Characterization of smoke and aerosol deliveries from combustible cigarettes, heated tobacco products and electronic nicotine delivery systems in the Vitrocell® Mammalian 6/48 exposure module☆

Brian M. Keyser a,*, Robert Leverette a,*, Michael Hollings b, Adam Seymour b, Randy A. Weidman c, Carlton J. Bequette c, Kristen Jordan a,1

a RAI Services Company, Scientific & Regulatory Affairs, 401 North Main Street, Winston-Salem, NC 27101, USA
b Labcorp Early Development Laboratories Ltd., Harrogate, North Yorkshire, UK
c RJ Reynolds Tobacco Company, 950 Reynolds Blvd., Winston-Salem, NC 27106, USA

ARTICLE INFO
Handling Editor: Dr. L.H. Lash

Keywords:
Whole smoke
Whole aerosol
Dosimetry
Nicotine

ABSTRACT
The rapid development associated with Next Generation Tobacco Products (NGTP) has necessitated the development of high throughput methodologies to test their genotoxic potential in vitro when compared to conventional cigarette smoke (CS). An assessment of two Vitrocell® Mammalian 6/48 exposure modules in three independent experiments was made by comparing results from multiple dosimetric techniques applied to aerosol generated from 3R4F Kentucky Reference cigarettes, commercially available electronically heated tobacco product (eHTP) and Electronic Nicotine Delivery System (ENDS) using the Vitrocell® VC10®. Real-time aerosol particle concentration was assessed by means of light scattering photometers and expressed as area under the curve (∑AUC). Nicotine concentrations were determined analytically by LC/MS. Humectant amount and distribution was assessed for eHTP and ENDS by the quantification of free glycerol in a phosphate buffered saline (PBS) trap, whereas total particulate matter (TPM) was assessed in the 3R4F cigarettes by the fluorescence of the particulate at 485 nm in anhydrous dimethyl sulfoxide (DMSO) trap within the exposure. Dose was adjusted by means of the addition of ambient air to dilute the whole smoke/aerosol in L/min and sampled into the system at a rate of 5 mL/min. Dilution of CS ranged from 8.0 to 0.5 L/min and for the eHTP and ENDS ranged from 4 to 0 L/min (undiluted). Dosimetric analysis of the system showed good concordance within replicates (p-values ranged from p = 0.3762 to p = 0.8926) and showed that the Vitrocell® Mammalian 6/48 is a viable means for genotoxic assessment of aerosol generated from both conventional cigarettes and NGTP. Results demonstrate the need to tailor dosimetry approaches to different aerosols due to variations in the physio-chemical composition, with a multi-dosimetry approach recommended.

1. Introduction
The chemical composition of aerosol generated from tobacco changes significantly depending on the degree to which it is heated. At temperatures > 650 °C [11,21] combustion occurs resulting in over 8000 known chemicals being generated [29]. By reducing the temperature below the point of combustion [33] to that of which nicotine volatilizes, electronically heated tobacco products (eHTP) have the ability to provide the end user with an aerosol containing nicotine but with a lower concentration of tobacco combustion related byproducts [31], potentially reducing the harm of these products [17,36,38].

Whilst there is a theoretical basis to assume that harm is reduced, for any product to make reduced risk claims in the United States of America the product must have been designated a modified risk tobacco product (MRTP) by the Food and Drug Administration (FDA) [5]. As such, it is important to ensure that any methodology by which a comparison is to be made against cigarettes is fully characterized and fit for purpose. Historically, assessments pertaining to the toxicity of cigarette smoke

☆ RAI Services Company performs regulatory compliance services for Reynolds American Inc.’s (“RAI”) subsidiary companies. RAI is an indirect, wholly owned subsidiary of British American Tobacco p.l.c.
* Corresponding authors.
E-mail addresses: keyserb@RJRT.com (B.M. Keyser), leverer@RJRT.com (R. Leverette).
1 senior author

https://doi.org/10.1016/j.toxrep.2022.11.001
Received 11 April 2022; Received in revised form 27 October 2022; Accepted 3 November 2022
Available online 4 November 2022
2214-7500/© 2022 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
(CS) have been those that incorporate the gaseous or particulate fractions (or a combination thereof) into assays primarily designed to assess the potential effects of single chemicals to human health and the environment [13,32,34,7,8]. While extremely robust, these assays were not conceived with the intention of assessing complex mixtures and as such there is interest in investigating approaches that look at the interaction of freshly generated aerosol with cellular assays that are exposed at the air liquid interface (ALI) or equivalent [10] rather than under submerged conditions [42]. By performing exposures at the air liquid interface, direct cell-aerosol interactions occur with both gaseous and particulate phases simultaneously and allow for a more biologically relevant approximation of exposure, without the impact of dissolution.

One well characterized system that is capable of generating fresh aerosol and delivering it to cells at the air liquid interface is the VC10®, produced by Vitrocell Systems GmbH [1,18,22,37,4,40]. Aerosol is generated via a fully automated rotary smoking machine from cigarettes, eTPs or Electronic Nicotine Delivery Systems (ENDS) and is diluted via a constant supply of compressed air as a means of adjusting dose. The diluted aerosol is then sampled at a constant rate under negative pressure over the test system where it is guided via a trumpet shaped inlet over a porous membrane on which the cells are grown. While exposure at the ALI is considered the gold standard for in vitro testing, there have historically been issues regarding low throughput. The emergence of a wide variety of ENDS on the market recently has resulted in the practical need to increase testing capability. In line with this requirement, a High Throughput Module has been developed by Vitrocell® that allows the simultaneous testing of 48 samples at the ALI with 6 replicates per dose. The primary aim of this study was to characterize the Vitrocell® 6/48 exposure module using a range of dosimetry techniques to ensure suitability and replicability for exposure of in vitro test systems (cell culture, tissue, etc.) to combustible cigarettes, ENDS and eHTP. The secondary aim was to demonstrate that the exposure can be replicated between two different modules.

2. Materials and methods

All chemicals and reagents were obtained from Merck KGaA (Darmstadt, Germany) unless otherwise stated. PBS was obtained from Gibco® via Thermo Fisher Scientific (Waltham, USA).

2.1. Test articles

The combustible cigarettes selected for this study were the University of Kentucky Reference Cigarette - 3R4F. The 3R4F reference cigarette was designed to replicate marketed “full flavored” American blended filtered cigarettes and has been widely used throughout the industry as a comparator against potentially reduced risk products [30]. Prior to their use, cigarettes were conditioned according to ISO standard 3402, namely loose cigarettes under forced airflow were held for at least 48 h and no more than 10 days at 22 ± 1 °C and 60 ± 3 % relative humidity [15].

EHTP was of a commercially available variety and was purchased in the UK direct from the manufacturer. eHTP consumables were conditioned packeted in cellophane (as purchased) for a minimum of 48 h and no more than 5 days at 22 ± 1 °C and 60 ± 3 % relative humidity [15]. ENDS was a commercially available pod variety (Mint flavor) and was purchased in the US direct from the manufacturer. ENDS consumables were stored at room temperature sealed in their original packaging until transferred to the laboratory for use. At the time of the experiments, the policy of RJ Reynolds, which funded this study, the authors are not able to share the brand name and trademarks of the devices used in this study.

2.2. Aerosol generation and exposure

Cigarette smoke was generated using a Vitrocell® VC10®, serial number VC10/210311 (Vitrocell® Systems GmbH, Waldkirch, Germany), and eHTP/ENDS aerosol was produced using a Vitrocell® VC10®, serial number VC10/200814. Different VC10® smoking machines were used for combustible cigarettes and eHTP/ENDS aerosol to avoid contamination, Mammalian 6/48 modules were used with all aerosol and a cleaning protocol of the modules was followed when moving from cigarettes to eHTP/ENDS aerosol. Different concentrations of whole smoke were achieved by altering the diluting airflow using mass flow controllers (Analyt-MTC GmbH, Mülheim, Germany). For all experiments, concentrations were expressed in terms of air flow in liters per minute (L/min). The vacuum rate was fixed at 5 milliliters per minute (mL/min). Operation and confirmation of functionality including installation, operational and performance qualification was performed as previously described [12]. All cigarettes were smoked according to the HCl smoking regimen (T-115, 1999) (namely; 55 mL puff, 2 s duration, 30 s frequency, with 100 % vent blocking) [35]. All eHTP consumables were smoked according to a modified HCl (mHCl) smoking regimen (T-115, 1999) [35], with no vent blocking as the consumables do not possess ventilation holes. All ENDS consumable were vaped according a modified ISO (mISO) method [16] with the following adaptations, a 60 s pause was taken after every 10 puffs.

This study utilized two Vitrocell® Mammalian 6/48 exposure module climatic chambers (serial numbers 10/17 and 43/17 for Mammalian 6/48 module (1) and (2) respectively). The Vitrocell® Mammalian 6/48 module utilizes the same technology as previously described [12,40,41], however this equipment allows higher throughput with up to seven doses and one air control all with up to six replicates (Fig. 1). Additional individual dosimetry modules were located on each of the seven dose rows to allow dosimetric assessment of delivered dose.

2.3. Photometers

Photometers were purchased from Vitrocell® Systems, GMBH. For combustible cigarettes, photometers were harmonized to whole smoke using the HCl regimen [19] and a diluting airflow of 1.5 L/min. For ENDS and eHTP consumables, photometers were harmonized to whole aerosol using a mHCl and mISO regimen, respectively. Eight photometers were harmonized for 3R4F and seven photometers were harmonized for the market ENDS and eHTP so that the voltage for each photometer was approximately 4.0 v (photometers are not suitable for an undiluted airflow). Harmonization was performed as previously described in [19]. Briefly, each photometer was connected to the VC Photometer Control Box, which was then connected to a computer. Photometer data was output as both comma-separated value (CSV) and virtual compact disk (VCD) file formats.

2.4. DMSO fluorescence

The fluorescence of the particulate of cigarettes in DMSO at Excitation (Ex)355 / Emission (Em)485 has been reported previously [3,27]. 3R4F pad-collected Total Particulate Matter (TPM) prepared in DMSO at 24 mg/mL was serial diluted (2-fold dilutions) in dimethyl sulfoxide (DMSO) (0.0024–5 mg/mL). The Ex355 / Em485 readings were taken from these dilutions to establish a standard curve of corrected relevant fluorescent units (RFU) versus TPM concentration (mg/mL). Immediately post exposure, all DMSO samples were retained and triplicate 100 µL aliquots from each well were plated into 96 well plates. Analysis was performed using a VERSAmax™ Microplate reader (Molecular Devices, San Jose, CA). Values were represented in RFU. The mean values for each smoke exposure were subtracted from the mean RFU for the air control to give relative fluorescence per smoke dose. An equivalent TPM concentration was determined using the standard curve.

2.5. Free glycerol determination assay

The quantity of glycerol deposited at the ALI was quantified by
enzymatic determination (F6428–40 mL) of glycerol when a PBS trap was placed in the module. Free glycerol reagent was reconstituted with 40 mL of ultrapure water. Glycerol standard (G7793–5 mL) solutions were prepared using a serial dilution from 130 to 2.03125 µg/mL, 100 µg/mL was also included in PBS. To ensure that the glycerol measurement was comparable to that of cells under exposure conditions, the quantity of PBS placed within the module was calibrated to ensure that meniscus height and permeable membrane location were equal. Optical density (OD) was measured at 540 nm on a Spectramax plate reader (Molecular Devices, San Jose, CA). Free glycerol absorbance was expressed in terms of absolute optical density (OD$_{540}$). The mean OD$_{540}$ values for each aerosol dilution were subtracted from the mean OD$_{540}$ obtained from the air control to give free glycerol absorbance per aerosol dose. Using this value and the glycerol standard curve, an equivalent glycerol concentration (µg/mL) was determined.

2.6. Nicotine

Samples in either PBS or DMSO were separated using a binary gradient and a Phenomenex Kinetex 2.6 µm EVO C18 HPLC column. Detection and analysis of nicotine was performed using a triple quadrupole tandem mass spectrometer utilizing electrospray ionization (ESI) and multiple reaction monitoring (MRM). The linear range of the method was approximately 5 ng/mL-500 ng/mL with a limit of quantification (LOQ) defined as the nominal concentration of the lowest level calibration standard and a limit of detection (LOD) defined as 1.5 ng/mL. Statistical analysis utilizing slope and standard deviation of the calibration curve (ICH Harmonized Tripartite Guideline, Q2(R1) Section 6) determined LOD values of 6 ng/mL (PBS) and 5 ng/mL (DMSO) and were determined experimentally as 1.5 ng/mL for both PBS and DMSO.
2.7. Exposures

In each whole smoke exposure experiment, each well exposed to the test article and flowing air control contained a stainless steel transwell (Vitrocell® Systems, GmBH) insert containing 3 mL of DMSO. In seven individual externally mounted dosimetry modules, a stainless steel Transwell insert containing 3 mL of DMSO was placed in each well. One ‘in-line’ photometer was placed between the dosimetry module and dilution system (Fig. 2).

3R4F studies were completed on two Mammalian 6/48 systems and each study consisted of three independent experiments which included seven whole smoke dilutions generated from eight cigarettes using the HCl smoking regimen. The smoke was serially diluted with the addition of diluting air via the air inlets to achieve the desired concentration for the diluted smoke doses (Fig. 2).

eHTP/ENDS studies were completed on the same two Mammalian 6/48 systems as the 3R4F and each eHTP or ENDS study of three experiments included an undiluted and six whole aerosol dilutions generated from ten consumables using the mHCl or mISO regimen, respectively. The whole aerosol was exhausted from two ports of the VC10® and directed to the dilution system. The aerosol directed to the first row was then serially diluted via the addition of diluting air via the air inlets to achieve the desired concentration for the diluted aerosol doses. (Fig. 3).

2.8. Data analysis

Photometer data was output as both comma-separated value (CSV) and virtual compact disk (VCD) file formats. Voltage for each airflow was recorded every second and from this, an area under the curve value was calculated for each experiment. For DMSO captured material an equivalent TPM concentration was calculated using the Ex355 / Em485 readings against a standard curve of known 3R4F HCl TPM concentrations (0.0024–5 mg/mL). To estimate free glycerol deposition in PBS, an equivalent glycerol concentration (mg/mL) was calculated using the corrected OD540 against a standard curve (130–2.03125 µg/mL). Nicotine values were reported in ng/mL.

Prior to analysis, results were subject to Iterative Grubbs’ test (significance α = 0.05) to identify outliers. This is a recommended outlier test and was used for DMSO captured material, free glycerol deposition and nicotine analysis [25]. For photometer data, Grubbs’ test (significance α = 0.05) was used due to the smaller sample size. From the results obtained, nonlinear regression analysis was used to make pairwise comparisons between the two Vitrocell® Ames 48 exposure modules.

3. Results

3.1. Photometers

Since the photometer data is connected to the module only at one of the wells for each row (Fig. 2), evaluation of the variation of the deposition between the wells of each row was determined to be ±15 % of the row mean for all of the test articles used here (data not shown). The variation seen was within the range stated by the manufacturer which is ±15 % of the row mean. AUC data from the photometers is presented in Table 1. Data are presented as the mean from three individual experiments. AUC data from eHTP were consistent between experiments as demonstrated by the low coefficient of variance, with all values less than 15 %. AUC data from 3R4F cigarettes demonstrated consistency at the lower airflows; however, at airflows higher than 4 L/min the increased airflow does appear to have some influence on the AUC values, as indicated by the increase in the %CV values in module 2. AUC data from ENDS showed the coefficient of variance was increased versus the other two product categories; however, inter-experimental comparisons are more variable and this could be attributed to the ENDS product variability. In all cases except for 1 L/min for ENDS in Mammalian 6/48 module (1) there is a concentration related increase in measured dose.

3.2. DMSO fluorescence

DMSO fluorescence values from all experiments are shown in Table 2. An equivalent TPM concentration was calculated using the corrected relative fluorescent units (RFU) extraplated against a standard curve of known concentrations. In all experiments and in both modules, there was a concentration related increase in deposited mass. The deposited mass was consistent between each module. There was higher variance observed for Mammalian 6/48 module (2) at the higher airflows, however this can be attributed to the lower deposition at these airflows.

3.3. Free glycerol determination assay

Free Glycerol deposition values from all experiments are shown in Table 3. An equivalent glycerol concentration (mg/mL) was calculated using the corrected OD540 against a standard curve from which the values were extrapolated. For eHTP, the free glycerol deposition values reported from both Mammalian 6/48 modules increased in a concentration related manner, with the exception of 4 L/min for Mammalian 6/48 module (1) where the coefficient of variance was less than 15 %. For ENDS, the free glycerol deposition reported from both Mammalian 6/48 modules increased in a concentration related manner. Higher variation is observed in Mammalian 6/48 module 2 than module 1 and no outliers

![Fig. 3. Vitrocell® VC10® and Mammalian 6/48 High throughput module schematic.](image-url)
were identified using Iterative Grubbs’ test (significance α = 0.05); however, one experiment was identified as driving the variance.

### 3.4. Nicotine

Nicotine concentrations from all experiments are shown in Table 4. In all conditions a concentration related increase was observed in deposited nicotine demonstrating consistency in dilution and vacuum rates of the Mammalian 6/48 system. For 3R4F cigarettes the coefficient of variance was less than 15 % for airflows of 4 L/min and below; for 6 L/min and above the coefficient of variance was higher, which can be attributed to the lower nicotine deposition due to the higher velocity of the higher airflows. Higher variability is observed for eHTP and ENDS; however, this is not un-expected due to the lower deposition and the nature of battery operated heating devices.

### 3.5. Comparison of dosimetry techniques

To compare the multiple dosimetry techniques, pairwise comparisons were made between the two Vitrocell® Mammalian 6/48 exposure modules for each of the three test articles. Linear regression (GraphPad Prism version 8.0.0, GraphPad Software, San Diego, California USA) was used to calculate the slopes of the individual dosimetry curves for each product type and these were compared to determine if any significant differences were observed between the two Mammalian 6/48 exposure modules. Y values were transformed (Y=Log(Y)) prior to linear regression. The following comparisons were made for each test article; equivalent TPM (mg/mL) (3R4F) or free glycerol (mg/mL) (eHTP or ENDS) versus photometer (ΣAUC) and nicotine (ng/mL) versus equivalent TPM (mg/mL) (3R4F) or free glycerol (mg/mL) (eHTP or ENDS) (Fig. 4). No statistical differences were observed in any of the comparisons; p-values ranged from 0.8926 demonstrating that the differences between the slopes are not significant.

### 4. Discussion

The Vitrocell® Mammalian 6/48 module in vitro exposure system was developed to increase the number of airflows or concentrations per
experiment from four in the standard Vitrocell® module system as previously described [12,39,41] to seven doses, allowing further discrimination and to better elucidate toxicity or mutagenic activity. The Vitrocell® Mammalian 6/48 module also allows each airflow to be exposed to six replicates versus only three for the standard module. The addition of three airflows and the increase in replicates also allows further compatibility and alignment of the whole aerosol neutral red uptake assay to the ICCVAM and OECD guidelines [14,24], which recommends eight concentrations and six replicates per concentration.

Understanding and characterizing a technically complex exposure system is crucial in order to decipher any results in terms of toxicological assessments. It was also critical to characterize this new exposure system with multiple tobacco product test article types due to the differences in aerosol composition between a combustible cigarette and next generation tobacco products (NGTP) [2]. The dosimetry techniques utilized for this study have been previously demonstrated as useful in the

Table 4

Nicotine values from Mammalian 6/48 modules 1 and 2 using 3R4F, eHTP and ENDS test articles (Seven replicates per airflow per experiment, three independent (n = 3) experiments). Dose was adjusted by means of the addition of ambient air to dilute the whole smoke/aerosol in L/min.

| Diluting Airflow (L/min) | Nicotine (ng/mL) | Diluting Airflow (L/min) | Nicotine (ng/mL) | Diluting Airflow (L/min) | Nicotine (ng/mL) |
|-------------------------|------------------|-------------------------|------------------|-------------------------|------------------|
|                         | Mean  | SD   | %CV | Mean  | SD   | %CV | Mean  | SD   | %CV |
| 3R4F HCI Mammalian 6/48 module (1) |       |      |     |       |      |     |       |      |     |
| 10                      | 1074  | 325  | 30  | 4     | 227  | 82  | 36  |
| 8                       | 1308  | 340  | 26  | 3     | 457  | 325 | 71  |
| 6                       | 1927  | 474  | 25  | 2     | 886  | 590 | 67  |
| 4                       | 3333  | 411  | 12  | 1     | 3543 | 935 | 26  |
| 2                       | 7305  | 726  | 10  | 0.5   | 7385 | 3484 | 47  |
| 1                       | 12808 | 1351 | 11  | 0.25  | 12881| 4794 | 37  |
| 0.5                     | 19665 | 2449 | 12  | 0     | 43782| 8999 | 21  |
| 3R4F HCI Mammalian 6/48 module (2) |       |      |     |       |      |     |       |      |     |
| 10                      | 626   | 229  | 37  | 4     | 734  | 405 | 55  |
| 8                       | 529   | 184  | 35  | 3     | 1177 | 690 | 59  |
| 6                       | 1121  | 325  | 29  | 2     | 2333 | 1233 | 53  |
| 4                       | 3462  | 498  | 14  | 1     | 4659 | 2041 | 44  |
| 2                       | 6517  | 716  | 11  | 0.5   | 7880 | 2679 | 34  |
| 1                       | 10902 | 881  | 8   | 0.25  | 11702| 2121 | 18  |
| 0.5                     | 14512 | 1392 | 10  | 0     | 37177| 9734 | 26  |

Fig. 4. Nonlinear regression comparing dosimetry measurements between two Vitrocell® Mammalian 6/48 exposure modules ((1) and (2)) using each of the three test articles.
characterization of exposure systems with cigarettes, eHTPs and ENDS [19]. Photometers were used to characterize the particle density of aerosols through the measurement of scattered light. As a laser passes through the aerosol stream, scattered light is then detected via an offset photodetector. Photometers are an important dosimetric tool as they can be used across product types, although a direct comparison in the output voltage cannot be made as each set of photometers is harmonized to the specific aerosol being measured. The harmonization is constant as long as the size distribution of the aerosol does not change; however, since the size of the particles in the aerosol changes over time, the photometers cannot give a mass concentration in this application. This is noted; however, photometers demonstrate aerosol movement throughout the system and have historically been used for different aerosol classes [18, 28, 43, 6, 9]. Enzymatic determination of glycerol and equivalent TPM concentrations via DMSO fluorescence offer an efficient and accurate assessment of aerosol deposition [19].

The data generated from the three product types used in this study (combustible reference cigarette, eHTP and ENDS) analyzed via the dosimetry techniques mentioned demonstrates the ability of the Vitrocell® Mammalian 6/48 module to differentiate the aerosol deliveries between different dilution airflows. In all experiments a concentration related increase in deposition was observed using photometers, nicotine and either glycerol or equivalent TPM. There were some sporadic occasions at the higher airflows where a higher diluting airflow resulted in a higher value for AUC and nicotine concentration. This was considered the result of lower deposition at the higher airflows and it was not seen consistently between the two Vitrocell® Mammalian 6/48 modules, and across all product types. This is considered to be an artefact and has been reported previously [18]. The data from the dosimetry techniques demonstrate a correlation between the whole smoke/aerosol delivered to the module as measured via photometer and deposition in the module with quantification of nicotine and either glycerol or DMSO fluorescence of particulate matter. For AUC values, a high degree of consistency between experiments and modules was observed for 3R4F and eHTP as demonstrated by the low coefficient of variance (<15%). Some variability for 3R4F were observed; however, this was only at the highest airflows in one module. ENDS results were more variable for AUC; however, this can be attributed to low number of cartridges used per exposure (1), unlike the other product types where multiple units of product were used per exposure. Only one cartridge was used per exposure in order to assess the generation and deposition across the complete usage of the product. This was considered to be more representative of actual usage rather than sampling a smaller range of puffs from multiple cartridges. Previous examples of where running a single ENDS consumable and device resulted in higher variability for AUC exist in the literature [23]. Moreover, as discussed, higher variability is observed with higher airflows across all product types. This is not unexpected due to the lower deposition at these concentrations [1].

Comparing the deposition of the aerosols from the three tobacco product types in both Vitrocell® Mammalian 6/48 exposure modules resulted in no statistically significant differences. All comparisons resulted in p > 0.05, with p-values ranging from p = 0.0610 to p = 0.9544 across the three comparisons per product type (Fig. 4). The diagrams in Fig. 4 show a non-linear trend and non-linear regression was used for this data. Previous characterization work with a similar module (Ames 48) using the same endpoints and comparisons was also performed using linear regression [28]. The data in Fig. 4 was also analyzed using linear regression and all comparisons (data not shown). The comparison of the 3R4F Kentucky Reference cigarettes resulted in the highest concordance with p = 0.4731 to p = 0.9544. This result is not surprising because 3R4F is a certified reference product designed for proficiency and monitoring studies. Having access to standardized eHTP and ENDS reference products (or systems) would enable more robust across-product category proficiency studies and enable direct comparison of different exposure systems through the measurement of select analytes, such as nicotine.

This study has demonstrated the importance of characterizing and understanding the aerosol delivery and deposition properties demonstrated by three different tobacco product types within an ALI exposure system. It also emphasizes the requirement to tailor dosimetry techniques to the product being used and the analyte(s) being examined [22, 26, 37]. The suitability and accuracy of the dosimetry measures have been shown for glycerol and equivalent TPM deposition. It is also noted that for a true comparison, having a common analyte across product types is useful for comparisons and nicotine is ideal for the test articles examined herein. Photometers are a useful real-time measure of aerosol distribution throughout the test system; however, without the ability to calibrate the photometers to a known standard prevents them from being used in a quantitative manner [19].

Overall, the experimental conditions with the dosimetry techniques employed for this characterization demonstrated reproducible and consistent delivery of whole aerosol generated from 3R4F Kentucky Reference cigarettes and commercially available eHTP and ENDS to both of the Vitrocell® Mammalian 6/48 modules used in this study. Further assessment is required to move beyond the characterization of aerosol deposition within this system to include the associated biological responses (i.e., cytotoxicity, genotoxicity) within this system.

CRediT authorship contribution statement

Brian M. Keyser: Conceptualization, Methodology, Formal analysis, Writing – review & editing, Visualization, Supervision, Project administration. Robert Leverette: Conceptualization, Methodology, Formal analysis, Writing – review & editing, Visualization, Supervision. Michael Hollings: Conceptualization, Methodology, Formal analysis, Writing – original draft. Adam Seymour: Conceptualization, Methodology, Investigation. Randy A. Weidman: Investigation, Writing – review & editing. Carlton J. Bequette: Investigation, Writing – review & editing. Kristen Jordan: Writing – review & editing, Funding acquisition.

Declaration of Competing Interest

Brian M. Keyser, Robert Leverette, and Kristen Jordan are full time employees of RAI Services company. Randy A. Weidman and Carlton J. Bequette are full time employees of RJ Reynolds Tobacco Company.

Data Availability

The data that has been used is confidential.

Acknowledgements

The authors acknowledge Hsiao-Pin Liu for his assistance in the statistical analysis of the data and Eric Scott Ph.D. for their critical review of the manuscript. The authors also acknowledge Kamal J. Rashmawi Ph.D., for his expertise and development of the analytical methodology.

Disclosure statement

The data presented in this manuscript was generated and analysed in studies commissioned by RAI Services Company and conducted under contract at Labcorp Early Development Laboratories Ltd. Brian M. Keyser, Robert Leverette, and Kristen Jordan are full time employees of RAI Services company. Randy A. Weidman and Carlton J. Bequette are full time employees of RJ Reynolds Tobacco Company.

References

[1] J. Adamson, D. Thorne, G. Errington, W. Fields, X. Li, R. Payne, T. Krebs, A. Dalrymple, K. Fowler, B. Dillon, et al., An inter-machine comparison of tobacco

1991
smoke particle deposition in vitro from six independent smoke exposure systems, Toxicol. Vitr. 28 (2014) 1320–1328.

[2] J. Adamson, D. Thorne, B. Zainuddin, A. Baxter, J. McAughey, M. Gaca, 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 (2016) 74.

[3] M. Auferheide, H. Gresmann, A modified Ames assay reveals the mutagenicity of native cigarette mainstream smoke and its gas vapour phase, Exp. Toxicol. Pathol.: Off. J. Ges. fur Toxikol. Pathol. 58 (2007) 383–392.

[4] H. Behrsing, M. Aragon, J. Adamson, D. Sheehan, M. Gaca, R. Curren, E. Hill, Characterization of a vitrocell VC1 using nicotine dosimetry; an essential component toward standardized in vitro aerosol exposure of tobacco and next generation nicotine delivery products, Appl. Vitr. Toxicol. 4 (2018) 159–166.

[5] R. Carvajal, D. Clisnold, J.J.P. Shapiro, D. Li, 2009. The family smoking prevention and tobacco control act: an overview, 64, 717.

[6] Y.S. Cheng, E.B. Barr, J.M. Benson, E.G. Damon, M.A. Medinsky, C.H. Hobbs, T.J. Goehl, Evaluation of a real-time aerosol monitor (RAM-S) for inhalation studies, Fundam. Appl. Toxicol. 10 (1988) 321–328.

[7] I. Crooks, D.M. Dillon, J.K. Scott, M. Ballantyne, C. Meredith, The effect of long term storage on tobacco smoke particulate matter in in vitro genotoxicity and cytotoxicity assays, Regul. Toxicol. Pharm. 65 (2013) 196–206.

[8] I. Crooks, L. Neilson, K. Scott, L. Reynolds, T. Oke, M. Forster, C. Meredith, P.J. Dacunto, K.C. Cheng, V. Acevedo-Bolton, R.T. Jiang, N.E. Klepeis, J.L. Repace, W.R. Ott, L.M. Hildemann, Real-time particle monitor calibration factors and PM2.5 emission factors for multiple indoor sources, Environ. Sci. Process Impacts 15 (2013) 1511–1519.

[9] B. Davis, V. To, P. Talbot, Comparison of cytotoxicity of IQOS aerosols to smoke from Marlboro Red and 3RF reference cigarettes, Toxicol. Vitr. 61 (2019), 104652.

[10] P. Ermola, L.R. Holtz, On the burning temperatures of tobacco, Cancer Res. 16 (1956) 490–495.

[11] W. Fields, K. Fowler, V. Hargreaves, L. Reeve, B. Bombick, Development, qualification, validation and application of the neutral red uptake assay in Chinese Hamster Ovary (CHO) cells using a VITROCELL(R) VC10(R) smoke exposure system, Toxicol. Vitr. 40 (2017) 144–152.

[12] T.L. Godec, I. Crooks, K. Scott, C. Meredith, In vitro mutagenicity of gas-vapour phase extracts from flavoured and unflavoured heated tobacco products, Toxicol. Vitr. Rep. 6 (2019) 1155–1159.

[13] M. Gaca, Assessment of tobacco heating product THP1.0. Part 5: In vitro dosimetric and cytotoxic assessment, Regul. Toxicol. Pharm. 93 (2018) 52–65.

[14] ICCVAM, 2006. The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM): ICCVAM test method evaluation report. In vitro cytotoxicity testmethods for estimating starting doses for acute oral systemic toxicity testing.

[15] ISO, 1992. ISO 3402-1992 Tobacco and tobacco products

[16] ISO, 2018. ISO 20768:2018 Vapour products

[17] T-115, H.C.O.M, 1999. Determination of Tar, Nicotine and Carbon Monoxide in Cigarette Smoke

[18] D. Thorne, A. Dalrymple, D. Dillon, M. Duke, C. Meredith, A comparative assessment of novel tobacco heating product THP1.0. Part 6: a comparative in vitro study using contemporary screening approaches, Regul. Toxicol. Pharmac. 93 (2018) 62–70.

[19] D. Thorne, D. Bre brey, C. Proctor, M. Gaca, Assessment of novel tobacco heating product THP1.0. Part 5: in vitro dosimetric and cytotoxic assessment, Regul. Toxicol. Pharmac. 93 (2018) 52–64.

[20] B.M. Keyser, R. Leverette, K. Fowler, W. Fields, V. Hargreaves, L. Reeve, B. Bombick, Development of a quantitative method for assessment of dose in in vitro aerosol exposure systems - exposure well chamber deposition efficiency, J. Aerosol Sci. 123 (2018) 1328–1339.

[21] B.M. Keyser, R. Leverette, M. Hollings, A. Seymour, R.A. Weidman, C.J. Bequette, K. Jordan, Characterization of aerosol deliveries from combustible cigarettes, heated tobacco products, and electronic nicotine delivery systems using the vitrocell Ames 48, Appl. Vitr. Toxicol. 8 (2022) 99–49.

[22] F. Lucci, N.D. Castro, A.A. Rostami, M.J. Oldham, J. Hoeng, Y.B. Pitahawalla, A. Kuczaj, Characterization and modeling of aerosol deposition in Vitrocell(R) exposure systems - exposure well chamber deposition efficiency, J. Aerosol Sci. 123 (2018) 141–160.

[23] J. Nicol, R. Frazer, L. Walker, C. Liu, J. Murphy, C.J. Proctor, Comprehensive chemical characterization of the aerosol emissions of a vaping product based on a new technology, Chem. Res. Toxicol. 33 (2020) 789–799.

[24] OECD, 2010. Test No. 129: Guidance document on using cytotoxicity tests to estimate starting doses for acute oral systemic toxicity tests.

[25] OECD 2018. Guidance Document on Good In Vitro Method Practices (GIVIMP).