Nicotine quantification *in vitro*: a consistent dosimetry marker for e-cigarette aerosol and cigarette smoke generation

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Introduction

Next generation tobacco and nicotine products (NGPs) such as electronic cigarettes (e-cigarettes) have appeared only within the last decade or so. In this short time (compared to cigarettes) they have evolved rapidly and changed in appearance and functionality (**Figure 1**).

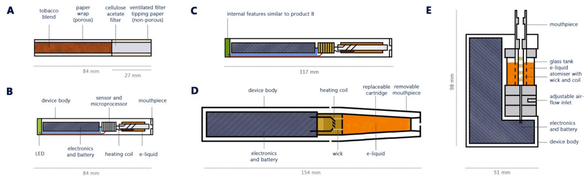

Figure 1 Different nicotine delivery products: cigarette (reference) [**A**]; commercially available first generation disposable e-cigarette (cig-a-like) [**B**]; commercially available rechargeable e-cigarette (cig-a-like) [**C**]; commercially available second generation closed modular e-cigarette [**D**]; commercially available open modular tank system [**E**]

Recent chemistry data have demonstrated the comparative simplicity of e-cigarette aerosol in comparison to tobacco cigarette smoke, with substantial differences between the levels of e-cigarette and cigarette emissions (88 to 99%)[1]. However, toxicological evaluation must follow, including the use of current and future *in vitro* test systems that can be exposed to the aerosols from these new and diverse devices. The basic requirement is that the test method should be able to generate and deliver the cigarette smoke or aerosol from different vapour products effectively and repeatedly to enable robust and reliable *in vitro* toxicological and biological evaluation. Dosimetric characterisation is an important foundation for *in vitro* exposure systems and is paramount in supporting and verifying the biological data obtained.

Objective

To compare NGP aerosol generation and nicotine quantification methods (**Figure 2**). Study 1: using a Vitrocell VC 10 Smoking Robot we compared aerosol generation from a cigarette and 4 different commercially available e-cigarettes at different regimes in the UK lab, showing that generated nicotine concentration and puff profiles can vary, even with the same base e-liquid in different e-cigarettes. Study 2: different nicotine quantification methods used by laboratories in the UK and China were employed to compare VC 10 aerosol generation from the same cigarette and e-cigarette, in an interlaboratory study.

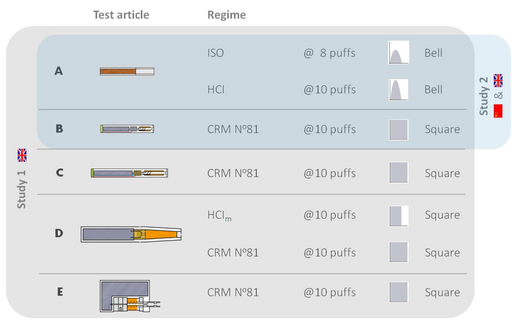

Figure 2 Test products and experimental parameters of Study 1 (product comparisons in UK lab) and Study 2 (nicotine quantification method comparison between UK and China)

Materials and Methods

All product aerosols in the UK and China were generated on the Vitrocell VC 10 Smoking Robot (Waldkirch, Germany). The aerosols of five different products (**A-E, Figures 1 & 2**) were assessed at source. Each puff was trapped on a fresh 44 mm diameter Cambridge filter pad (CFP) installed directly after the robot's mouthpiece (**Figure 3**). Nicotine products were smoked or vaped at different regimes and for different durations, based on product specifications (**Table 1**).

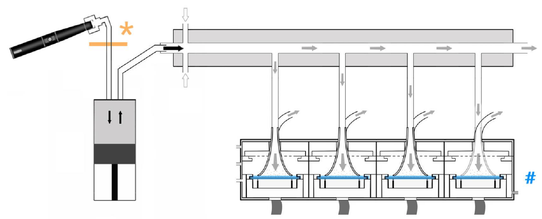

Figure 3 Nicotine measurements were made on the VC 10 at source with a CFP (indicated*), undiluted at the point of generation; product D is shown in this case. Dose measurements can also be made at the ALI (indicated#) with real-time quartz crystal microbalance monitoring[2] or with other analytical quantification methods post-exposure. Figure adapted from Adamson *et al.* 2016[3]

Table 1 Standardised puffing regimes for cigarettes and e-cigarettes

Regime name	Puff volume (ml)	Puff duration (s)	Puff frequency (s)	Puff profile
ISO	35	2	60	Bell
HCI	55	2	30	Bell
HCIm	55	2	30	Square
CRM N°81	55	3	30	Square

Exposed CFPs were placed in clean stoppered flasks and extracted in solvent. In the UK lab, CFPs were extracted in 20 ml HPLC methanol, shaken for 30 minutes at 180 rpm, and spiked with 10 ng/ml d_4-nicotine. Solvent extracts were condensed, re-suspended in 5% acetonitrile in water and nicotine concentration was quantified by ultra-high performance liquid chromatography triple quad mass spectrometry (UPLC-MS/MS).

In the Chinese lab, CFPs were extracted in 10 ml HPLC methanol, shaken for 30 minutes at 180 rpm, and spiked with 50 ng/ml n-Heptadecane. Solvent extracts were syringe filtered into auto sampler vials and nicotine concentration was quantified by gas chromatography mass spectrometry (GC-MS).

The main method differences between the two labs are described in Table 2, and the methods are reported in full in Adamson *et al*. 2017[4].

Table 2 Nicotine quantification: summary of the differences between labs

	UK	China
Analytical method	UPLC-MS/MS	GC-MS
Extraction solvent	HPLC methanol	HPLC methanol
Volume of solvent (ml)	20	10
Internal standard	d_4-nicotine	Heptadecane
[Internal standard] for the samples (ng/ml)	10	50
Concentration range for calibration (ng/ml)	10-10,000	5-100
Points on calibration curve	10	5
Usage of e-cigarette	Same device, three successive runs (no cooling between runs)	Same device, cooled to room temperature between runs

Results

STUDY 1 – Comparing [nicotine] at source on the VC 10 from a cigarette and a number of different e-cigarettes, at different regimes in the UK lab (**Figure 4**):

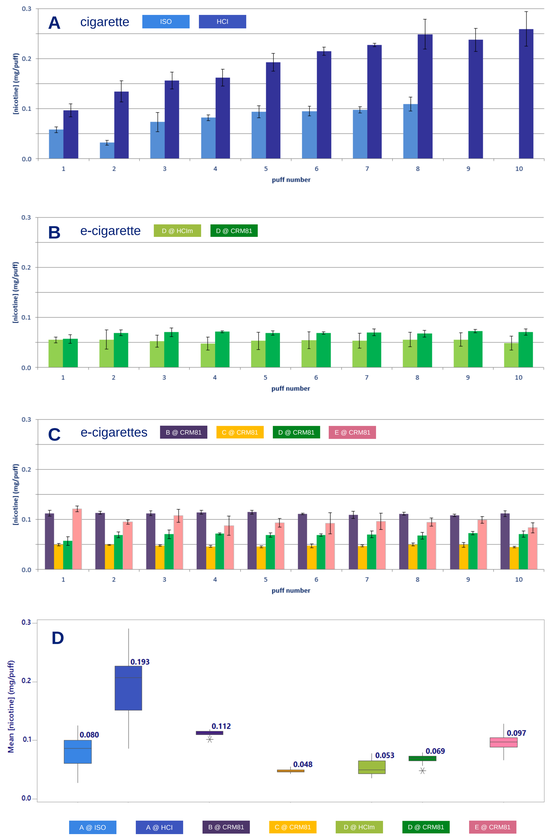

Figure 4 Product and puffing regime nicotine comparisons. Puff-by-puff analysis of reference cigarette nicotine concentration at the ISO and HCI regime [**A**]; comparison of the same e-cigarette (product D) at the HCIm and CRM N°81 regimes [**B**]; comparison of four different e-cigarettes at the same regime (CRM N°81) (products B-E) [**C**]; mean nicotine concentration per puff; asterisks denote outliers [**D**]. All products/regimes were repeated 3 times (n=3)

STUDY 2 – Comparing aerosol generation on the VC 10 from a cigarette and an e-cigarette, and comparing different nicotine quantification methods, in the UK and China (**Figure 5 & 6**):

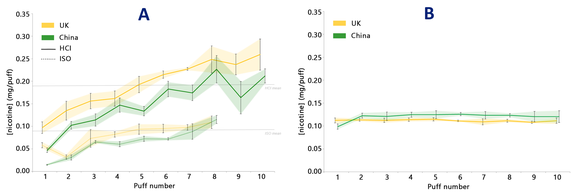

Figure 5 Interlaboratory nicotine at source. Puff-by-puff analysis of nicotine concentration from a cigarette at the ISO and HCI regime (product A) [**A**], and an e-cigarette at the CRM N°81 regime (product B) [**B**]; (n=3/product/lab)

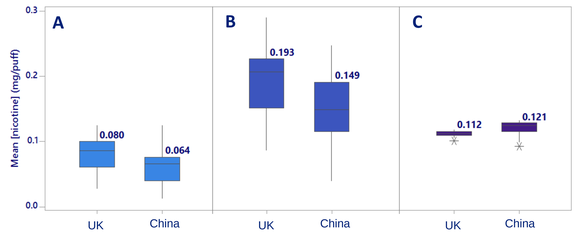

Figure 6 Mean interlaboratory nicotine at source from the two products tested in two labs: cigarette following the ISO regime [**A**], cigarette following the HCI regime [**B**], e-cigarette following the CRM N°81 regime [**C**]. Outliers are denoted by asterisks (n=3/product/lab)

Table 3 Mean interlaboratory nicotine at source

Product	UK (mg/puff)	China (mg/puff)
A at ISO (cigarette)	0.080±0.026	0.064±0.031
A at HCI (cigarette)	0.193±0.055	0.149±0.054
B at CRM N°81 (e-cigarette)	0.112±0.004	0.121±0.010

Conclusions

Dosimetry plays an important role in understanding the dose delivery of test article aerosols to *in vitro* cultures during exposure. There is a key requirement to understand how the exposure system works to generate, dilute and deliver aerosols to *in vitro* systems. It is paramount to understand what the exposure system is generating at source, and delivering to the cells thereafter. This study addressed the first part of such characterisation: are aerosols generated consistently, is regime important, and do all NGPs (e-cigarettes) generate the same aerosol? In addition, two different nicotine quantification methods were compared in labs in UK and China.

The key findings from this *in vitro* dosimetry study demonstrated:

- Reference cigarette smoke delivers different nicotine concentrations across ISO and HCI smoking regimes, and across puff numbers in accordance with known smoke formation and delivery mechanisms (**Figure 4A**)
- Nicotine assessment across 4 different e-cigarettes showed consistent delivery of nicotine per puff within products, but with different levels of nicotine across products, even with the same base e-liquid (**Figure 4B**)
- Puffing regime affects e-cigarette nicotine delivery particularly puff duration and puff flow profile (2 vs. 3 seconds) (**Figure 4C**)
- There was good overlap in nicotine results obtained in two laboratories utilising different methods for nicotine quantification (**Figure 5**)
- Interlaboratory assessment of nicotine generated at source from an e-cigarette and a cigarette in two labs, with different analytical quantification methods showed agreement in the values obtained (**Figure 6** and **Table 3**):
- When all variables were combined in a GLM ANOVA, the interlaboratory difference was **p=0.067** and the interaction of 'lab * puff number' was **p=0.960**

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3. Adamson *et al*. 2016. **Application of dosimetry tools for the assessment of e-cigarette aerosol and cigarette smoke generated on two different *in vitro* exposure systems**. *Chem. Cent. J.*, 10:74
4. Adamson *et al*. 2017. **Nicotine quantification *in vitro*: a consistent dosimetry marker for e-cigarette aerosol and cigarette smoke generation**. *Applied In Vitro Toxicol.*, 3:1

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The e-cigarette category is evolving rapidly providing consumers with a variety of formats, ranging from ‘cig-a-like’ products to larger, high powered modular devices. When generating an *in vitro* assessment approach across such diverse products, dosimetry considerations are paramount. In this investigation we have compared nicotine quantification techniques in two studies using a Vitrocell VC 10 Smoking Robot to generate aerosols from different e-cigarettes.

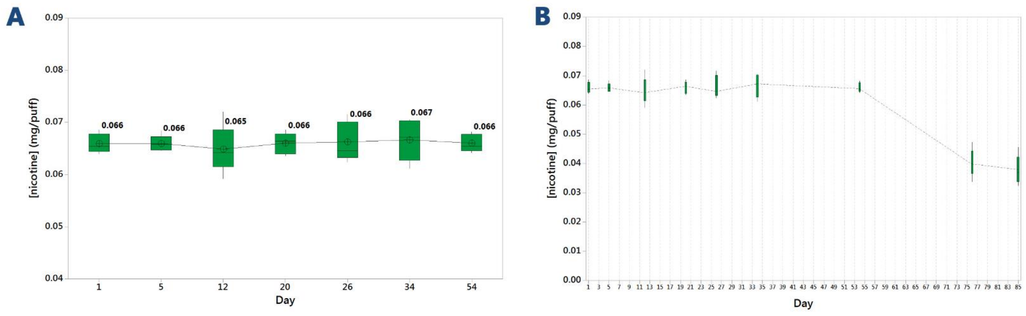
In Study 1, a 3R4F reference cigarette and four different commercially available e-cigarettes were compared: puff-by-puff nicotine concentration was quantified, at the same e-cigarette puffing regime (CRM Nº81) or with different puff durations, (2 or 3 seconds), comparing 3R4F puff-by-puff yields at ISO and HCI smoking regimes. In Study 2, 3R4F and one e-cigarette were assessed for puff-by-puff nicotine concentration in different locations (China and UK) comparing different nicotine quantification methods by GC/MS and UPLC-MS/MS used in the two laboratories.

Study 1 showed that 3R4F cigarette delivers different nicotine concentrations across the different regimes and puff number, supporting the nicotine methodology; e-cigarettes tested generated different amounts of nicotine across the devices tested, but showed consistent puff-by-puff delivery per device. Study 2 showed positive agreement between results across two different laboratories utilising different methods for nicotine quantification; statistical analysis, combining all interlaboratory variables indicated laboratory differences and the interaction of ‘lab and puff number’ were not significant (p=0.067 and 0.960 respectively).

These studies will add further knowledge to support the *in vitro* assessment of novel nicotine products, providing reliability and assurance around *in vitro* dosimetry.

Supplementary data:

In an additional investigation the UK lab, a nicotine stability study was conducted to assess the shelf life of extracted CFP nicotine (from an e-cigarette) over time. This was to support future studies where samples may be exchanged and analysed by different laboratories. Results of these studies suggest that assessment of nicotine extract samples may provide a more reproducible and reliable range of diluted effluents for toxicology testing.



Nicotine extracts from an e-cigarette show no degradation in nicotine day 1 to 54 [**A**] but then a decline thereafter up to day 85 (where no further measurements were taken) [**B**]; (n=5/timepoint)

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Conflict of interest statement: The authors declare that there are no conflicts of interest. All of the authors are full time employees of British American Tobacco or the Zhengzhou Tobacco Research Institute of CNTC.