experimental and control cigarettes generated under ISO machine smoking conditions. All data expressed to 1 DP or 3 SF.

Cigarette code	SS yield		Δ %[a]	*P* value
	N324	R817		
% TSS inclusion	0	60	–	–
SS constituent				
NFDPM (mg)	16.9	23.9	41.4	<0.01
Ammonia (mg)	5.9	4.2	−30.1	<0.01
1-Aminonaphthalene (ng)	191	149	−22.0	<0.01
2-Aminonaphthalene (ng)	145	113	−22.1	<0.01
3-Aminobiphenyl (ng)	37.0	33.0	−10.8	0.05
4-Aminobiphenyl (ng)	19.0	16.0	−15.8	<0.05
Benzo[a]pyrene (ng)	160	106	−33.8	<0.01
Formaldehyde (ng)	519	1510	191	<0.01
Acetaldehyde (μg)	1270	2090	64.6	<0.01
Acetone (μg)	761	792	4.1	NS
Acrolein (μg)	441	647	46.7	<0.01
Propionaldehyde (μg)	245	199	−18.8	NS
Crotonaldehyde (μg)	126	118	−6.3	NS
Methyl ethyl ketone (μg)	226	258	14.2	NS
Butyraldehyde (μg)	196	158	−19.4	NS
Hydrogen cyanide (μg)	141	52.0	−63.1	<0.01
NNN (ng)	29.0	13.0	−55.2	<0.01
NAT (ng)	11.0	7.0	−36.4	0.05
NAB (ng)	23.0	6.0	−73.9	<0.01
NNK (ng)	135	54.0	−60.0	<0.01
Phenol (μg)	234	124	−47.0	<0.01
o-Cresol (μg)	23.2	16.0	−31.0	<0.05
m-Cresol (μg)	22.9	16.3	−28.8	<0.05
p-Cresol (μg)	43.6	26.0	−40.4	<0.01
Catechol (μg)	100	61.1	−38.8	<0.01
Resorcinol (μg)	1.1	0.4	−65.5	<0.01
Hydroquinone (μg)	112	60.8	−45.7	<0.01
Pyridine (μg)	279	159	−43.0	<0.01
Quinoline (μg)	12.2	5.0	−59.0	<0.01
1,3-Butadiene (μg)	148	77.0	−48.0	0.08
Isoprene (μg)	3280	1780	−45.7	<0.01
Acrylonitrile (μg)	192	93.0	−51.6	<0.01

[a] Increased yields are shown in bold.

Ames' tests were carried out using the dissolved particulate matter from the experimental cigarettes and control cigarettes using all 5 responder strains, with and without S9 metabolic activation. Only the results for the three responders that typically show positive activity with cigarette smoke fractions are presented in Table 8. The results are further summarised by presenting the number of revertants counted expressed on a per microgram basis, calculated for TPM, NFDPM and NHFDPM.

For TA98 and TA1537, calculated on a TPM and NFDPM basis, the revertants per microgram obtained for experimental cigarettes were lower than those of matched control cigarettes. All differences were statistically significant except for S620, which contained 30% TSS. With the TA100 strain, experimental cigarettes were not statistically significantly lower than control cigarettes, except for R817 but this strain typically gives lower overall responses than TA98 and so differences are difficult to observe. Once again, when the revertants per microgram were calculated on an NHFDPM basis, there were no statistically significant differences between any of the samples, for any responder strain. These results are typical of those found with all experimental cigarettes that contain TSS and release glycerol into the MS. That is, Ames activity in TA98 responder strain is always reduced in proportion to the glycerol content of the NFDPM and the difference typically reaches statistical significance with 5 replicates at approximately 30% TSS inclusion in the experimental cigarette blend.

The possible aneugenic and clastogenic effects of particulate phases from experimental cigarettes were examined by the induction of micronuclei using V79 cells. According to the established methods, micronuclei are only scored in bi-nucleate cells (i.e. those in the process of cell division, arrested with a spindle cell poison) after 3 and 20 h pulsing with test material, with and without S9 metabolic activation. Representative results for S619, under the conditions which give the greatest micronucleus induction (20 h exposure without S9 activation), are shown in Fig. 2.

Table 7
Cytotoxicity of mainstream smoke particulates expressed as IC50 values for experimental and control cigarettes.

Sample	Description	TPM (ng/ml)		NFDPM (μg/ml)		NHFDPM (μg/ml)	
		Mean	SD	Mean	SD	Mean	SD
S 618	Tobacco control/CA filter	27.0	7.0	21.0	6.0	21.0	6.0
S 620	60% TSS/C A filter	48.0	7.0*	36.0	6.0*	23.0	5.5
S 619	30% TSS/C A filter	35.0	3.0	28.0	2.0	22.5	2.0
N324	Tobacco control/CA filter	26.0	5.0	21.5	4.4	21.6	4.1
R817	60% TSS/CA filter	46.3	7.4*	36.8	5.5*	21.8	3.2
S 513	Tobacco control/DC filter	25.0	3.2	20.9	2.7	22.3	3.5
P462	60% TSS/DC filter	49.5	11.1*	39.9	8.6*	20.9	2.7
T291	50% TSS + glycerol/DC filter	44.9	7.3*	35.4	5.6*	21.9	3.9

* $p < 0.05$ compared to appropriate control cigarette.

Table 8
Ames activity of mainstream smoke particulates from experimental and control cigarettes. Values are expressed as a mean ± SD of 5 replicates and are shown to 2SF.

Sample	Responder strain + S9 description	Induced revertants μg^{-1}								
		TPM			NFDPM			NHFDPM		
		TA98	TA100	TA1537	TA98	TA100	TA1537	TA98	TA100	TA1537
S618	Tobacco control/CA filter	0.51 ± 0.1	0.17 ± 0.1	0.08 ± 0.04	0.64 ± 0.1	0.21 ± 0.1	0.10 ± 0.05	0.64 ± 0.13	0.21 ± 0.13	0.10 ± 0.05
S619	60% TSS/CA filter	0.29 ± 0.1*	0.09 ± 0.1	0.05 ± 0.03*	0.39 ± 0.1*	0.12 ± 0.2*	0.06 ± 0.04*	0.65 ± 0.20	0.19 ± 0.31	0.10 ± 0.06
S620	30% TSS/CA filter	0.43 ± 0.1	0.13 ± 0.2	0.08 ± 0.04	0.57 ± 0.2	0.17 ± 0.2	0.11 ± 0.06	0.67 ± 0.19	0.20 ± 0.28	0.12 ± 0.06
N324	Tobacco control/CA filter	0.83 ± 0.2	0.35 ± 0.2	0.13 ± 0.05	0.99 ± 0.2	0.41 ± 0.3	0.15 ± 0.06	0.99 ± 0.2	0.41 ± 0.3	0.15 ± 0.06
R817	60% TSS/CA filter	0.47 ± 0.1*	0.18 ± 0.1*	0.10 ± 0.05	0.59 ± 0.1*	0.22 ± 0.1*	0.13 ± 0.07	0.96 ± 0.2	0.37 ± 0.2	0.21 ± 0.11
S513	Tobacco control/DC filter	0.93 ± 0.2	0.27 ± 0.2	0.16 ± 0.05	1.11 ± 0.2	0.32 ± 0.2	0.19 ± 0.06	1.11 ± 0.2	0.32 ± 0.3	0.19 ± 0.06
P462	60% TSS/DC filter	0.49 ± 0.1*	0.18 ± 0.1	0.09 ± 0.05*	0.62 ± 0.2*	0.23 ± 0.2	0.11 ± 0.06*	1.07 ± 0.3	0.40 ± 0.3	0.19 ± 0.10
T291	50% TSS + Glyerol/DC filter	0.51 ± 0.3*	0.17 ± 0.2	0.09 ± 0.4*	0.64 ± 0.3*	0.21 ± 0.2	0.11 ± 0.5*	1.02 ± 0.5	0.33 ± 0.3	0.18 ± 0.08

* $p < 0.05$ compared to appropriate control cigarette.

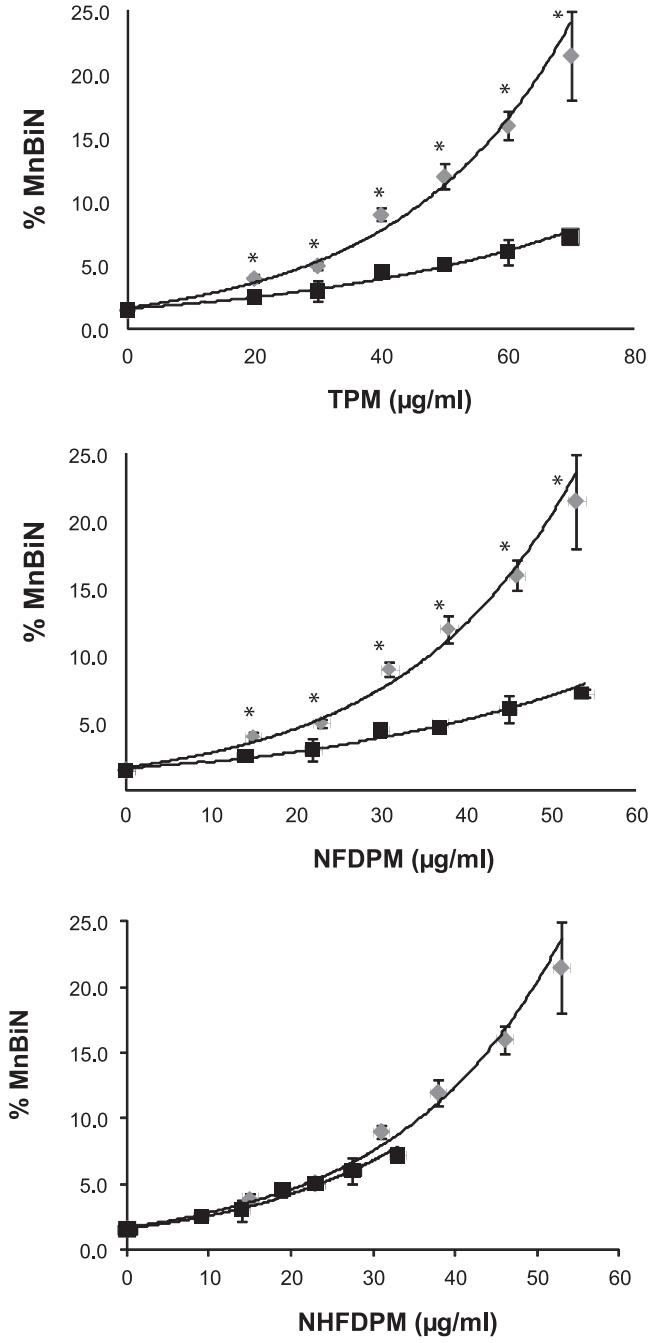

Fig. 2. Micronucleus induction in V79 cells exposed to mainstream smoke particulates from experimental or control cigarettes after 20 h exposure without S9 metabolic activation. Points plotted are the mean ± SEM of duplicate observations. Where error bars are not visible, they are contained within the symbol for the mean value. *$p < 0.05$ compared to control sample. ♦ S618 and ■ S619.

When the results are expressed on a TPM or an NFDPM basis, there is a clear and statistically significant lowering of micronucleus induction from the experimental cigarette compared to the control cigarette at each concentration of particulate matter (upper and middle panels). However, when the particulate matter solution is corrected for the amount of water, nicotine and glycerol present, by calculating the NHFDPM, there was no difference between the test and control (lower panel). These data suggest that the NHFDPM from both of the test and control cigarettes are part of the same dose–response continuum. However, it was not possible to continue this dose response curve for the experimental cigarette because of the practical limits of solubility, delivery to the culture system and the degree of cytotoxicity observed at the higher concentrations of both the test and control TPM solutions.

The potential for mutation induction in mammalian cells was also examined using the *in vitro* Mouse Lymphoma Assay. All assays were performed in the presence or absence of metabolic activators and were conducted for 3 or 24 h exposures. Results of a representative experiment are shown for S619, in Fig. 3. To simplify this figure, only the results expressed on a TPM and NHFDPM basis are shown at 3 h with and without metabolic activation and at 24 h without metabolic activation.

When the L5178Y tk +/− mutation frequency is plotted against the concentration of TPM added, under all conditions tested the experimental cigarette induced fewer mutations than a control cigarette (Fig. 3, upper panels). However, when the results were recalculated on an NHFDPM basis and the mutation frequency was expressed allowing for the water, nicotine and glycerol content of the particulate matter, under all conditions the dose–response curves moved together and the responses for experimental and control cigarettes lie together, within the overall uncertainty of measurement of the bioassay procedure (Fig. 3, lower panels).

3.3. Evaluation of experimental cigarettes in human subjects

By intention, the human evaluations were performed on a limited number of experimental cigarettes. With the exception of single item central location sensory evaluations, human studies were performed with co-ordination through a clinical centre, after local ethical committee approval. Regardless of the nature of the study, all subjects gave written informed consent, prior to commencement.

3.3.1. Sensory evaluation

Sensory evaluation of several experimental cigarettes was conducted over the course of these studies and, in most cases, the experimental cigarettes were found to be inferior to smokers' usual brands (data not shown). Sensory parity was only achieved for a limited number of experimental cigarettes and one example is given for U271 in Supplementary Table 2 (available in the online version of the paper).

3.3.2. Human exposure evaluation

Two approaches were taken to the estimation of human exposure to smoke constituents in short-term evaluation of the experimental cigarettes. The first approach was the analysis of used cigarette filters from smokers of commercial cigarettes and an experimental cigarette of similar NFDPM yield in a limited home-use study. The second approach was the analysis of biomarkers in 24 h urine samples collected from smokers of these products.

A limited home-use study of an experimental cigarette (J473), which contained 60% TSS in a cigarette with a CA filter, was performed in comparison with SCKS in smokers of that commercial product. The SCKS commercial cigarette was chosen to facilitate smoker subject recruitment because it was a popular brand on the UK market. The study was designed as a double cross-over, open label study, with each arm of the study (i.e. experimental cigarette or usual commercial cigarette smoking) lasting 7 days. Twenty-one subjects were recruited (13 male and 8 female) but one male subject withdrew on day 2 and his data were removed from the analysis. Subjects' age was 33.6 ± 10.7 years (mean ± sd) and their mean duration of smoking was 15.1 ± 8.4 years, with a mean cigarette consumption of 18.7 ± 3.5 cigarettes per day, as reported on recruitment.

The day by day numbers of cigarettes smoked, daily nicotine, NFDPM and NHFDPM exposure estimates based on filter analysis, are shown in Table 9.

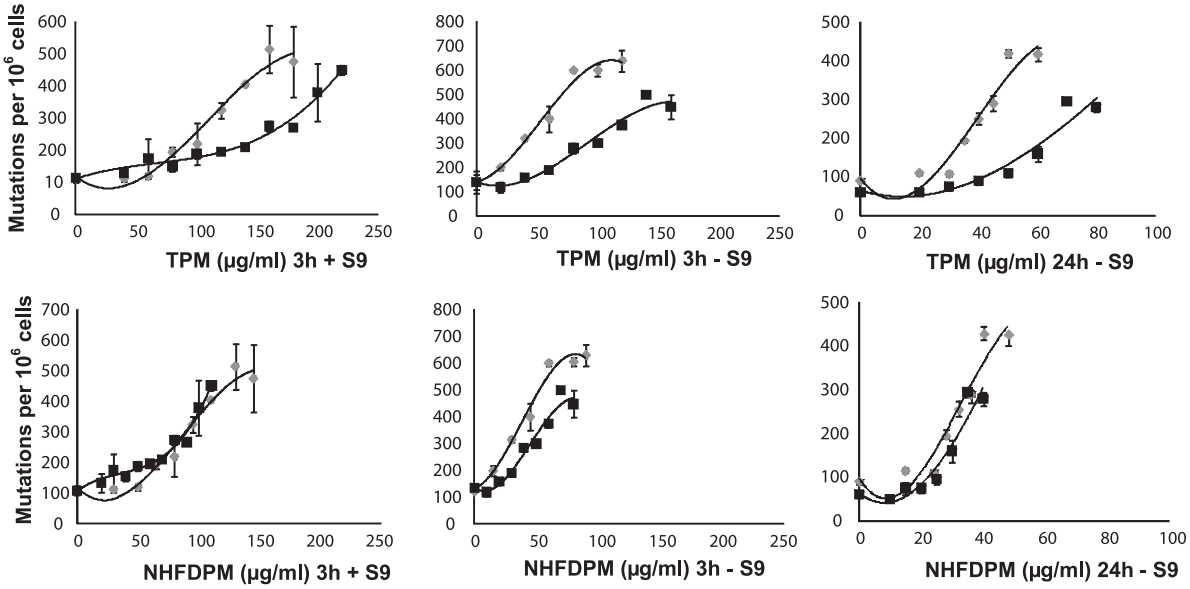

Fig. 3. Mammalian cell mutation frequency after exposure to mainstream smoke particulates from experimental and control cigarettes using L5178Y tk +/− cells with and without metabolic activation. Mean values of duplicate assays ± SEM are shown. Where error bars are not visible, they are contained within the symbol for the mean value. All upper panel results are shown on a TPM basis and all lower panel values are expressed on an NHFDPM basis. In the lower panels, for S619 some of the intermediate dose data symbols have been omitted for the clarity of the figures. ♦ S618 and ■ S619.

Table 9
Estimated nicotine, NFDPM and NHFDPM mouth level exposure based on filter analysis. Cigarettes smoked per day were recorded by subjects and the numbers cross-checked with the returned used cigarette filters on each daily visit to the clinical centre. The group mean ± SD for each parameter is shown (n = 20 for all).

Day	Cigarettes smoked per day (number)			Daily nicotine mouth level exposure (mg)			Daily NFDPM mouth level exposure (mg)			Daily NHFDPM mouth level exposure (mg)		
	SCKS	J473	p-value	SCKS	J473	p-value	SCKS	J473	p-value	SCKS	J473	p-value
1	18.5 ± 5.0	16.5 ± 5.2	0.130	23.2 ± 6.5	17.1 ± 5.7	<0.001	254.9 ± 71.2	291.4 ± 97.2	0.081	254.9 ± 71.2	161.9 ± 54.2	<0.001
2	18.4 ± 5.2	16.1 ± 4.6	0.044	21.9 ± 8.4	17.3 ± 5.8	0.016	241.1 ± 92.8	294.7 ± 98.0	0.026	241.1 ± 92.8	164.0 ± 54.6	0.001
3	17.7 ± 5.6	17.8 ± 3.6	0.922	20.7 ± 7.1	18.4 ± 5.4	0.026	228.1 ± 77.9	314.8 ± 92.1	<0.001	228.1 ± 77.9	175.3 ± 51.2	<0.001
4	18.6 ± 5.1	18.4 ± 4.0	0.849	22.4 ± 8.5	19.1 ± 5.9	0.091	246.4 ± 93.8	325.1 ± 100.4	0.004	246.4 ± 93.8	181.1 ± 55.9	0.004
5	18.6 ± 5.4	17.9 ± 4.3	0.489	22.2 ± 7.3	18.3 ± 5.4	0.019	244.1 ± 80.1	312.3 ± 91.4	0.003	244.1 ± 80.1	174.0 ± 50.8	<0.001
6	19.3 ± 5.4	17.7 ± 4.4	0.151	23.1 ± 7.4	18.4 ± 6.3	0.025	254.1 ± 80.9	313.5 ± 107.8	0.04	254.1 ± 80.9	174.4 ± 59.8	0.001
7	18.9 ± 4.9	18.0 ± 5.3	0.342	22.4 ± 7.3	18.8 ± 7.1	0.035	247.1 ± 80.7	321.0 ± 120.9	0.006	247.1 ± 80.7	178.6 ± 67.3	0.001
Mean	18.5 ± 5.1	17.5 ± 4.5	0.007	22.3 ± 7.4	18.2 ± 5.9	<0.001	245.1 ± 81.4	310.4 ± 99.9	<0.001	245.1 ± 81.4	172.7 ± 55.6	<0.001
Change[a]	−6%			−18%			27%			−29%		

[a] Change calculated from the overall mean values and expressed as a whole number. Smoking machine measured yields (ISO conditions) for nicotine, NFDPM and NHFDPM were 0.5, 5.0 and 5.0 mg (SCKS) and 0.33, 5.3 and 3.0 mg (J473) respectively.

On each day, with the exception of day 2, the number of cigarettes smoked was not different between the experimental cigarette and the smokers' usual commercial cigarette. Over the entire 7-day period, however, the mean number of cigarettes smoked per day was one fewer for the experimental cigarette compared to the usual commercial cigarette. The filter analysis data presented in Table 9 shows clear and consistent differences in smoke exposure estimates, from day 1 of the study onward. The daily mean nicotine mouth level exposure estimates were statistically significantly lower when smoking the experimental cigarette compared to their usual commercial cigarette, and the overall average daily reduction was 18%. This difference is not as large as the smoking machine yield difference between these cigarettes, which was 34% lower for the experimental cigarette. The NFDPM mouth level exposures were statistically significantly higher for the experimental cigarette smokers but the NFDPM for the experimental cigarette includes glycerol. When the amount of glycerol was subtracted from the NFDPM, giving an estimate of NHFDPM exposure, there was a statistically significant reduction in the

Table 10
24 h urinary excretion of biomarkers in smokers of an experimental cigarette compared to their usual commercial cigarettes.

	Total biomarker excreted in 24 h period			
	SCKS Mean ± SD	J473 Mean ± SD	Change (%)	p-value
Day 6				
NNAL (ng)	261 ± 132	209 ± 110	−20	0.032
1-hydroxypyrene (ng)	222 ± 127	259 ± 147	17	0.162
Nicotine metabolites* (mg)	13.5 ± 6.7	12.1 ± 5.5	−10	0.141
Day 7				
NNAL (ng)	298 ± 148	187 ± 70	−37	<0.001
1-hydroxypyrene (ng)	283 ± 150	240 ± 110	−15	0.116
Nicotine metabolites* (mg)	14.7 ± 5.3	12.6 ± 5	−14	0.021

* The total of nicotine, cotinine, hydroxycotinine and their glucuronide conjugates, calculated on a molar basis and expressed as a mass of nicotine. Smoking machine measured yields (ISO conditions) for nicotine, NFDPM and NHFDPM as stated in Table 9. Yields for NNK and pyrene were 20 and 29 ng (SCKS); 7 and 19 ng (J473) respectively.

estimate of mouth level exposure for the experimental cigarette compared to usual commercial cigarette smoke exposure. The group mean reduction over the entire 7 days was 29% for NHFDPM. Again this difference is less than the smoking machine yield difference, which was 40% lower NHFDPM for the experimental cigarettes. Statistical analysis of the test period showed that smokers of SCKS but not of the experimental cigarettes had a significantly lower daily nicotine exposure (filter estimation) in period 2 (23.4 mg/day in period 1 and 20.9 mg/day in period 2, $p < 0.05$). For both periods, these nicotine exposure estimates were significantly higher ($p < 0.05$) than the estimated exposure from the experimental cigarette (18.7 mg/day period 1 and 18.4 mg/day in period 2).

Exposure estimation was also based on biomarker analysis. This was performed in the 24 h urine samples collected on a daily basis from each subject. No subject's data were excluded from the analysis due to variation in total creatinine excretion. Although samples were collected for each 24 h period, it was expected that there would be a time delay for each subject to approach steady state when using the experimental cigarette and so, at the level of urinary biomarker measurement, the values for days 6 and 7 are most likely to show differences between the test and control products, if any difference exists. The 24 h urinary total excretions of the three urinary biomarkers of smoke exposure that were measured in this study are shown in Table 10 for days 6 and 7 only.

From Table 10, the mean urinary excretion of nicotine metabolites and of NNAL is decreased on both days 6 and 7 while subjects were smokers of the experimental cigarette compared to the period smoking the commercial cigarette. Using the paired Student's *t*-test, these values were statistically significantly different for NNAL (days 6 and 7) and for nicotine metabolites (day 7 only). There was wide variation in both individual subject's and group mean levels of 1-hydroxypyrene on both days 6 and 7 and none of the differences could be distinguished statistically. Analysis of test period also showed no statistically significant differences between period 1 or 2 use of commercial or experimental cigarette for any of the biomarkers (data not shown).

4. Discussion

This paper summarises a range of investigations that were performed on experimental cigarettes made with a novel TSS, designed to produce an overall smoke dilution, when used by smokers in the same way as conventional cigarettes. At the outset, it was recognised that smoking machine yields and laboratory testing would not be sufficient to establish the technical success of such a product and a major challenge was to achieve a reduction in exposure to smoke constituents under a smoker's typical circumstances; that is, in home use tests.

Each step in the process of making and testing these experimental cigarettes required a decision to be made and the approaches taken are discussed below. The first decision concerned the nature of the novel TSS itself. The concept of substituting the tobacco in a cigarette blend is not new. In 1977, 12 commercial cigarettes containing 25% TSSs were made available in the UK. They did not prove popular with smokers and were eventually withdrawn from the market (Waller and Froggatt, 1996). There were two different substitute sheets used in the 1970s cigarettes ('Cytrel' or 'New Smoking Material') and both were based on non-tobacco sources of combustible materials. The experimental cigarettes described here have extended this approach in two main directions. The first conceptual direction was to replace as much of the organic material in the substitute sheet as possible but to retain properties that allow it to be manufactured into cigarettes using conventional machinery. The resulting experimental cigarettes were designed to burn with uniform speed and then to produce an ash that behaves like that of a conventional cigarette. This was achieved using calcium carbonate and sodium alginate as a binder, as described in this paper and by Dittrich et al. (2003a, 2003b). The second conceptual development from the 1970s tobacco-substitute was a substantial increase in the amount of glycerol added to the TSS, with the intention that it would distil into the MS and dilute all of the other particulate smoke constituents, including any smoke toxicants. It was predicted that the use of alginate with additional glycerol would produce MS more acceptable to smokers than the MS from the 1970s tobacco-substitutes.

Glycerol was chosen for use in the TSS because it is a widely-used tobacco humectant that transfers into MS and because its use is permitted in tobacco ingredients legislation in countries which use positive ingredients lists. Further, the level of use in experimental cigarettes is below the permitted level in the UK for pipe tobaccos. Glycerol is a normal product of cellular metabolism in all animal species and it has been tested in many standard regulatory toxicology assays both as a stand-alone chemical and in a tobacco matrix. Thus, bacterial mutagenicity assays (Ishidate et al., 1984), mammalian cytogenetic studies (Lee et al., 1990) and long-term carcinogenicity studies (JECFA, 1976) on glycerol did not report glycerol-related adverse findings. Inhalation studies in rodents following exposure to smoke generated from cigarettes containing glycerol did not report any adverse events related to glycerol (Renne, 1992; Vanscheeuwijck et al., 2002, Heck et al., 2002). In mouse skin painting assays, the addition of high levels of glycerol to cigarette smoke condensate reduced the number of skin lesions (Wilson et al., 1978).

The yields of smoke constituents are a key feature in the development of an experimental cigarette intended to reduce exposure to toxicants; therefore, machine smoked yields of smoke constituents were used to make early decisions about the performance of the TSS. Although the ISO machine smoking test and its former FTC counterpart in the USA were not developed to mimic human smoking (Federal Trade Commission, 1967; ISCSH, 1988), smoking behaviour varies considerably and there is a trend in the literature to also report smoke yields measured under more intense machine smoking conditions. For these reasons different machine smoked yields, including those with partial filter ventilation blocking and with larger puff volumes (Commonwealth of Massachusetts, 1997), were also used when evaluating experimental cigarettes in this study. In general, these different smoking machine settings altered the yields of smoke constituents but had little impact on the ratio or yield relationships between various smoke constituents as reported by others; for example, Counts et al. (2005). Nonetheless, the wider analysis of smoke chemistry, including SS measurement, did show that some volatile smoke constituents were not reduced by the expected amounts based on TSS inclusion levels in the blend and that formaldehyde yields in SS were elevated in a reproducible manner.

The association of an elevation of formaldehyde in MS with the addition of saccharides to tobacco has been reported by Baker (2006b) but this was not seen in SS; although the generation of formaldehyde from sugars and its potential interaction with ammonia in MS and SS has been described (Baker et al., 2006). Therefore, several explanations for the elevated SS formaldehyde yield are possible: its increase might be a consequence of the decreased SS ammonia yield; it may represent a thermal degradation of the alginate at inter-puff smouldering temperatures; or it might reflect the lower tobacco packing density due to the presence of the TSS in the experimental cigarettes. The mechanism for the elevation of SS formaldehyde yields observed in the present studies remains to be established.

The variable reduction in formaldehyde and other volatile carbonyl yields that was found when comparing TSS containing experimental cigarettes with blend control cigarettes without

TSS, suggests that other cigarette design changes will be required to develop a combustible cigarette with lower overall toxicant yields. Activated carbon is an effective adsorbent for volatile chemicals and it is used in cigarette filters for this purpose (Tokida et al., 1985). The incorporation of activated carbon into a DC filter proved to be effective at reducing the volatile carbonyl yields in MS in the present study and represents a potential area for development in experimental cigarettes, to reduce toxicant yields; although a recent review was unable to reach a firm conclusion about the likely overall health effect of carbon use in cigarette filters (Coggins and Gaworski, 2008).

The evaluation of tobacco products using toxicological tests and the interpretation of their outcome, presents particular challenges. Nonetheless, the current experimental cigarettes were evaluated using guidelines developed for other chemical exposures (OECD, 1979; ICH, 2008; NIH, 2001). It would not be scientifically appropriate to evaluate an experimental cigarette as a stand alone product and so all tests were made as comparisons with appropriate control cigarettes, which were either made in the same manner as the experimental cigarettes but without addition of specific novel materials ('scientific controls') or were commercial cigarettes with similar design features and target NFDPM yields ('commercial controls'). This comparative toxicological approach would be appropriate under circumstances in which a PREP might be used as part of a harm-reduction strategy (Stratton et al., 2001). From the data presented in this paper, it was concluded that the experimental cigarettes generate the same spectrum of toxicological endpoints as control cigarettes but the dose–response curves are moved to the right; that is, on an equivalent concentration of smoke particulate phase basis, the experimental cigarettes were less active in these tests. There are no predictive tests available that would allow this conclusion to be interpreted further, regarding the potential short-term or long-term toxicity that might be expected in smokers of such products. Of equal importance, using the *in vitro* toxicity endpoints described in this study, when the concentration of smoke particulate materials from the experimental cigarettes was adjusted to take into account the amount of glycerol water and nicotine present, the dose–response curves could not be separated from those generated with control cigarettes. This shows that there was no gross distortion of the toxicological activity of the smoke: a conclusion supported by the smoke chemistry yields and the additional particulate and vapour phase scans that were described. Together these findings suggest that no additional burden of toxicity would be generated for a smoker of these experimental cigarettes. The question of whether the observed dilution effect would have any biological relevance to the health risks of smokers remains unknown.

To address the potential level of reduction of smoke constituent exposure that could be experienced by a smoker of an experimental cigarette, a variety of short-term, limited exposure studies were conducted with human smokers. Based on the past experience with tobacco-substitute materials, it was believed to be important to establish that smokers of commercial cigarettes would smoke experimental cigarettes without immediate rejection based on perceived sensory characteristics and so initial human evaluations with experimental cigarettes addressed their sensory properties. Sensory parity between an experimental cigarette and a commercial cigarette was achieved after many design iterations but this was not found on every occasion. The sensory properties of experimental cigarettes intended to reduce human smoked toxicant yields remain as a significant challenge for any future work in this area. Nonetheless, the data obtained were sufficient to suggest that seven-day trials with experimental cigarettes could be completed successfully, without outright rejection by volunteer smokers or observed modification of smoking behaviour due to grossly unacceptable sensory properties.

The estimation of mouth-level exposure for experimental cigarettes based on used filter analysis also confirmed that reductions in nicotine and smoke particulates were seen. The daily mouth level exposures to nicotine were statistically significantly reduced compared to the levels obtained from the smokers' usual brand but this was less than the reduction expected based on smoking machine yields. The daily mouth level exposures to NHFDPM were also statistically significantly reduced compared to smokers' usual brand cigarettes, suggesting that glycerol dilution of particulate phase smoke toxicants was achieved. In an attempt to determine whether these mouth level exposure reductions translate into reduced MS constituent absorption by smokers, 24 h urine samples were collected and analysed for biomarkers. The statistically significant reductions of 14% for nicotine metabolites and 37% for NNAL found on day 7 samples of 24 h urine, support the observations from the filter studies. Hecht et al. (1999) reported that the clearance of NNAL is bi-phasic, with a distribution (α) phase half-life of 3–4 days and a clearance (β) phase half-life of 40–45 days. Despite these long half-lives, Hecht et al. (1999) were able to see differences of approximately 65% in urinary NNAL concentrations from smokers after seven days of smoking cessation. Therefore, although this time period is not sufficient to reach a new steady-state NNK/NNAL concentration, it should be sufficient to observe a decrease in smokers switching to the use of cigarettes with relatively lower NNK yields. The 37% decrease in urinary NNAL concentration after 7-days of switching in the present study, is consistent with the half-life values calculated by Hecht et al. (1999).

The results observed with 1-hydroxypyrene were less clear-cut: the difference between the urinary biomarkers in the experimental arm and commercial cigarette arm of the study, measured on day 7 was not statistically significant. The clearance half-life for 1-hydroxypyrene (approximately 18 h: Buchet et al., 1992) is considerably shorter than NNK/ NNAL and so, it might be expected that smokers of the experimental cigarettes would have achieved a steady-state by day 7, if exposure were only due to smoking. The parent molecules for both of these urinary biomarkers, NNK and pyrene, are present in the particulate phase of MS, and so it is not clear how great the overall reduction in exposure to smoke toxicants was in this short cross-over study. However, NNAL is believed to be a more specific marker for tobacco (Carmella et al., 2003) than 1-hydroxypyrene (Alexandrie et al., 2000; Apostoli et al., 2003) and Hecht (2002) concluded that the lack of specificity for tobacco exposure detracts from the overall utility of 1-hydroxypyrene as a biomarker of exposure to tobacco smoke. Taken together, the results of the present study suggest that a longer switch period and possibly the use of more subjects might be required to evaluate urinary biomarkers in smokers of experimental cigarettes.

Overall, the results described in this study have confirmed that it is technically possible to make combustible cigarettes in which a large proportion of the tobacco is replaced with calcium carbonate and for the cigarettes to be manufactured successfully. The inclusion of glycerol at 12.5% in the TSS, with the direct addition of up to 2.5% in the final tobacco blend, was also technically successful as an approach to tobacco smoke dilution, offering a possible alternative to air dilution approaches for the design and development of cigarettes with reduced toxicant yields. The experimental cigarettes generated for this study were not ideal because not all MS constituents were diluted by the amount expected based on TSS inclusion and glycerol dilution calculations. Typically, volatile MS constituent yields were not reduced and SS formaldehyde yield was reproducibly increased. Greater reductions of the volatile MS constituent yields were obtained with the use of DC filters, suggesting that further developments in filter technology could play a role in reduced toxicant cigarette design. It is also recognised that a wider range of biomarkers and other tests must become available

for the complete evaluation of experimental cigarettes (Stratton et al., 2001; Gregg et al., 2006; Hatsukami et al., 2009) and attempts to qualify and validate biomarkers for such purposes have commenced (Lowe et al., 2009; Calapai et al., 2009).

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The work described in this paper is a summary of several years of research at Group R&D, British American Tobacco, and the following were major contributors to the studies described:

R.R. Baker, C. Barber, P. Bevan, P. Biggs, L. Bishop, D. Breheny, A. Bushby, S. Coburn, M. Coleman, W. Cooper, H. Digard, M. Dixon, A. Griffiths, L. Guy, E. John, K. Kalarai, F. Lowe, J. McAughey, A. Manson, D.C. Mariner, E.D. Massey, A.L. Parry, J. Philips, S.K. Prasad, G.A. Read, A. Richter, C.J. Shepperd, S. Slayford, G. Smith, J.P. Sutton, N. Warren, J. Whittle, A. Williams, J. Williamson.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fct.2011.04.002.

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