Inhalation Toxicology

http://informahealthcare.com/iht
ISSN: 0895-8378 (print), 1091-7691 (electronic)

Inhal Toxicol, 2015; 27(12): 629–640
 DOI: 10.3109/08958378.2015.1080773

Taylor & Francis
Taylor & Francis Group

RESEARCH ARTICLE

A comparative assessment of cigarette smoke aerosols using an *in vitro* air–liquid interface cytotoxicity test

David Thorne, Annette Dalrymple, Deborah Dillon, Martin Duke, and Clive Meredith

British American Tobacco, Group R&D, Southampton, Hampshire, UK

Abstract

This study describes the evaluation of a modified air-liquid interface BALB/c 3T3 cytotoxicity method for the assessment of smoke aerosols *in vitro*. The functionality and applicability of this modified protocol was assessed by comparing the cytotoxicity profiles from eight different cigarettes. Three reference cigarettes, 1R5F, 3R4F and CORESTA Monitor 7 were used to put the data into perspective and five bespoke experimental products were manufactured, ensuring a balanced and controlled study. Manufactured cigarettes were matched for key variables such as nicotine delivery, puff number, pressure drop, ventilation, carbon monoxide, nicotine free dry particulate matter and blend, but significantly modified for vapor phase delivery, via the addition of two different types and quantities of adsorptive carbon. Specifically manufacturing products ensures comparisons can be made in a consistent manner and allows the research to ask targeted questions, without confounding product variables. The results demonstrate vapor-phase associated cytotoxic effects and clear differences between the products tested and their cytotoxic profiles. This study has further characterized the *in vitro* vapor phase biological response relationship and confirmed that the biological response is directly proportional to the amount of available vapor phase toxicants in cigarette smoke, when using a Vitrocell® VC 10 exposure system. This study further supports and strengthens the use of aerosol based exposure options for the appropriate analysis of cigarette smoke induced responses *in vitro* and may be especially beneficial when comparing aerosols generated from alternative tobacco aerosol products.

Keywords

Aerosol, BALB/c 3T3, cigarette comparison, neutral red, 3R4F, tobacco smoke, vapor phase, whole smoke

History

Received 17 June 2015
Revised 24 July 2015
Accepted 29 July 2015
Published online 3 September 2015

Introduction

Cigarette smoking is a major risk factor for many adverse health conditions, including vascular disease, respiratory disease and lung cancer (IARC, 2004; Stratton, et al., 2001). Given this, the tobacco industry has spent many years investigating reduced exposure technologies, cigarettes and devices, which ultimately may limit exposure in those that continue to smoke tobacco products. The concept of tobacco harm reduction is defined by the Institute of Medicine (IOM) as ''decreasing total morbidity and mortality without completely eliminating tobacco and nicotine use'' (Institute of Medicine, 2011), through the use of potentially reduced exposure products (Stratton et al., 2001). The use of such products may reduce the risk of one or more specific diseases or other adverse health effects compared with the risks associated with the use of traditional tobacco products (Institute of Medicine, 2011; Stratton et al., 2001).

Address for correspondence: David Thorne, British American Tobacco, Group R&D, Southampton SO15 8TL, UK. Tel: 02380 58 8088. Fax: 02380 58 8856. E-mail: David_Thorne@bat.com

The chemical composition of smoke from any product results from the choice of tobacco blend, the design/format, the presence or absence of a filter and the respective filter components, such as charcoal and/or other selective adsorptive materials. Recent examples of technologies used to reduce toxicant profiles include, but are not limited to; substitute tobacco sheet, which acts as a tobacco diluent (McAdam et al., 2011); the development and refinement of cigarette design, format and selective filtration (Bombick et al., 1997; Branton et al., 2011a,b; Dittrich et al., 2014; Norman, 1999); treatment of tobacco prior to cigarette manufacturing (Liu et al., 2011); agronomic practices (Lewis et al., 2008) and the development of alternative products such as electronic cigarettes and heat not burn devices (Doolittle et al., 1990; Goniewicz et al., 2014; Smith et al., 1996).

It has been widely accepted that short term *in vitro* studies may provide valuable information (Andreoli et al., 2003) on the toxicity of cigarette smoke and may even be useful to measure the mechanistic and biological end-points linked to chronic disease states, biomarkers of disease or biological effect. *In vitro* genotoxicity and cytotoxicity tests are used world-wide for new chemicals as an initial screen to determine their mutagenic and cytotoxic potential. International guidelines have also been developed (e.g. Organization for Economic Cooperation and Development;

International Conference on Harmonization [ICH]) to ensure uniformity of testing procedures prior to submission of data to regulatory agencies. Currently, no such guidelines exist for the testing of smoke aerosols derived from tobacco products *in vitro*. This limits the capability of the tobacco industry and others involved in tobacco-related research to assess and compare current products with potentially reduced exposure products and other tobacco aerosols such as electronic cigarettes and products that heat tobacco as opposed to burning it. As a result there is a clear need and drive to develop *in vitro* approaches for aerosol testing.

The efficiency with which *in vitro* assays can be conducted and the relevance of the results depends solely on the test article. In the context of human risk to smoking and smoke related products, the test article (smoke aerosol) should mimic as close as possible, human exposure. With the advent of aerosol air–liquid interface (ALI) exposure devices and technologies, such as those supplied by CULTEX®, Vitrocell®, Borgwaldt, Burghart and even one off bespoke systems (Thorne & Adamson, 2013a), there is focus on optimizing existing *in vitro* techniques and to develop new ones to work alongside these aerosol-exposure devices. With the development of these new exposure technologies comes a necessity to understand aerosol dilution, delivery and exposure principles, which may differ between smoke exposure technologies. This will ultimately ensure that any conclusions derived from data are accurate and appropriately considered and will facilitate cross-platform comparisons.

This study describes the evaluation of a Mouse fibroblast (BALB/c 3T3 clone A31) cytotoxicity technique to discriminate between cigarette smoke aerosols *in vitro* using reference and specially designed products, aimed at the modification of the vapor phase, whilst balancing particulate and puffing parameters. The applicability and functionality of this protocol was confirmed with the use of reference cigarettes, 1R5F, 3R4F and the CORESTA Monitor (CM7), all delivering different smoke toxicant yields. Biological responses from 3R4F reference cigarette smoke, generated 12 months apart were directly compared, for assay variability and robustness and found to be statistically comparable. A more structured approach was also investigated, where different experimental cigarettes were manufactured, matched for variables such as particulate and nicotine delivery, puff number, pressure drop (PD), carbon monoxide (CO), ventilation and blend, but significantly modified for vapor phase delivery through the incorporation of two different adsorptive carbons. This strategy allows cigarettes to be directly compared with each other in a consistent manner, whilst investigating vapor phase-only associated effects. The vapor phase is made up of known smoke toxicants (Fowles & Dybing, 2003; Hoffmann et al., 1997), with clear biological activity, it is also theorized to be the driving contributor of cytotoxicity. This is supported by extensive *in vitro* and *in vivo* data all showing the cytotoxicity and mutagenicity of the vapor phase of cigarette smoke, indicating this as a significant driver of potential adverse health effects (Azzopardi et al., 2015; Bombick et al., 1997; Fukano et al., 2004; Witschi et al., 1997).

This study demonstrates clear differences between these manufactured products and their associated dilution IC_{50} (defined as the dilution at which 50% cytotoxicity is observed). It concludes that this *in vitro* test combined with an aerosol exposure device such as the Vitrocell® VC 10 can be further used to assess the potential cytotoxicity of future aerosol related tobacco products, which may be especially beneficial when comparing aerosols generated from potentially reduced exposure products and alternative and emerging tobacco categories, particularly those aimed at the reduction of vapor phase components of the smoke which are not easily captured or assessed *in vitro*.

Materials and methods

Chemicals and reagents

All chemicals and reagents were obtained from Sigma-Aldrich (Gillingham, UK) unless otherwise stated. All cell culture media was obtained from Gibco® via Life Technologies (Paisley, UK).

Cigarettes

Kentucky reference cigarettes, 1R5F and 3R4F were obtained from the University of Kentucky (Kentucky, USA), CM7 was supplied by Cerulean, UK. All other cigarettes used in this study were manufactured at British American Tobacco's Research and Development facility (Southampton, UK). Prior to analysis, all cigarettes were conditioned for at least 48 h at 22 ± 1°C and 60 ± 3 % relative humidity in accordance with the International Organization of Standardisation (ISO) guideline (ISO 3402:1999). Cigarettes were smoked exclusively to the ISO puff regime, defined as one 35 ml puff per 60 s, over 2 s (ISO 3308:2012) with an 8-s exhaust.

Manufactured cigarettes were made to the same specification [length, circumference, blend, ventilation, filter configuration, PD (often referred to as the draw resistance of a cigarette)], but modified for filter additive, using either activated coconut charcoal (CC) or activated synthetic carbon (SC). Cigarettes were manufactured with a dual filter with different quantities of either CC or SC (45, 80, 80 or 110 mg SC). A control product was manufactured to the same technical specification with a mono cellulose acetate (CA) filter, termed Control.

Test cigarettes were constructed to a cigarette circumference of 24.6 mm with a 56 mm tobacco rod containing 43% Virginia, 25% Burley, 9% oriental tobacco blend, with 10% stem, 5% extruded and 8% reconstituted tobacco. Cigarettes were manufactured to a tobacco rod density of 240 mg/cm^3 with a moisture content of 13.5%, with a 27 mm filter segment (either a mono acetate or a dual filter carbon segment) to a total cigarette length of 83 mm. The only marked difference in these cigarettes was the filter additive. Carbon particles were interspersed amongst the CA set in a commercially based dual filter configuration next to the tobacco rod, with 7% triacetin plasticizer (Figure 1).

The amount of and type of carbon added to test cigarette filters was selected to produce a range of different vapor phase yield products. Irrespective of carbon type and loading, cigarettes were matched for key characteristics such as, nicotine free dry particulate matter (NFDPM) delivery (mg/cig), open cigarette PD, CO (mg/cig), puff number (/cig),

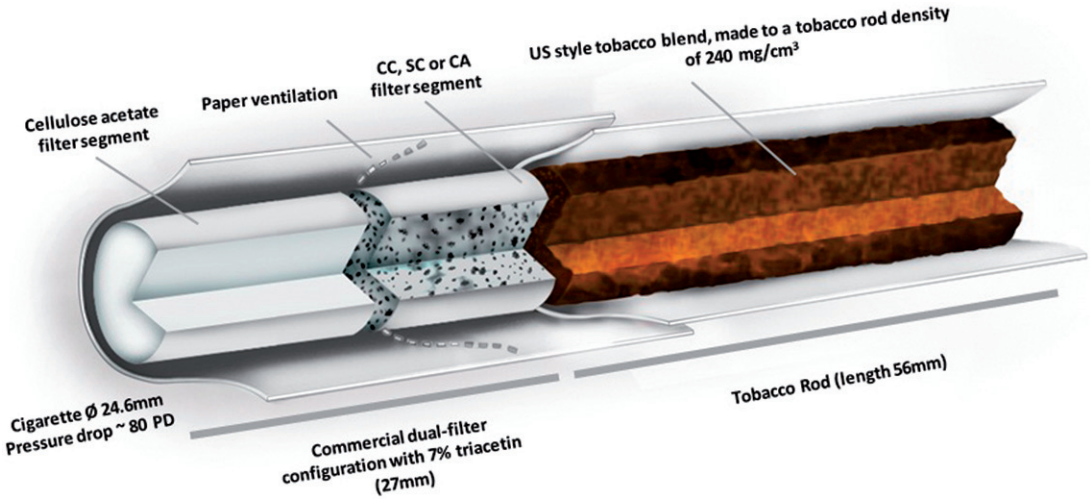

Figure 1. Schematic design and specification of the manufactured test cigarettes. CC, coconut carbon; SC, synthetic carbon.

Table 1 Product specification and parameter breakdown for manufactured cigarettes.

Cigarette code	Control	45 mg CC	80 mg CC	80 mg SC	110 mg SC
Filter type and additive	Mono CA	Dual CA/Carbon	Dual CA/Carbon	Dual CA/Carbon	Dual CA/Carbon
Carbon type	N/A	Coconut	Coconut	Synthetic	Synthetic
Carbon weight (mg)	N/A	47	81	81	106
Filter length (mm)	27	27	27	27	27
Filter PD (mm WG)	75	77	75	73	71
Filter ventilation (%)	43	46	45	45	44
Carbon segment length (mm)	N/A	11.8	12.1	12.1	11.6
Open cigarette PD (mm WG)	83.8	84.1	83.1	80.9	85.3
Tobacco rod length (mm)	56.1	56.3	56.0	55.7	56.3
Tobacco density (mg/cm^3)	244.6	248.1	256.4	254.2	258.3
Tobacco weight (mg)	657.8	693.1	688.1	672.3	687.4
Paper porosity (CORESTA units)	44.4	42.9	44.7	44.1	45.2

CA, cellulose acetate.

ventilation (%) and nicotine (mg/cig), to create a balanced comparable study (Table 1).

The use of activated carbons in cigarette filters has been widely established. This is due to the relative ease in which coconut shell-derived carbon can be manufactured, and the understanding of increased cigarette filtration and adsorption efficiencies (Branton et al., 2011b; Coggins & Gaworski, 2008; Tokida et al., 1985). Activated coconut carbon was obtained from Jacobi carbons Ltd, UK. Generation of carbon activity was achieved by charring raw coconut shell at ~300–500 °C and then activating it in a rotary kiln at 900–950 °C using steam, prior to crushing and grinding. Synthetic polymer-derived carbon was produced in batches (Branton et al., 2011b; Von Blücher et al., 2006). Polymer feed-stock was thermally stabilized using oleum, and heated to 500 °C resulting in carbonization of the polymer material. An activated porous system was created in the carbon by heating the material to 900–1000 °C (Table 2).

Chemical analysis

Chemical analyses were conducted by Labstat International, ULC. Chemicals were selected for measurement due to their status as proposed toxicants by the World Health Organization for mandatory lowering in cigarette smoke (Burns et al., 2008). Five independent analyses were conducted per analyte. A brief account of the techniques employed is described in the Supplementary Data. For a more detailed account of the techniques please refer to Wright (2015).

Cell culture

Mouse fibroblasts (BALB/c 3T3 clone A31) were obtained from the European Collection of Cell Cultures. BALB/c 3T3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM; containing 4 mM glutamine and 4.5 g/l glucose supplemented with 10% foetal calf serum and penicillin/streptomycin) at 37 ± 1 °C in an atmosphere of 5.0% CO_2 in air. For whole smoke exposure, monolayer cultures were prepared on permeable membranes (24 mm Transwells®, Fisher Scientific, UK) by seeding 5×10^5 cells in 1 ml DMEM into each Transwell® (pre-equilibrated by soaking in DMEM for at least 1 h). Cells were incubated for ~24 h at 37 °C in a humidified atmosphere of 5% CO_2 in air to achieve ~90% confluent monolayers.

Smoke generation and exposure

A Vitrocell® VC 10 Smoking Robot (Vitrocell® Systems, Waldkirch, Germany), serial number VC10/090610 was used

Table 2. Characteristics of coconut-shell compared with synthetic derived carbon.

	Carbon type	
Characteristics	Coconut shell-derived	Polymer/synthetic-derived
Abbreviation	CC	SC
Carbon shape	Irregular	Spherical
Carbon size distribution	0.2–0.60 mm	0.25–0.58 mm
Activated carbon precursor. Activation media	Coconut shell derived. Steam activated to 300–950 °C, crushed and ground to size	Synthetic polymer derived. Feed-stock prepared, stabilized, heated to 500 °C and activated using steam and CO_2
Pore size volume	Predominantly microporous Micropore volume 0.40 cm^3/g Total pore volume 0.42 cm^3/g	Bimodal pore size distribution Micropore volume 0.76 cm^3/g Total pore volume 1.22 cm^3/g
Surface area (m^2/g)	980	1780
Density (cm^3/g)	0.46	0.40

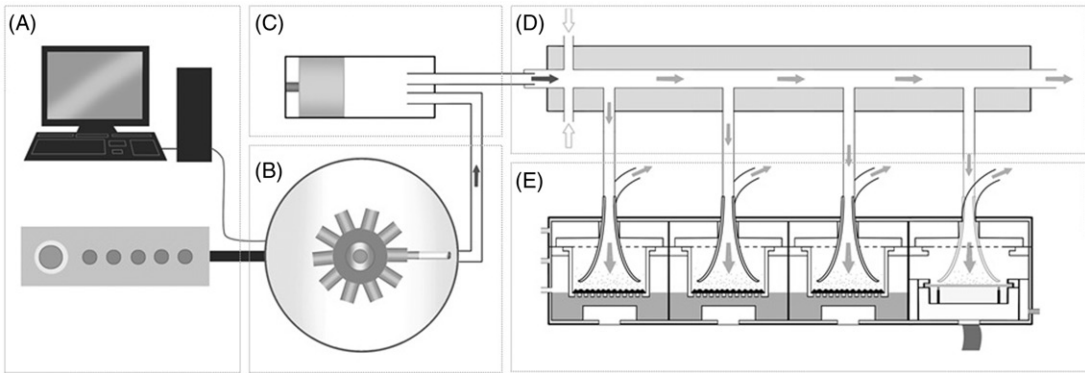

Figure 2. A schematic representation of the Vitrocell® VC 10 smoke exposure system. **(A)** Computer, software controller and air-flow controller. **(B)** Smoking Robot carousel and ventilation hood where cigarettes are smoked. **(C)** Piston/syringe which draws and delivers smoke to the dilution system. **(D)** Dilution, transit and delivery of aerosol occurs in the dilution bar. **(E)** Smoke exposure module (Vitrocell® 6/4 CF Stainless Steel module) which holds the Transwells® maintained at the ALI. Smoke is sampled from the dilution system into the exposure module via negative pressure applied through a vacuum pump at 5 ml/min/well. The central islands can be removed and QCMs can be installed into each position or as shown in position 4 [Taken from Thorne et al. (2013a).].

to generate, dilute and deliver cigarette smoke to BALB/c 3T3 cells housed in 6/4 CF stainless steel exposure modules and maintained at the ALI at 37 °C. The VC 10 is a rotary style smoking machine that has a single syringe which transfers the tobacco smoke to an independent continuous flow dilution bar. Different smoke concentrations are achieved by increasing or decreasing the diluting airflow (l/min). In this system increasing airflow, decreases the available smoke concentration and vice-versa. A vacuum sub-samples smoke (via negative pressure) from the dilution bar into the module, which docks directly under the dilution system. Vacuum flow rate was maintained at 5.0 ml/min/well for all treatments. Diluting airflow rates within this system were controlled using mass flow controllers and vacuum rates were set by mass flow meters (Analyt-MTC GmbH, Mülheim, Germany) (Figure 2).

For each experiment, triplicate Transwells® were housed in a Vitrocell® 6/4 CF stainless steel module. Trumpet heights within the module were set at 2 mm above the Transwell® membrane. BALB/c 3T3 cells were exposed for 3 h on three independent occasions at the ALI to varied concentrations (eight doses) of either whole smoke or gas vapor phase (GVP). The GVP was generated by capturing the particulate material on a Cambridge filter pad (CFP) positioned between the smoking head and syringe.

Neutral red uptake cytotoxicity test

The neutral red uptake assay was performed as previously described by Thorne et al. (2014) and is based on the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) BALB/c 3T3 test method, with modifications for aerosol ALI exposure. Briefly, cells were incubated in DMEM culture media containing 50 µg/ml Neutral Red for 3 h. Neutral Red dye was released by the addition of Neutral Red de-stain solution [ethanol: acetic acid: distilled water; (50:1:49)] and was measured by absorbance at 540 nm. For each condition a dilution IC_{50} was calculated on a l/min diluting airflow rate (defined as a smoke dilution at which 50% cytotoxicity is achieved). A concurrent air control, included in each exposure, which provided a base-line normalization factor on which analysis was conducted. Experiments were deemed valid if they met the following acceptance criteria; OD_{50} coefficient of variance values were <15%; positive control treatments caused >50% decrease in viability relative to the air control, and the air control cell survival was ≥70%.

Quartz crystal microbalances

Quartz crystal microbalances (QCMs) were used in this study to assess smoke deposition (µg/cm^2) *in situ.* QCM technology

Table 3. Smoke analyte yields for reference cigarettes, measured under ISO smoking conditions.

Group	Analytes	Units	Yields (±SD) 1R5F	3R4F[a]	CM7
Ammonia		µg/cig	2.5 (0.5)	10.3 (0.2)	19.3 (0.9)
Aromatic amines	1-aminonaphthalene	ng/cig	3.9 (0.1)	15.1 (0.9)	24.2 (1.3)
	2-aminonaphthalene	ng/cig	2.7 (0.1)	9.8 (0.5)	13.7 (1.1)
	3-aminobiphenyl	ng/cig	0.7 (0.0)	1.9 (0.1)	2.4 (0.2)
	4-aminobiphenyl	ng/cig	0.6 (0.0)	1.4 (0.0)	1.6 (0.2)
Benzo[a]pyrene		ng/cig	1.3 (0.2)	6.3 (0.3)	14.9 (1.0)
Carbonyls	Formaldehyde	µg/cig	3.8 (0.4)	22.8 (1.7)	43.4 (1.3)
	Acetaldehyde	µg/cig	115.9 (9.8)	447.5 (16.5)	564.7 (27.1)
	Acetone	µg/cig	69.7 (4.8)	238.2 (8.2)	283.6 (15.7)
	Acrolein	µg/cig	10.2 (0.7)	51.5 (0.8)	68.5 (3.3)
	Propionaldehyde	µg/cig	11.6 (1.2)	42.1 (2.1)	51.5 (3.1)
	Crotonaldehyde	µg/cig	NQ	11.5 (0.7)	20.6 (0.8)
	MEK	µg/cig	20.4 (1.9)	65.8 (3.2)	76.6 (5.7)
	Butyraldehyde	µg/cig	9.7 (0.9)	29.3 (1.7)	42.4 (3.2)
HCN		µg/cig	16.6 (0.9)	88.5 (4.0)	148.7 (7.8)
Nitric oxides	NO	µg/cig	90.2 (8.9)	204.5 (8.9)	88.0 (4.2)
	NOx	µg/cig	96.9 (9.7)	219.1 (9.6)	95.3 (4.9)
Tobacco-specific nitrosamines	Nitrosonornicotine (NNN)	ng/cig	44.9 (2.1)	98.8 (2.2)	17.6 (1.2)
	Nitrosoanatabine (NAT)	ng/cig	43.6 (2.5)	106.7 (2.5)	34.2 (2.5)
	Nitrosoanabasine (NAB)	ng/cig	7.9 (0.7)	14.0 (0.1)	4.9 (0.4)
	4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK)	ng/cig	23.7 (2.0)	85.7 (2.9)	21.7 (1.7)
Nitrogen heterocyclics	Pyridine	µg/cig	1.9 (0.1)	7.1 (0.2)	13.0 (0.3)
	Quinoline	µg/cig	0.0 (0.0)	0.2 (0.0)	0.5 (0.0)
	Styrene	µg/cig	1.9 (0.1)	5.7 (0.4)	10.6 (0.5)
Phenolic compounds	Hydroquinone	µg/cig	7.5 (0.3)	33.1 (2.1)	93.3 (5.1)
	Catechol	µg/cig	NQ	35.2 (2.0)	94.4 (4.4)
	Phenol	µg/cig	NQ	9.9 (0.9)	35.8 (2.2)
	m-cresol	µg/cig	NQ	1.8 (0.1)	4.0 (0.2)
	p-cresol	µg/cig	0.8 (0.1)	5.7 (0.3)	14.0 (0.8)
	o-cresol	µg/cig	BDL	2.4 (0.2)	5.0 (0.2)
	Resorcinol	µg/cig	BDL	NQ	NQ
Volatiles	1,3-butadiene	µg/cig	12.1 (0.6)	34.9 (0.9)	51.0 (2.6)
	Isoprene	µg/cig	113.3 (4.5)	306.7 (12.2)	413.5 (20.8)
	Acrylonitrile	µg/cig	1.8 (0.1)	6.7 (0.5)	10.7 (0.7)
	Benzene	µg/cig	11.5 (0.4)	31.6 (2.0)	42.4 (1.8)
	Toluene	µg/cig	18.4 (1.2)	56.6 (4.6)	73.9 (3.5)
ISO smoke yields	NFDPM	mg/cig	1.7 (0.2)	8.1 (0.4)	13.4 (0.6)
	Nicotine	mg/cig	0.1 (0.0)	0.7 (0.0)	1.2 (0.1)
	CO	mg/cig	2.5 (0.4)	10.5 (0.3)	11.3 (1.1)
	Puff number	/Cig	6.5 (0.2)	8.0 (0.2)	8.1 (0.3)

[a]Values consistent with Roemer et al. (2012).
BDL, below limit of detection; NQ, not quantifiable.

has been previously described in conjunction with *in vitro* whole smoke exposure systems (Adamson et al., 2013, 2014; Majeed et al., 2014; Thorne et al., 2013b). In this study a QCM was installed into position 4 (furthest position within the module) and recorded mass values every 2 s in a real-time format.

Data presentation and statistics

GraphPad Prism 6 (2012) statistical software, version 6.0 was used to generate a dilution IC_{50} and to assess the curve using regression analysis. Best fits were generated using a sigmoidal four-parameter-logistic curve for all data with statistical analysis. Statistical analysis was conducted using GraphPad Prism and/or in Minitab® version 16.1.0 with a two-sample one-way analysis of variance, using Dunnett's test. All assessments were conducted on three independent occasions/cigarette, with three replicates/dose/occasion and assessed to a 95% confidence limit.

Results

Smoke chemical analysis was conducted on all cigarettes assessed in this study. Tables 3 and 4 show a detailed breakdown of the smoke chemistries for the reference cigarettes and manufactured cigarettes, respectively. Mainstream cigarette smoke chemistry data obtained for 3R4F in this study are consistent with that previously published in (Roemer et al., 2012).

Clear cytotoxicity profiles (defined as a cytotoxic range, from 100 to 0% viability) were generated for all cigarettes tested within this study (Table 5).

Reference cigarettes

The response of 3R4F (3R4F-A) in this study was directly compared and analyzed against previously published 3R4F data (3R4F-B) (Thorne et al., 2014). When comparing between the two datasets no statistical difference was observed ($p = 0.703$). 3R4F data generated in this study

Table 4. Smoke analyte yields for bespoke manufactured cigarettes, measured under ISO smoking conditions.

			Yields (±SD)				
Group	Analytes	Units	Control	45 mg CC	80 mg CC	80 mg SC	110 mg SC
Ammonia		µg/cig	8.6 (0.6)	8.3 (0.5)	8.4 (0.6)	8.4 (0.5)	9.0 (0.2)
Aromatic amines	1-aminonaphthalene	ng/cig	14.0 (1.0)	15.5 (1.4)	14.3 (0.6)	19.5 (2.3)	20.1 (1.1
	2-aminonaphthalene	ng/cig	9.4 (0.7)	10.2 (0.9)	10.3 (0.6)	11.8 (0.7)	12.4 (0.9)
	3-aminobiphenyl	ng/cig	1.9 (0.1)	2.2 (0.2)	2.2 (0.1)	2.4 (0.1)	2.6 (0.1)
	4-aminobiphenyl	ng/cig	1.5 (0.1)	1.6 (0.1)	1.7 (0.1)	1.9 (0.1)	2.0 (0.1)
Benzo[a]pyrene		ng/cig	6.2 (0.9)	6.3 (0.5)	6.4 (0.5)	6.5 (0.7)	7.2 (0.7)
Carbonyls	Formaldehyde	µg/cig	17.8 (1.8)	15.1 (1.1)	14.2 (2.0)	13.8 (1.4)	12.8 (0.7)
	Acetaldehyde	µg/cig	416.0 (11.4)	347.9 (24.8)	244.8 (24.1)	72.2 (9.0)	29.2 (10.9)
	Acetone	µg/cig	180.9 (5.1)	136.6 (12.4)	68.4 (7.6)	6.2 (0.8)	4.1 (0.7)
	Acrolein	µg/cig	32.6 (2.2)	22.8 (3.4)	10.6 (1.6)	2.8 (0.3)	NQ
	Propionaldehyde	µg/cig	32.4 (1.3)	24.1(2.0)	13.5 (1.5)	NQ	BDL
	Crotonaldehyde	µg/cig	7.7 (0.7)	NQ	NQ	BDL	BDL
	MEK	µg/cig	44.3 (1.3)	28.2 (3.6)	11.3 (1.7)	NQ	NQ
	Butyraldehyde	µg/cig	25.0 (1.2)	18.5 (1.7)	12.0 (0.8)	3.4 (0.1)	NQ
HCN		µg/cig	83.3 (3.8)	61.7 (4.8)	35.1 (2.0)	22.7 (2.4)	15.6 (2.0)
Nitric oxides	NO	µg/cig	115.4 (11.0)	134.7 (6.7)	128.0 (5.9)	122.1 (10.8)	114.0 (9.3)
	NOx	µg/cig	125.2 (11.5)	144.6 (7.0)	136.8 (6.6)	127.5 (11.0)	119.2 (9.2)
Tobacco-specific nitrosamines	NNN	ng/cig	84.9 (5.7)	94.4 (4.3)	97.4 (4.0)	107.8 (8.6)	115.4 (10.4)
	NAT	ng/cig	53.8 (4.2)	61.0 (1.2)	62.3 (3.9)	68.4 (4.7)	72.8 (2.8)
	NAB	ng/cig	7.8 (0.8)	9.2 (0.4)	9.1 (1.0)	10.6 (1.0)	10.9 (1.0)
	NNK	ng/cig	27.6 (1.9)	31.6 (2.6)	33.1 (1.6)	34.9 (1.2)	38.0 (3.1)
Nitrogen heterocyclics	Pyridine	µg/cig	6.6 (0.2)	2.4 (0.2)	1.2 (0.2)	NQ	NQ
	Quinoline	µg/cig	0.3 (0.0)	0.2 (0.0)	0.2 (0.0)	0.2 (0.0)	0.2 (0.0)
	Styrene	µg/cig	4.3 (0.2)	1.6 (0.2)	0.9 (0.1)	NQ	NQ
Phenolic compounds	Hydroquinone	µg/cig	36.0 (2.1)	37.4 (1.3)	39.2 (1.0)	40.8 (1.7)	45.0 (1.4)
	Catechol	µg/cig	42.8 (2.9)	44.6 (2.7)	47.8 (0.9)	48.5 (1.5)	54.1 (2.2)
	Phenol	µg/cig	14.2 (1.6)	12.9 (1.3)	13.2 (0.3)	10.1 (0.5)	12.2 (0.4)
	m-cresol	µg/cig	2.7 (0.2)	2.6 (0.2)	2.7 (0.0)	2.3 (0.1)	2.7 (0.1)
	p-cresol	µg/cig	6.8 (0.6)	6.6 (0.5)	6.9 (0.1)	5.7 (0.3)	6.8 (0.1)
	o-cresol	µg/cig	3.5 (0.4)	3.1 (0.3)	3.1 (0.1)	2.4 (0.1)	2.8 (0.1)
	Resorcinol	µg/cig	NQ	NQ	NQ	NQ	NQ
Volatiles	1,3-butadiene	µg/cig	23.2 (2.5)	19.0 (1.7)	15.6 (1.5)	1.1 (0.1)	NQ
	Isoprene	µg/cig	204.2 (12.6)	160.5 (12.4)	113.7 (8.4)	NQ	NQ
	Acrylonitrile	µg/cig	6.0 (0.6)	4.0 (0.2)	2.2 (0.4)	NQ	NQ
	Benzene	µg/cig	23.7 (1.7)	14.5 (1.2)	7.7 (1.0)	NQ	NQ
	Toluene	µg/cig	36.3 (2.9)	19.1 (1.5)	8.7 (3.4)	NQ	NQ
ISO smoke yields	NFDPM	mg/cig	7.5 (0.6)	7.4 (0.3)	7.9 (0.5)	7.6 (0.4)	8.3 (0.6)
	Nicotine	mg/cig	0.7 (0.0)	0.6 (0.0)	0.7 (0.0)	0.7 (0.0)	0.8 (0.1)
	CO	mg/cig	6.7 (0.5)	6.6 (0.3)	6.8 (0.6)	6.6 (0.3)	7.2 (0.7)
	Puff number	/Cig	7.2 (0.5)	7.5 (0.2)	7.6 (0.3)	7.7 (0.4)	7.7 (0.2)
Vapor phase reductions[a]		%	**0**	**23.4**	**51.1**	**89.3**	**94.6**

[a]Reductions based on percentage difference of vapor and semi-volatile chemicals normalized against Control (Calculation based on reductions of carbonyls, HCN, nitrogen hetrocyclics, volatiles, through carbon filtration).
BDL, below limit of detection; NQ, not quantifiable.

Table 5. Dilution IC_{50} ranges and curve analysis based on 95% confidence limits.

Cigarette	Dilution IC_{50} (l/min)	Dilution IC_{50} range (l/min)	R^2 curve fit	p value	Statistical analysis
3R4F–A	6.72	6.25–7.23	0.90	0.703	*
3R4F–B[a]	6.09	5.27–6.72	0.89	0.703	*
1R5F	1.49	1.29–1.74	0.96	∼	N/A
CM7	8.12	7.15–9.22	0.93	∼	N/A
Control	5.65	5.30–6.02	0.94	∼	N/A
Control + CFP	3.64	3.23–4.11	0.90	0.028	**
45 mg CC	4.01	3.20–5.02	0.91	0.316	NS
45 mg CC + CFP	0.80	0.40–1.58	0.81	0.012	**
80 mg CC	3.39	2.96–3.90	0.96	0.002	**
80 mg SC	2.41	1.93–3.02	0.94	0.001	** (***)
110 mg SC	1.94	1.63–2.32	0.96	0.009	**

[a]Data taken from Thorne et al. (2014).
CFP, Cambridge filter pad; N/A, statistical analysis not applied; NS, not statistically different compared with Control.
*No statistical difference between 3R4F datasets (A and B) when compared with each other ($p = 0.703$).
**Statistically lower cytotoxicity when compared with Control.
***Statistically lower cytotoxicity when compared with 80 mg CC ($p = 0.016$).

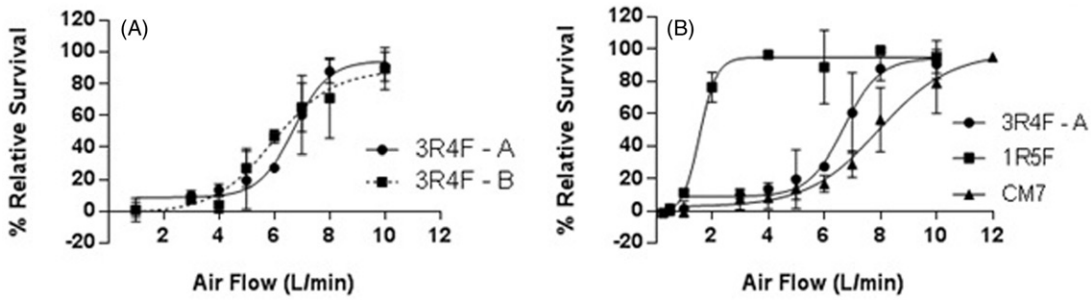

Figure 3. Cytotoxicity analysis of reference cigarettes. **(A)** Comparison of 3R4F reference data (3R4F-A) generated in this study compared with historical 3R4F data (3R4F-B) previously published in Thorne et al. (2014). **(B)** Cytotoxic comparison of three different reference cigarettes, CM7. 3R4F and 1R5F.

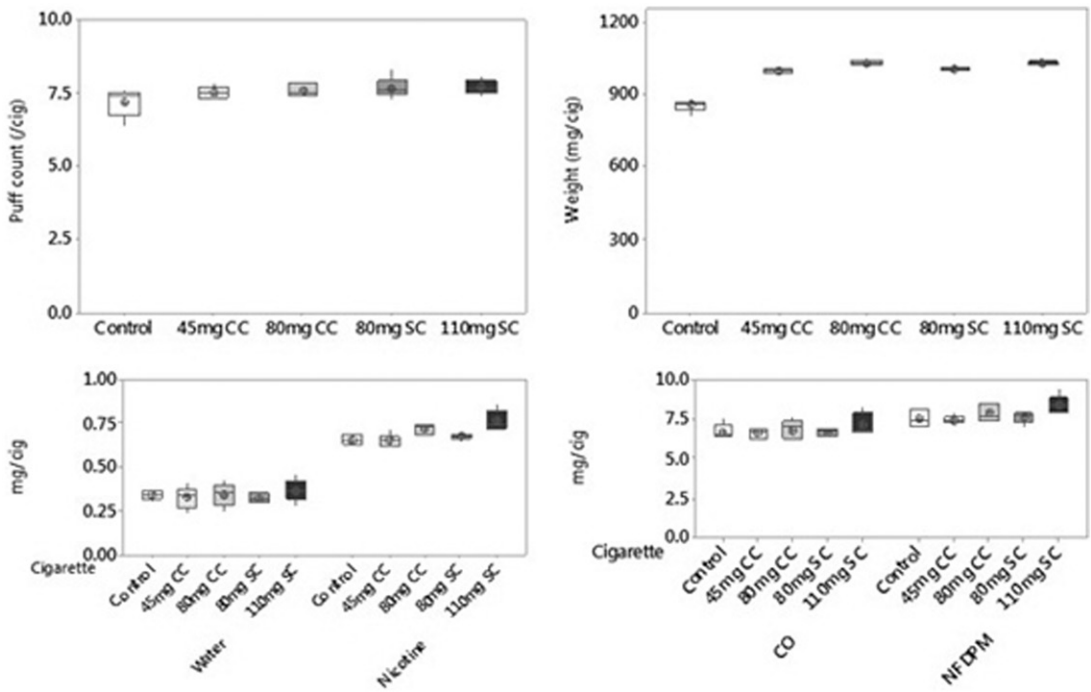

Figure 4. Manufactured cigarettes showing comparable specifications compared with Control.

demonstrated a dilution IC_{50} of 6.70 l/min with a range of 6.25–7.23 l/min and a curve fit of $R^2 = 0.90$. This supports the data published in Thorne et al. (2014), of a dilution IC_{50} of 6.09 l/min. All reference products produced a cytotoxic response. CM7 was deemed the most cytotoxic in this study, followed by 3R4F, with 1R5F producing the lowest cytotoxic response. For the purpose of this study, reference cigarettes were not directly compared, rather these products were used as a 'reference point' to contextualize the study (Figure 3).

Manufactured cigarettes

For a more comprehensive and direct comparison, five cigarettes were specially manufactured, matched for key cigarette variables, such as; NFDPM (mg/cig), nicotine (mg/cig), puff number (/cig) PD, ventilation (%) and CO (mg/cig) (Figure 4).

Manufactured cigarettes were modified with varying filter additives to selectively remove vapor phase chemicals, such as acetaldehyde, acetone, acrolein, benzene, isoprene, hydrogen cyanide (HCN), propionaldehyde and methyl ethyl ketone (MEK) and to produce different vapor phase chemical profiles (Figure 5 and Table 4).

The manufactured cigarette 110 mg SC showed the highest reductions in vapor phase chemistries followed by 80 mg SC, 80 mg CC and finally 45 mg CC, when compared with the Control product. Cigarettes 110, 80 and 80 mg CC, and all showed a statistically lower cytotoxic response compared with the Control. The only cigarette in this study not to show a statistical difference when compared with the Control was 45 mg CC product. Based in order of increasing cytotoxicity the products were ranked as follows, 110 mg SC, 80 mg SC, 80 mg CC and 45 mg CC/Control, with IC_{50} dilutions of 1.94, 2.41, 3.39, 4.01 and 5.65 l/min, respectively. The ranking of the cytotoxicity, mirrors that of the vapor phase profiles in that the higher the chemical reductions the lower the observed cytotoxicity. For example 110 mg SC produced the lowest cytotoxic response, with the highest vapor phase reductions. This trend was observed throughout the five test articles.

When the two types of carbon (coconut and synthetic) were directly compared, with matched loading in the cigarette filter, SC was shown to produce a lower cytotoxic response compared with coconut carbon, when matched for weight (80 mg), also following the vapor phase chemical/cytotoxicity reduction trend (Figure 6).

Smoke phases

To assess the contribution of the smoke phases, a CFP was installed in-line and particulate material filtered, thus giving a cytotoxic measure of the vapor phase. The 110 mg SC product which gave the highest vapor phase reductions (~95%) was used to assess the contribution of the particulate phase. No reductions in cell viability were observed in an experiment using a 110 mg SC with a CFP in-line (data not shown), which confirmed that vapor phase chemicals were present below the levels required to elicit a biological response in this assay.

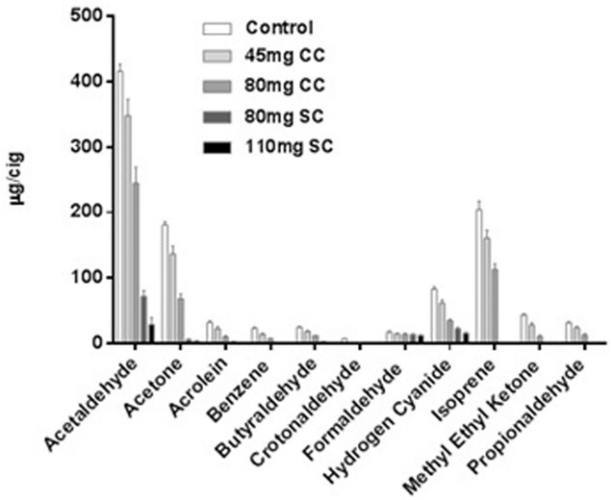

Figure 5. A snap-shot of the chemistry profile of 11 vapor phase chemicals for the manufactured cigarettes.

Removal of the particulate phase via a CFP demonstrated a significant reduction in cytotoxicity, from a dilution IC_{50} of 5.65 to 3.64 l/min (Control compared with the Control + CFP). Removal of the vapor phase using 110 mg SC, also demonstrated a significant reduction in cytotoxicity from a 5.65 to 1.94 l/min. The reductions in cytotoxicity were more pronounced when the vapor phase was removed compared with that of the particulate. Based on these observations, it appears that the vapor phase contributes to ~65% of the cytotoxicity, with the remaining 35% associated with the particulate fraction (Figure 7).

When a CFP was installed in-line of a 45 mg CC product, it barely produced a full cytotoxic response with a dilution IC_{50} of 0.80 l/min, the lowest cytotoxic response observed in this study. Previous experiments demonstrated that removal of the particulate phase produced an approximate reduction in cytotoxicity of 35%. However, in this instance by removing the particulate fraction from the mainstream smoke of a modified product (45 mg CC), with only mild vapor phase reductions (~23%), a 80% reduction in cytotoxicity was observed. This result differs from the observed 65:35% vapor/particulate ratio. Although the modified 45 mg CC product was not statistically different from the control in terms of cytotoxicity and had only mild vapor phase reductions, this altered vapor has significantly lower impact without the

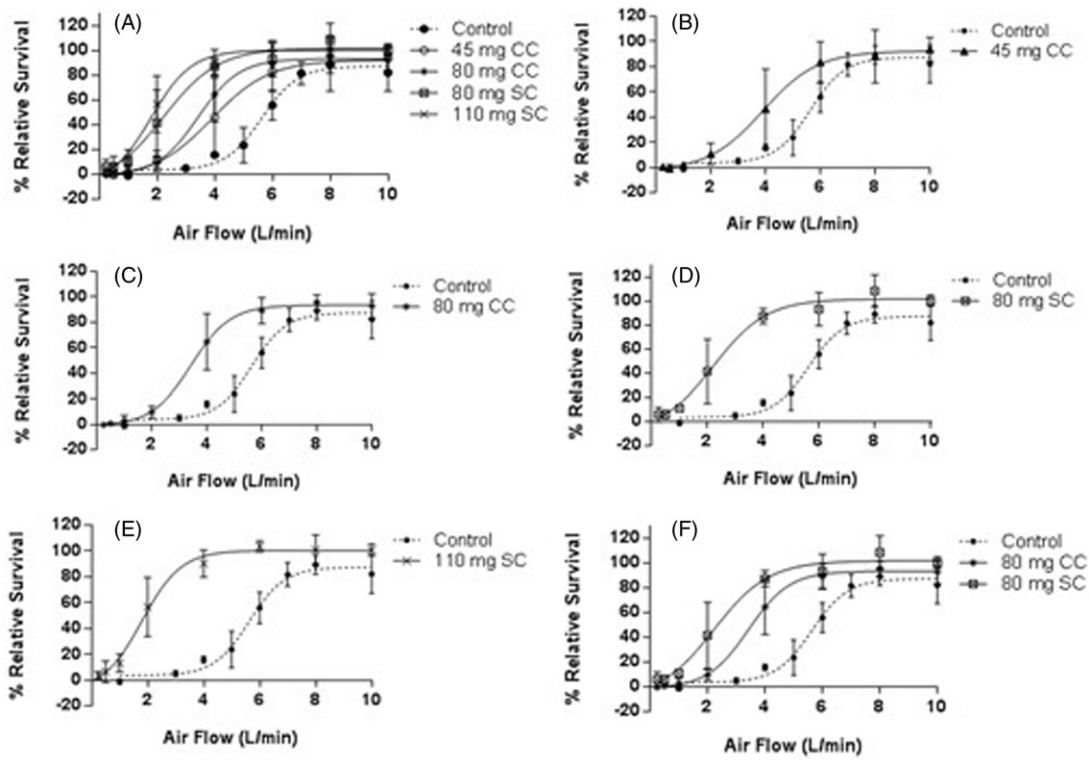

Figure 6. Comparative cytotoxicity analysis of manufactured cigarettes. **(A)** Comparison of all cigarettes tested. **(B)** Comparison of Control versus 45 mg CC. **(C)** Comparison of Control versus 80 mg CC. **(D)** Comparison of Control versus 80 mg SC. **(E)** Comparison of Control versus 110 mg SC and **(F)** Comparison of Control versus 80 mg CC versus 80 mg SC.

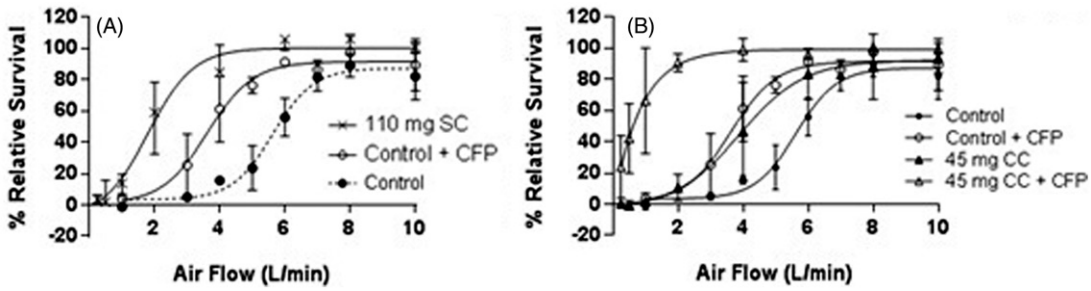

Figure 7. **(A)** Analysis of smoke phase contribution. Whole smoke (Control), vapor (Control + CFP) and particulate (110 mg SC). **(B)** Analysis of smoke phases using carbon filtered products and altered chemistry dynamics.

Table 6. Cigarette smoke phase contributions and proposed vapor phase activity.

Cigarette	Smoke phase assessed	Dilution IC_{50} (l/min)[a]	% Vapor phase activity[b]	% Measured vapor reductions[c]
Control	Whole smoke	5.65	100	0
Control + CFP	Vapor[d]	3.64	100	0
45 mg CC	Vapor reductions and particulate	4.01	60	23.4
80 mg CC		3.39	40	51.1
80 mg SC		2.41	10	89.3
110 mg SC	Particulate[e]	1.94	0	94.6

[a]Obtained dilution IC_{50} response.
[b]Percentage biological activity attributed to the vapor phase, normalized against Control.
[c]Reductions based on percentage difference of vapor and semi-volatile chemicals in each product normalized against Control.
[d]Particulate phase filtered using CFP.
[e]Vapor phase filtered using performance carbon.

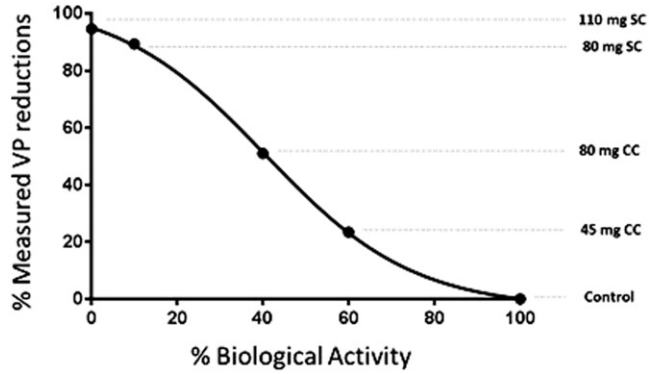

Figure 8. Analysis of vapor phase contribution to biological activity. % chemical reductions for vapor and semi-volatiles calculated against Control. % biological activity based on cytotoxic shift, normalized against Control. % biological activity shows a statistical correlation with reductions in vapor and semi-volatile toxicants ($R^2 = 0.99$).

associated particulate fraction. This potentially indicates a synergistic/additive effect of the smoke fractions.

By comparing responses from particulate and vapor (whole smoke), with particulate only (vapor removed via 110 mg SC), with vapor only (particulate removed via CFP) and in the presence of known vapor phase reductions, the vapor phase biological relationship was identified (Table 6).

By calculating percentage vapor phase reductions against the Control product (Table 4), and by determining the associated reduction in biological activity (Table 6), the two variables were compared. Figure 8 demonstrates the highlighted relationship between vapor phase reductions and % biological effect ($R^2 = 0.99$), showing that reductions in the vapor phase (and semi-volatile) compounds have a direct and proportional effect on biological activity. Biological activity (%) was measured against the Control product and reduced activity (%) refers to the reduced cytotoxic profiles of the manufactured products when normalized against the Control.

Deposition

Deposited mass values obtained for the reference cigarettes (1R5F, 3R4F and CM7) *in situ* of exposure and were statistically different when compared on a per puff basis ($p = 0.000$). When investigated further, a direct relationship was identified between deposited mass, nicotine and NFPDM for each product ($R^2 = 0.97$ and $= 0.96$, respectively). When deposited mass values were compared between the five bespoke manufactured products, no statistical differences were observed between products ($p = 0.147$), despite significant toxicant reductions, when compared at a consistent dilution (1 l/min) (Figure 9).

Discussion

This study describes the evaluation of a BALB/c cytotoxicity technique employed alongside a Vitrocell® VC 10 smoke exposure system for the assessment of mainstream cigarette smoke *in vitro*. The BALB/c technique employed is a modification of the ICCVAM acute toxicity test optimized for ALI aerosol exposure. Essentially, this protocol allows for the assessment of semi-volatiles and vapor phase compounds *in vitro*. This is especially important, as tobacco smoke is a complex aerosol consisting of >6000 chemicals (Rodgman & Perfetti, 2013), distributed between both the particulate and vapor phase, with semi-volatiles transiting between phases. Analysis of the particulate material only, omits any interactions or responses generated by the vapor phase. Furthermore, the vapor phase makes up the majority smoke fraction and contains known toxicants responsible for adverse health effects (Fowles & Dybing, 2003; Hoffmann et al., 1997). Finally, separating smoke fractions may lead to alterations or chemical changes which may not be representative of the complete smoke aerosol. For these reasons, ALI technologies for aerosol exposure *in vitro* are becoming more widely used and adopted.

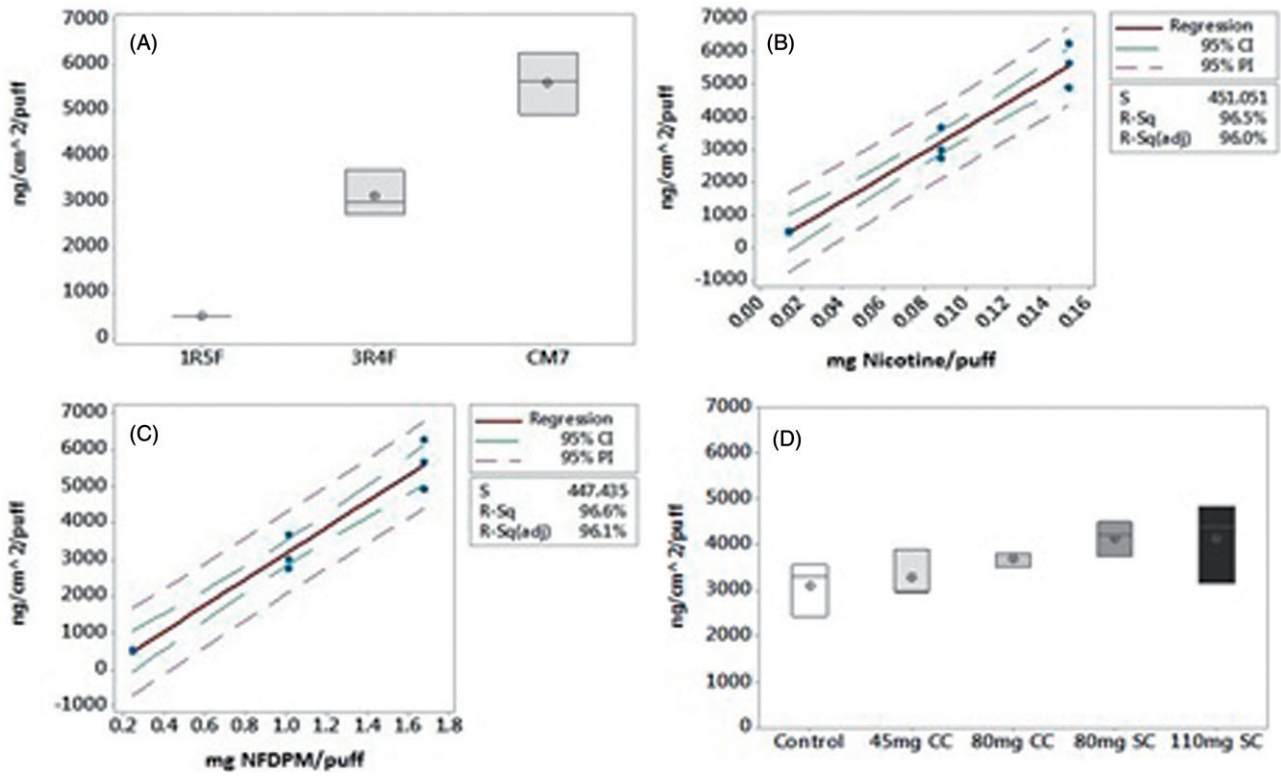

Figure 9. Analysis of deposited mass obtained *in situ* of exposure. **(A)** deposited mass per puff for 1R5F, 3R4F and CM7, showing a statistical difference between products ($p = 0.000$). **(B)** relationship between ng/cm^2/puff and nicotine/puff ($R^2 = 0.97$). **(C)** relationship between ng/cm^2/puff and NFDPM/puff ($R^2 = 0.96$). **(D)** deposited mass obtained from the bespoke manufactured cigarettes, showing no statistical differences for deposited mass measured between products ($p = 0.147$), measured at the 1 l/min dilution.

Eight different cigarettes were assessed, three reference products (1R5F, 3R4F and CM7) and five experimental products. Analysis of cigarette cytotoxic responses using 3R4F reference cigarette smoke demonstrated no statistical difference between data obtained in this study compared with that obtained 12-month prior. This analysis gives confidence in the robustness of the assay, exposure and the biological responses obtained.

For a direct comparative analysis, five experimental products were specifically manufactured and matched for PD, NFDPM yields, Nicotine, Puff number and blend, but significantly altered for vapor phase delivery.through the use of coconut or synthetic carbon (SC). In the context of whole smoke exposure, understanding the contribution of the smoke phases and their interaction is fundamental. In order to obtain a broad spectrum of vapor phase deliveries, two carbons previously described by Branton et al. (2011b) and a range of loadings were investigated, all using the same tobacco-blend and cigarette parameters to eliminate potential variability. Clear statistical differences were observed between the cigarette test pieces and the cytotoxic profiles (obtained through a dilution IC_{50}) when compared with the Control product. For example, cigarettes 80 mg SC, 80 mg CC and 110 mg SC produced a statistically lower cytotoxic profiles, when compared with the Control. In order of increasing cytotoxicity, the cigarettes are ranked as follows; 110 mg SC, 80 mg SC, 80 mg CC and 45 mg CC/Control, which corresponds directly with the amount of vapor phase toxicants removed per product.

SC has previously been demonstrated to be more efficient at vapor phase removal compared with traditional coconut carbon (Branton et al., 2011b), and the chemistry data from this study support this. Directly comparing the cytotoxic response between the two different carbon products, demonstrated that matched for loading, SC cigarettes produced a significantly different ($p = 0.016$) and lower cytotoxic response compared with traditional coconut carbon. Although other studies have demonstrated the effect of carbon loadings on cigarette induced cytotoxicity (Bombick et al., 1997; Coggins & Gaworski., 2008; Phillips et al., 2005; Shin et al., 2009), none have balanced the tobacco blend, NFDPM yields and puffing characteristics of the particulate phase, whilst demonstrating reductions in the vapor phase yields. By calculating % vapor and semi-volatile toxicant reductions and, by calculating the % cytotoxic shift of each product normalized against the Control, the relationship between cytotoxicity and vapor phase and semi-volatile chemicals was defined. The data show a clear direct and statistical relationship ($R^2 = 0.99$) between vapor phase reductions and reduced biological response. The relationship suggests that reductions in vapor phase toxicants have a direct and proportional impact on the observed biological response in the set-up under these experimental conditions.

Additionally, analyses of the contribution of the particulate and vapor smoke phases towards cytotoxicity were examined. Removal of the particulate phase from the Control product via a CFP produced a significant reduction in cytotoxicity, as did removal of the vapor phase (through 110 mg SC). The reduction in cytotoxicity was more pronounced when the vapor phase was removed compared with that of the particulate fraction. Based on these observations, the vapor

phase contributes ~65%. This differs from previous estimations and puts more emphasis on the vapor phase. For example previous studies have estimated an even split between the particulate and vapor contribution towards cytotoxicity, using a comparable set-up (Thorne et al., 2014). The disparity between these observations can be attributed to the fact that previous studies did not consider both the complete removal of the particulate or the vapor phase, as this study has done, thus allowing more accurate interpretations of the data.

In addition to assessing the various smoke phases, a CFP was installed in-line of a 45 mg CC product, which barely produced a full cytotoxic dose response. Previous experiments demonstrated that removal of the particulate phase produced an approximate reduction in cytotoxicity of 35%. By removing the particulate fraction from the mainstream smoke of a modified product (45 mg CC), with only mild vapor phase reductions (~23%), a 80% reduction in cytotoxicity was observed. This result differs from the observed 65:35% estimated vapor/particulate ratio. This altered vapor phase demonstrated significantly lower impact without the associated particulate fraction, potentially indicating a synergistic/additive effect. This reduced cytotoxic response may be attributed to the change in vapor phase characteristics from the filtered product and the lower levels of interactions in the vapor phase and semi-volatile chemicals present. In addition, by removing the particulate and modifying the vapor phase delivery through a carbon filtered cigarette, the resulting cytotoxic profile can be attributed to chemicals within the complex cigarette smoke mixture, such as formaldehyde, acetaldehyde, crotonaldehyde, acrolein, acetone, propionaldehyde, acrylonitrile and HCN. These chemicals have all previously been associated with lung injury, tissue damage and have distinct modes of action (Fowles & Dybing, 2003; IARC 2004, 2008, 2012).

QCMs were included in this study to assess the deposited material from each product. Due to the nature of the study, in that chemistries were obtained and that cigarettes were balanced for particulate and modified for vapor and semi-volatile deliveries, the QCM data obtained was far more valuable and informative. Based on a per puff delivery (ng/cm^2) significant differences were observed in the deposited material obtained between the three reference products tested (1R5F, 3R4F, CM7). This is explained as these products differ in both vapor and particulate deliveries and have clear nicotine and NFDPM yield differences. QCM data for the experimental cigarettes showed a contrasting pattern. No statistical differences were observed for deposited material between any of the bespoke products tested when compared with the Control at a set dilution (1 l/min). This data suggest that QCMs are only detecting the particulate fraction of the cigarette smoke and not any deposited vapor or semi-volatiles, as previously hypothesized. Additionally, if QCMs were measuring an element of the semi volatile or vapor phase deposited or adsorbed material, this study would have expected to see a reduction in QCM readings for the modified products, given that the highest vapor phase modified product (110 mg SC), showed ~95% reduction in vapor and semi volatiles compared with Control; no such reductions were observed in QCM data. QCM readings from the reference products were compared with NFDPM and nicotine chemistries obtained. A clear relationship between deposited mass per puff and NFDPM and Nicotine per puff was identified ($R^2 = 0.97$ and 0.96, respectively).This supports the theory that QCMs are only measuring the particulate phase of cigarette smoke, thus helping us understand their importance and capacity in *in vitro* exposure scenarios.

Conclusions

The data presented here is based on a modified BALB/c protocol and the Vitrocell® VC 10 whole smoke exposure system, which has demonstrated the ability to distinguish between altered vapor phase products and produced consistent and robust responses for 3R4F reference cigarette smoke data generated over 12 months. This study has demonstrated a clear, direct vapor phase cytotoxic effect dose response relationship, derived from mainstream cigarette smoke from bespoke manufactured cigarettes. It has clarified the contribution of the respective smoke phases, vapor and particulate, towards cytotoxicity in ~65:35% ratio in favor of the vapor phase. In addition the study has potentially highlighted an *in vitro* vapor particulate phase interaction that is altered once cigarette smoke has been filtered. It has also highlighted that key vapor phase chemicals, such as the carbonyls, can drive cytotoxicity without the corresponding particulate fraction of smoke. Finally, the data shown here demonstrates the essential requirement to develop ALI-based techniques for the appropriate analysis of the complete smoke aerosol *in vitro*, which will be of particular and of growing importance as new aerosol based tobacco products become more widely accepted and used.

Acknowledgements

The authors would like to acknowledge Joanne Kilford and Michael Hollings for managing all experimental work through Covance Laboratories Ltd, Harrogate, UK. The authors would also like to acknowledge Marcos Vinicius Gama for his generous help in obtaining chemistry data, Paul Blachford for his tireless support, Peter Branton for his help regarding Carbon specifics, Jason Adamson for his insight with Figures 1 and 7 and Sophie Larard for QC checking all data and finally, Marianna Gaça for her technical review.

Declaration of interest

The authors declare there are no competing interests and are employees of British American Tobacco or contracted by British American Tobacco. Covance Laboratories Ltd, Harrogate, UK, conducted all biological experimental work, which was funded by British American Tobacco. Labstat International ULC conducted all cigarette chemical analysis and was funded by British American Tobacco.

References

Adamson J, Thorne D, Dalrymple A, et al. (2013). Assessment of cigarette smoke particle deposition within the Vitrocell® exposure module using quartz crystal microbalances. Chem Cent J 7:50.

Adamson J, Thorne D, Errington G, et al. (2014). An inter-machine comparison of tobacco smoke particle deposition *in vitro* from six independent smoke exposure systems. Toxicol In Vitro 28:1320–28.

Andreoli C, Gigante D, Nunziata A. (2003). A review of *in vitro* methods to assess the biological activity of tobacco smoke with the aim of reducing the toxicology of smoke. Toxicol In Vitro 17:587–94.

Azzopardi D, Haswell L, Foss-Smith G, et al. (2015). Evaluation of an air–liquid interface cell culture model for studies on the inflammatory and cytotoxic responses to tobacco smoke aerosols. Toxicol In Vitro 29:1720–8.

Bombick D, Bombick R, Ayres P, et al. (1997). Evaluation of the genotoxic and cytotoxic potential of mainstream whole smoke and smoke condensate from a cigarette containing a novel carbon filter. Fund Appl Toxicol 39:11–7.

Branton P, McAdam K, Winter D, et al. (2011a). Reduction of aldehydes and hydrogen cyanide yields in mainstream cigarette smoke using an amine functionalised ion exchange resin. Chem Cent J 5:15.

Branton P, McAdam K, Duke M, et al. (2011b). Use of classical adsorption theory to understand the dynamic filtration of volatile toxicants in cigarette smoke by active carbons. Adsorp Sci Technol 29:117–38.

Burns D, Dybing E, Gray N, et al. (2008). Mandated lowering of toxicants in cigarette smoke: a description of the World Health Organization TobReg proposal. Tobacco Control 2008;17:132–41.

Coggins C, Gaworski C. (2008). Could charcoal filtration of cigarette smoke reduce smoking-induced disease? A review of the literature. Regul Toxicol Pharmacol 50:359–65.

Dittrich D, Fieblekorn R, Bevan M, et al. (2014). Approaches for the design of reduced toxicant emission cigarettes. SpringerPlus 3:374.

Doolittle D, Lee C, Ivett J, et al. (1990). Comparative studies on the genotoxic activity of mainstream smoke condensate from cigarettes which burn or only heat tobacco. Environ Mol Mutagenesis 15: 93–105.

Fowles J, Dybing E. (2003). Application of toxicological risk assessment principles to the chemical constituents of cigarette smoke. Tob Control 12:424–30.

Fukano Y, Ogura M, Eguchi K, et al. (2004). Modified procedure of a direct in vitro exposure system for mammalian cells to whole tobacco smoke. Exp Toxicol Pathol 55:317–23.

Goniewicz M, Knysak J, Gawron M, et al. (2014). Levels of selected carcinogens and toxicants in vapour from electronic cigarettes. Tobacco Control 23:133–9.

Hoffmann D, Hoffmann I. (1997). The changing cigarette, 1950–1995. J Toxicol Environ Health 50:307–64.

IARC. (2004). Monograph on the evaluation of carcinogenic risks to humans. Tobacco smoke and involuntary smoking, vol. 83. France, Lyon: IARC.

IARC. (2008). Monograph on the evaluation of carcinogenic risks to humans. 1,3-Butadiene, Ethylene Oxide and Vinyl Halides (Vinyl Fluoride, Vinyl Chloride and Vinyl Bromide), vol. 97. Lyon: IARC.

IARC. (2012). Monograph on the evaluation of carcinogenic risks to humans. Chemical Agents and Related Occupations, vol. 100F. Lyon, France: IARC.

Institute of Medicine. (2011). Scientific standards for studies on modified risk tobacco products. Washington, DC: National Academies Press.

International Organization for Standardization. (1999). Tobacco and tobacco products – Atmosphere for conditioning and testing. ISO 3402:1999. International Organization for Standardization, Geneva.

International Organization for Standardization. (2012). Routine analytical cigarette-smoking machine – Definitions and standard conditions. ISO 3308:2012. International Organization for Standardization, Geneva.

International Conference on Harmonization (ICH). Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use. Available from: http://www.ich.org/products/guidelines/safety/safety-single/article/guidance-on-genotoxicity-testing-anddatainterpretation-for-pharmaceuticals-intended-for-human-use.html. [Last accessed: 25 Aug 2015].

Lewis R, Jack A, Morris J, et al. (2008). RNA interference (RNAi)-induced suppression of nicotine demethylase activity reduces levels of a key carcinogen in cured tobacco leaves. Plant Biotechnol J 6: 346–54.

Liu C, DeGrandpre Y, Porter A, et al. (2011). The use of a novel treatment process to reduce toxicant yields in cigarette smoke. Food Chem Toxicol 49:1904–17.

Majeed S, Frentzel S, Wagner S, et al. (2014). Characterization of the Vitrocell® 24/48 *in vitro* aerosol exposure system using mainstream cigarette smoke. Chem Cent J 8:62.

McAdam K, Gregg E, Liu C, et al. (2011). The use of a novel tobacco-substitute sheet and smoke dilution to reduce toxicant yields in cigarette smoke. Food Chem Toxicol 49:1684–96.

Norman A. (1999). Cigarette design and materials. In: Davis DL, Nielsen MT. (eds.) Tobacco production, chemistry, and technology 1999. Malden, MA: Blackwell Science, 353–87.

Phillips J, Kluss B, Richter A, Massey D. (2005). Exposure of bronchial epithelial cells to whole cigarette smoke: assessment of cellular responses. Altern Lab Animals 33:239–48.

Rodgman A, Perfetti T. (2013). The chemical components of Tobacco and Tobacco smoke. 2nd edition. Chapter 28. ISBN: 978-1-4200-7883-1.

Roemer E, Schramke H, Weiler H, et al. (2012). Mainstream smoke chemistry and *in vitro* and *in vivo* toxicity of reference cigarettes 3R4F and 2R4F. Beiträge zur Tabakforschung International/Contrib Tob Res 25:316–35.

Shin H-J, Sohn H-O, Han J-H, et al. (2009). Effect of cigarette filters on the chemical composition and in vitro biological activity of cigarette mainstream smoke. Food Chem Toxicol 47:192–7.

Stratton K, Shetty P, Wallace R. (2001). Clearing the smoke: assessing the science base for tobacco harm reduction. In: Institute of Medicine (eds). Washington, DC: National Academies Press.

Smith C, McKams S, Davis R, et al. (1996). Human urine mutagenicity study comparing cigarettes which burn or primarily heat tobacco. Mutat Res Environ Mutagenesis Relat Sub 361:1–9.

Thorne D, Adamson J. (2013a). A review of cigarette smoke exposure systems. Exp Toxicol Pathol 65:1183–93.

Thorne D, Kilford K, Payne R, et al. (2013b). Characterisation of a Vitrocell® VC 10 *in vitro* smoke exposure system using dose tools and biological analysis. Chem Cent J 7:146.

Thorne D, Kilford J, Payne R, et al. (2014). Development of a BALB/c 3T3 neutral red uptake cytotoxicity test using a mainstream cigarette smoke exposure system. BMC Res Notes 7:367.

The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). ICCVAM test method method evaluation report. In vitro cytotoxicity test methods for estimating starting doses for acute oral systemic cytotoxicity. Available from: http://ntp.niehs.nih.gov/iccvam/docs/acutetox_docs/brd_tmer/at-tmer-complete.pdf. [Last accessed: 25 Aug 2015].

Tokida A, Atobe I, Maeda K. (1985). Adsorption of the vapor phase components in cigarette smoke by activated carbon in vented cigarettes. Agric Biol Chem 49:3109–13.

Von Blücher H, Böhringer B, Giebelhausen J-M (2006). Pat. No. EP1918022 - High performance adsorbents based on active carbon with high micro-porosity.

Witschi H, Espiritu I, Maronpot R, et al. (1997). The carcinogenic potential of the gas phase of environmental tobacco smoke. Carcinogenesis 11:2035–42.

Wright C. (2015). Standardized methods for the regulation of cigarette-smoke constituents. Trends Anal Chem 66:118–27.