Regulatory Toxicology and Pharmacology 122 (2021) 104917

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

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

Nicotine dosimetry and stability in cambridge filter PADs (CFPs) following different smoking regime protocols and storage conditions

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ARTICLE INFO

Handling Editor: Dr. Martin Van den berg

Keywords:
Nicotine
Electronic cigarette
Methodology
Tobacco constituents
Cambridge filter pad
Smoking regimes

ABSTRACT

Despite the growing numbers of studies on cigarettes and electronic nicotine delivery products (ENDs), no standard assessment of nicotine stability in various matrix post exposure is currently available. The aim of the present study was to evaluate the optimal standard condition to store Cambridge Filter Pads (CFPs) before chemical analysis in order to guarantee the titer of nicotine.We further performed data normalization according to different smoking or vaping runs. Smoke and vapor generated respectively by a reference tobacco cigarette (1R6F) and ENDs under different exposure regimes (ISO, HCI and CRM81) were collected on CFPs as total particulate matter (TPM) and subsequently analyzed for nicotine content. For each exposure, some CFPs were analyzed at time zero, whereas the others were stored under different conditions for nicotine assessment after 30 days. Principal Component Analysis (PCA) showed the best correlation between nicotine on CFPs and TPM for normalization. This study suggests that different exposure regimes and products can affect the preservation of nicotine titer on CFPs while samples storage at −80 °C may prevent the loss of nicotine. Finally, normalization of nicotine with TPM is strongly recommended for regulatory purpose.

1. Introduction

Nicotine is an alkaloid extracted from tobacco leaves. It is a dibasic compound with pyridine and pyrrolidine rings and a pKa of 8.5. Nicotine is a colorless and water-soluble substance, separated preferentially by organic solvents depending on the pH of the solution. Its degradation mechanisms include photolysis, thermolysis, oxidation, and hydrolysis (Benowitz et al., 2009; George Ngwa, 2010) when exposed to light or air, turning to a brown color (Mishra et al., 2015). Therefore, pharmaceutical formulations containing nicotine must be stored in the dark and at temperatures not exceeding 25 °C (American Society of Health System Pharmacists, 2009). Moreover, some environmental bacteria and fungi may be also responsible for nicotine degradation (Brandsch, 2006).

Nicotine exhibits a wide spectrum toxicological profile also related to its thermal degradation metabolites. To this regard, following heating, nicotine is degraded producing nitrogen oxides, carbon monoxide and other highly toxic compounds. Therefore, over the last decade newer consumer products able to deliver nicotine by a combustion-free process have significantly increased. These products, known as electronic cigarettes (e-cigs) and tobacco heating products (THPs) seem to be a less harmful alternative to smoking providing at the same time a 'smoking experience without smoking' (Biener and Hargraves, 2015; Caponnetto et al., 2013; Farsalinos et al., 2014).

The potential benefits and risks of using combustion-free nicotine delivery technologies, (e-cig and THP) also known as Electronic Nicotine Delivery Products (ENDs) have been the subject of intense scientific debate (Polosa et al., 2019ZZ). *In vitro* studies allow to carry out a quick and easy evaluation of the potential human health impact of these products. A number of toxicological tests is required to establish the reduced harm potential compared to combustible cigarettes and to ensure protection of individual and public health from the adverse effects of harmful exposures (Bals et al., 2019; Polosa et al., 2019).

In order to compare and assess previous work on the toxicological

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https://doi.org/10.1016/j.yrtph.2021.104917
Received 16 October 2020; Received in revised form 10 February 2021; Accepted 9 March 2021
Available online 18 March 2021

List of Abbreviations

ENDs	Electronic Nicotine Delivery products
CFPs	Cambridge Filter Pads
ISO	International Organization for Standardization
HCI	Health Canada Intensive
CRM81	CORESTA Recommended Method n. 81
PCA	Principal Component Analysis
TPM	Total Particulate Matter
THPs	Tobacco Heating Products
E-cigs	Electronic Cigarettes
GC-FID	Gas Chromatography coupled with Flame Ionization Detector
RDA	Regularized Discriminant Analysis

effects between ENDs and combustible cigarettes, confirmatory and, preferably multicenter studies are considered necessary. With this in mind, we set up an inter-laboratory collaboration between seven international centers in order to replicate the most relevant *in vitro* studies on toxicology of cigarette smoke and ENDs vapor (Replica study).

Standardized protocols allowing to reproduce *in vitro* assays are demanded in order to obtain consistent results in terms of biological effect. For this purpose, it is required that exposure runs are consistent and reproducible across all participating laboratories. As suggested by CORESTA *in vitro* Toxicology Task Force (Jordan and Wieczorek, 2019), when TPM is tested by *in vitro* assays, concurrently smoked samples should be used for chemical analysis of nicotine content, as well as possibly other constituents, in order to confirm the acceptable functioning of the smoking machine and for consistency within smoking samples. The comparison between the TPM and the nicotine trapped on CFPs obtained through their exposure to the same product under the same exposure regimes can be a reliable method to assess the repeatability and reproducibility of the smoking machine systems across collaborating laboratories from multiple geographical locations.

In order to minimize bias and variability of results due to individual nicotine extraction procedures from the CFPs, we centralized analysis of nicotine dosimetry samples to our testing lab which performed both extraction and chemical analysis. Shipment logistics entails variables such as temperature and travel time which demands protocols for proper storage during transit. Hence, it is necessary to identify the optimal conditions to preserve samples requiring environmental control. No studies, to our knowledge, report details on the stability of nicotine trapped on CFPs post exposure. To establish a reliable method able to guarantee the preservation of the nicotine trapped on CFPs we performed a series of smoke and vapor exposure runs on CFPs under different storing conditions for a period of thirty days to evaluate repeatability of exposure and then stability.

Moreover, since this study was aimed to test different nicotine-containing products smoked at different regimes across different laboratories, it was important to assess if the smoke/vapor TPM produced by each device under different regimes could have a different yield or stability.

Various smoking regimes and approaches have been developed and implemented to measure smoke constituents of tobacco products including the International Organization of Standardization ("ISO - ISO 3308:2012 - Routine analytical cigarette-smoking machine — Definitions and standard conditions," n.d.) and Health Canada Intensive (HCI) smoking regimes ("ISO/TR 19478–2:2015 (en), ISO and Health Canada intense smoking parameters — Part 2: Examination of factors contributing to variability in the routine measurement of TPM, water and NFDPM smoke yields of cigarettes," n.d.). Each of these smoking regimes has been developed for different reasons. ISO regime is considered a non-intense smoking regime whereas HCI is considered an intense smoking regimen. The different intensities of the smoking regimes are used to understand and quantify the various levels of harmful constituents to which consumers may be exposed (Marian et al., 2009). Since 2000 the Health Canada Intense (HCI) regime, with vent holes blocked, was used to collect cigarette smoke components and, recently, for the collection of aerosol components from THPs. However, for these latter products, the hole vents are not-blocked in order to avoid the overheating of electronic devices. In 2014, the CORESTA E-cigarette Task Force drafted a Technical Report outlining the necessary requirements for the generation and collection of e-cigarette aerosol for analytical testing purposes and defined the exposure regime for e-cigarettes, the "CORESTA Reference Method n. 81 " (CRM81) (CORESTA Recommended METHOD N^{o} 81 Routine Analytical Machine for e-Cigarette Aerosol Generation and Collection-Definitions and Standard Conditions, 2015).

Although tobacco cigarettes and ENDs exhibit several differences regarding released substances during operation, they present as well some similarities. They share similar release of nicotine in smoke and aerosols. Thus, the comparison between these two types of products is often made on the basis of the nicotine released during their use. For this reason, it is relevant to establish a standard for nicotine dosimetry and to determine its stability in order to schedule *in vitro* experiments accordingly. Currently there is no data available on the stability of nicotine when it is subjected to various stressors produced, for example, by different smoking regimes. Moreover, the comparison between nicotine on CFPs could be affected by high variability due to different smoking or vaping runs, and this aspect could influence the interpretation of data for nicotine stability. By employing a normalization process the nicotine data can be conformed with respect to variations in sample preparation. Normalized data can be directly compared, regardless of the details of the experiment. Nicotine could be normalized through ratio with either TPM values or the number of puff or with other empirical variables.

The aims of the present study were to assess the best way to normalize the nicotine amount trapped on CFPs during exposure to ensure the repeatability of TPM and nicotine production across all tested products, and to evaluate the best conditions to store nicotine containing CFPs post exposure for sample shipment guidelines and long term intra- and inter-laboratory *in vitro* studies.

2. Material and methods

2.1. Cambridge Filter Pads (CFP) exposure

Three test products were selected for CFP exposure: 1R6F reference cigarettes (University of Kentucky), Vype ePen3 and Vype eStick Maxx electronic cigarettes (Nicoventures Trading Ltd). Borgwaldt LM1 smoking machine and LM4E vaping machines (Borgwaldt KC, Hamburg – Germany) were used to collect total particulate matter (TPM) from smoke and vapor respectively (Fig. 1).

The 1R6F reference cigarettes are unflavored blended cigarettes (83 mm length) characterized by 0.721 mg/cigarette of nicotine following International Organization for Standardization (ISO) smoking regime, and 1896 mg/cigarette of nicotine following Health Canada Intense (HCI) smoking regime, as reported by University of Kentucky (Center for Tobacco Reference Products - University of Kentucky, 2018). Vype ePen3 is a button-activated "closed-modular" e-cigarette, while Vype eStick Maxx is a puff-activated cigarette-like product. Both devices consist of two modules, a rechargeable battery section and a replaceable liquid ("e-liquid") containing cartridge ("cartomizer"). "Master Blend" flavored variant containing 18 mg/mL nicotine was used for Vype ePen3, and "Toasted Tobacco" flavored variant containing 18 mg/mL nicotine was used for Vype eStick Maxx.

1R6F cigarettes were conditioned for at least 48h (60 ± 3% relative humidity, 22 ± 1 °C) before smoke generation, and smoked in a test atmosphere of 60 ± 5% relative humidity, 22 ± 2 °C according to ISO 3402:1999. Nineteen reference cigarettes 1R6F were smoked to the

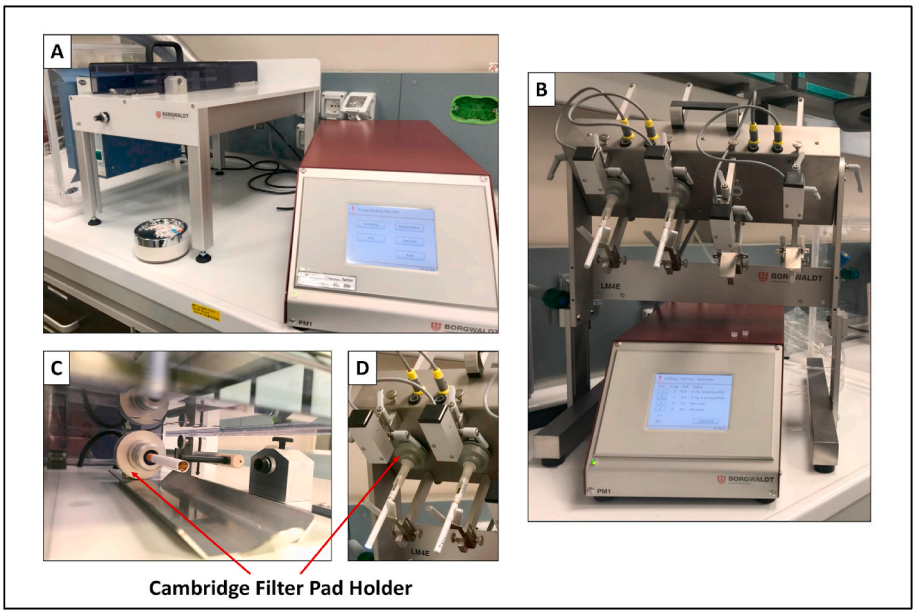

Fig. 1. (A) The Borgwaldt LM1 smoking machine and (B) LM4E button automated vaping machine. Undiluted aerosol is generated from a single syringe (within the red box) and delivered from each product to in-line Cambridge Filter Pad (CFP – 44 mm diameter) placed in the pre-syringe Cambridge Filter Pad holders (C and D).

length of the filter overwrap +8 mm under ISO regime (35 mL puff volume, drawn over 2 s, once every minute with ventilation holes unblocked) according to reference method described in ISO 4387:2000, and nineteen reference cigarette 1R6F were smoked following the HCI regime (55 mL puff volume, drawn over 2 s, once every 30 s with ventilation holes blocked) to the length of the filter overwrap +8 mm. The smoke generated by each cigarette was captured in line on 44 mm diameter CFPs. Vype ePen3 was vaped following a modified HCI regime (55 mL puff volume, drawn over 2 s, once every 30 s with square shape profile) plus 1 s of pre-activation, for 15 puffs/CFP. Vype eStick Maxx was vaped following CRM81 regime (55 mL puff volume, drawn over 3 s, once every 30 s with square shape profile) for 15 puffs/CFP.

2.2. Sample preparation

Sample preparation and analysis were performed according with the international standard ("No. 7 - Determination of Nicotine in the Mainstream Smoke of Cigarettes by Gas Chromatographic Analysis | CORESTA," n.d.). After collection of TPM, each CFP was cut into small pieces and transferred into a 15 mL plastic tube containing 10 mL of extraction solvent consisting of isopropanol (LC/MS grade, Carlo Erba) with N-decane (purity 99%, Sigma-Aldrich) (50 μg/mL) as internal standard. Tubes were shaken for 30 min by vortex at 200 rpm. The samples were then sonicated for 5 min in an ultrasonication bath. Subsequently, 1 ml of each sample was filtered with cellulose acetate filters (mm 25; μm 0.45) and 100 μl of each extract was transferred in a vial with a conical insert for auto-sampler.

2.3. GC-FID analysis

Analysis was performed by a gas chromatography Shimadzu (model GC, 2010 AF) coupled with Flame Ionization Detector. An Agilent J&W DB-HeavyWAX Intuvo GC column (30 m × 0.25 mm, 0.25 μm) was used. The GC-FID operating condition and the column oven temperature program are reported respectively in Table 1 and Table 2 of supplementary material section.

2.4. Calibration curve

Nicotine stock solution at concentration of 100 μg/μL was prepared weighing 1 g of nicotine at purity of 99% (Sigma Aldrich) into a 10 mL volumetric flask and diluted to volume with acetone. The solution was stored between 0 °C and 4 °C in the dark. Nicotine calibrating standard solutions were prepared at concentration levels 0, 100, 200, 500 and 1000 μg/mL in 1 mL of extraction solution consisting to propan-2-ol with heptadecane at purity of 99% (Sigma Aldrich, cod. 128503-100G) at concentration of 50 μg/L.

2.5. Dosimetry performance assessment

For linearity assessment, calibration curve was performed, and linear correlation coefficient was assessed (r^2): the acceptability criterion was $r^2 > 0.98$. For the precision and accuracy assessment, eight filters were spiked each with 100 μg of nicotine and others eight filters with 500 μg. For each nicotine level, precision was assessed on the basis of the Relative Standard Deviation (RSD%) as the percental ratio between Standard Deviation and the mean value: the acceptability criterion was RSD%<10%. The accuracy was estimated on the base of the Recovery (R %) as the percent ratio between the main value and the true value (100 μg or 500 μg): the acceptability criterion was 80%<R%<120%.

2.6. Nicotine normalization assessment

The Principal Component Analysis (PCA) was used to verify the correlation of variables (nicotine on filters, TPM, cartridge weight differential or number of puff) and to determine if the normalization of the nicotine on filters for TPM was relevant. We performed one PCA for the electronic devices (Vype ePen and eStick) and one for 1R6F reference cigarettes. For the latter including the two regimes of exposure ISO and HCI.

The Principal Components Analysis (PCA) was performed using RStudio software Version 1.2.5033. The regularized discriminant analysis (RDA) function was used for PCA. Data were standardized before analysis and the results were displayed in a biplot of correlation. The filter samples were unscaled and the weighted dispersion was equal on all dimensions. The variables were scaled proportionally to eigenvalues. Although the small number of variables, the sample size was considered appropriate for this purpose (Zuccarello et al., 2019).

2.7. Comparison between the different storing conditions

RStudio Software was used to perform the statistical analysis aimed to assess stability of nicotine under different storing conditions. Data were collected in five groups based on the storage conditions for each test product and each exposure regime. Three CFPs, analyzed at zero time without any conditioning, were included into the control groups (Group 0). Four filters for each group were stored for 30 days, under different conditions: (i) at room temperature (25 °C) in extraction solution (Group 1), (ii) at room temperature (25 °C) dry (Group 2), (iii) at the temperature of −20 °C dry (Group 3) and (iv) at the temperature of −80 °C dry (Group 4). The CFPs from all four groups were analyzed for nicotine content according to the selected time point and condition. For each CFP, the amount of nicotine was normalized for the weight of TPM both for reference cigarettes and for electronical devices.

For each group, normality was assessed by Shapiro-Wilk test. A p-value >0.05 was considered for a normal distribution. Moreover, one-sample *t*-test was performed for each storing condition group in order to assess the difference of the average value of the group from the average value of the control group.

Finally, the non-parametric test of Kruskal-Wallis was performed to investigate the differences of the storing conditions compared to the control group. Dunn's-test for multiple comparisons of independent samples was used as post-hoc test. A p-value <0.05 was used for a statistically significative difference.

3. Results

3.1. Dosimetry performance assessment

The linear correlation coefficient r^2 of calibration curve was 0.9999. The results showed a relative standard deviation (RSD%) and a recovery (R%) at nicotine level of 100 μg equal to 6.0% and 94.6%, respectively, while RSD% and R% at nicotine level of 500 μg equal to 4.4% and 109.7%, respectively.

3.2. Vape/smoke exposure in CFP

Data on weight of both cartridges and CFPs before and after each electronic cigarette aerosol exposure run were collected. Concurrently data on weight of CFPs were collected before and after each 1R6F reference cigarette smoke exposure run under ISO and HCI regimes. The difference between the final and initial weight of the CFPs exposed to smoke/vapor was used to calculate the amount of trapped TPM. Data collected from CFPs exposed to e-cigs vapor are shown in Tables 3 and 4 reported in the supplementary material section. Data on CFPs exposed to 1R6F reference cigarette smoke under ISO and HCI regimes, are shown respectively in Tables 5 and 6 in the supplementary material section. The results show that the means ± SD weights of TPM collected from ePen and eStick are 82.7 ± 5.1 mg and 48.0 ± 7.4, respectively. Whereas, the means ± SD weights of TPM collected from 1R6F following ISO and HCI regimes are 7.9 ± 0.4 mg and 30.8 ± 2.2 mg.

3.3. Nicotine normalization assessment

We performed a PCA analysis by linear transformation of four variables, including nicotine amount on CFP, TPM and cartridge weight differential or number of puffs, in order to retain the maximal information from individual variables simultaneously. The resulting two variables, principal component 1 (PC1) and principal component 2 (PC2), represent respectively the direction along which the samples show the largest variation, and the direction uncorrelated to PC1 along which the samples show the largest variation.

Correlation biplot for e-cigarettes (PC1 73% and PC2 1.5%) showed a high correlation between nicotine amount on filters and TPM (Fig. 2). PCA highlights a minor correlation with the cartridge weight differential. Samples between 1 and 19 are relative to ePen exposure while samples between 20 and 38 are relative to eStick exposure. The two groups of exposure are divided in two different areas of the biplot.

Correlation biplot for 1R6F (PC1 45% and PC2 9%) shows also a high correlation between nicotine amount on filters and TPM. PCA highlights a minor correlation with the number of puffs. Samples between 1 and 19 are relative to ISO-regime exposure while samples between 20 and 38 are relative to HCI-regime exposure. The two groups of exposure regimes are located in two different longitudinal bands (Fig. 3).

3.4. Comparison between different storing conditions

All data of nicotine normalized to TPM are reported in table 7 of supplementary material. Data distribution was assessed for each storing condition group of tested products. Also, each average value of the storing condition groups for each product was evaluated in order to verify whether this value was different from the average of the control group. The distributions of TPM-normalized nicotine data for Vype ePen were normal in groups 1 (p = 0.5719), 2 (p = 0.6298), 3 (p = 0.2742) and 4 (p = 0.3741) whereas the normality for the control group (0) was not verified (p = 2.2E-16). Moreover, the mean values of groups 2, 3 and 4 were not significantly different from the mean of the control group (group 2, p = 0.1319; group 3, p = 0.7277; group 4 p = 0.7408). Conversely, the mean of group 1 was slightly different from the mean of the control group (p = 0.04016). Comparison of TPM-normalized nicotine concentration on CFPs exposed to Vype e-Pen by Kruskal-

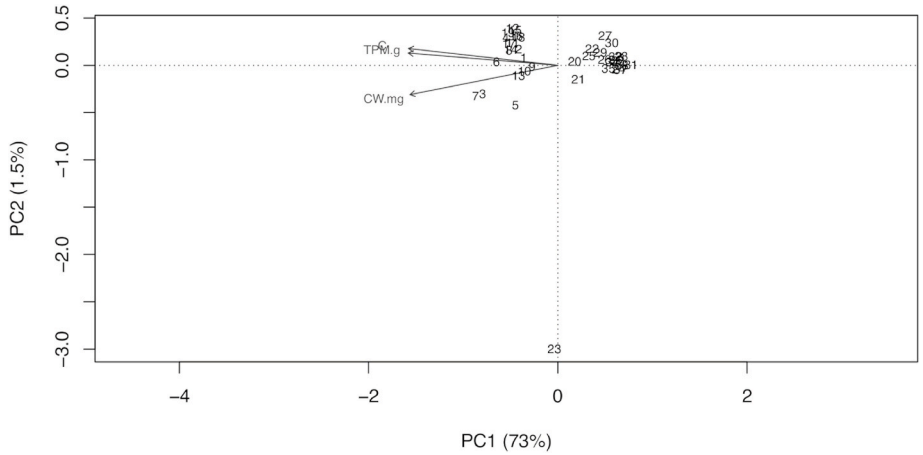

Fig. 2. Correlation biplot for e-cigarettes: samples between 1 and 19 are relative to ePen exposure, while samples between 20 and 38 are relative to eStick exposure.

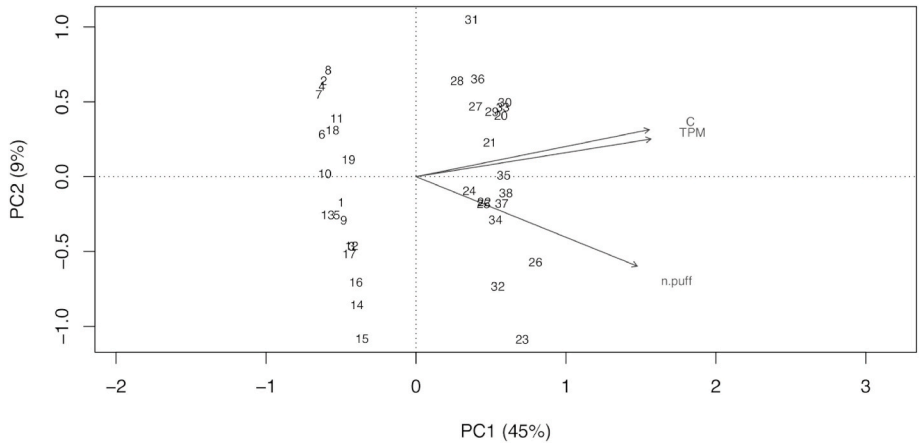

Fig. 3. Correlation biplot for 1R6F: samples between 1 and 19 are relative to ISO-regime exposure while samples between 20 and 38 are relative to HCI-regime exposure.

Wallis test did not show significant difference among the storing conditions and the control group with an overall p-value of 0.1439 (Fig. 4A).

The distributions of TPM-normalized nicotine data for eStick were normal in groups 1 ($p = 0.4679$), 2 ($p = 0.7553$), 3 ($p = 0.85$) and 4 ($p = 0.9886$) whereas the normality of the control group data was not verified ($p = 2.2E\text{-}16$). Furthermore, the mean values of each storing condition group were not significantly different from the mean value of the control group ($p = 0.9816$ for group 1; $p = 0.136$ for group 2; $p = 0.09252$ for group 3; $p = 0.6973$ for group 4). Likewise, the comparison of TPM-normalized nicotine concentration on CFPs exposed to Vype e-Stick by Kruskal-Wallis test did not show a significant difference among the storing conditions and the control group ($p = 0.2851$) (Fig. 4B).

The distributions of TPM-normalized nicotine data for CFPs exposed to 1R6F under ISO regime were normal in the control group ($p = 0.7952$), and groups 1 ($p = 0.5436$), 2 ($p = 0.2066$) and 4 ($p = 0.2188$). Instead, the normality for group 3 was not verified ($p = 0.01162$). The mean values of groups 1 and 4 were not different from the mean value of the control group ($p = 0.5448$ for group 1; $p = 0.85$ for group 4), whereas the mean values of groups 2 and 3 were significantly different from the mean of the control group ($p = 0.01266$ for group 2; $p = 0.02784$ for group 3). Comparison of TPM-normalized nicotine concentration on CFPs exposed to 1R6F under ISO regime by Kruskal-Wallis

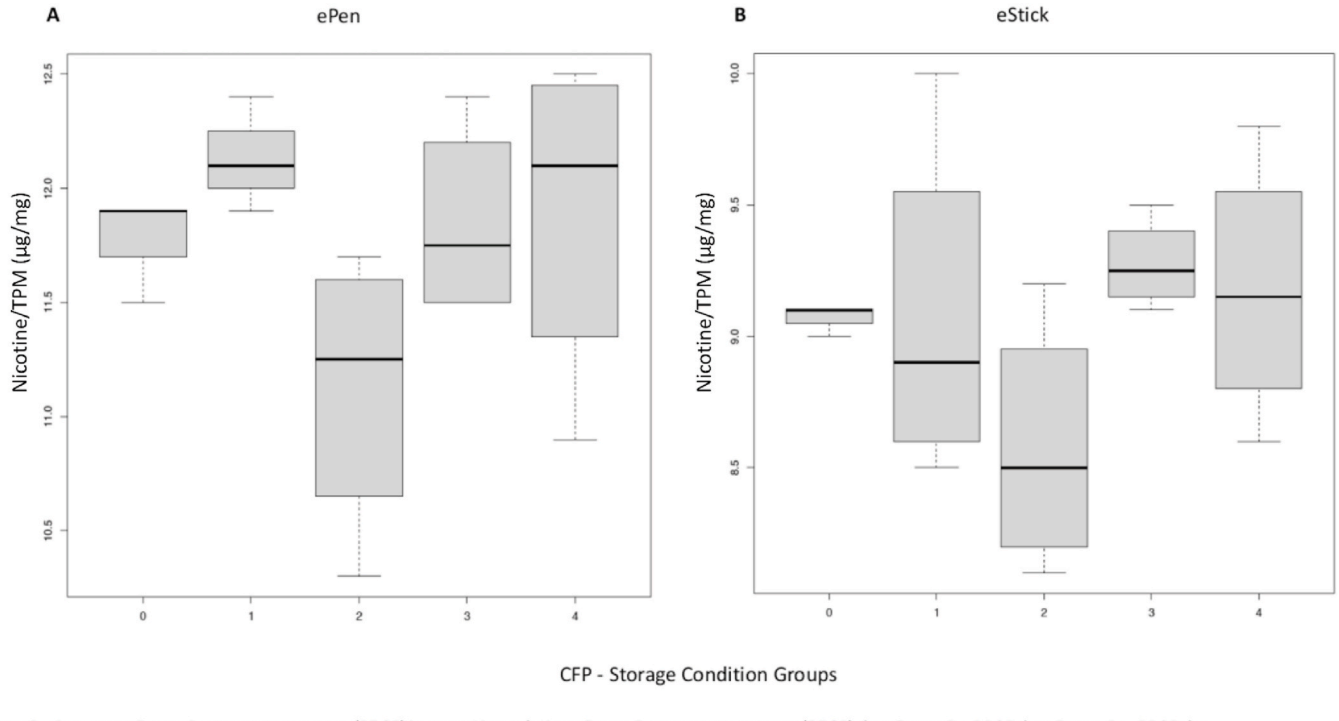

Fig. 4. (A) Comparison of TPM-normalized nicotine on CFPs exposed to Vype e-Pen among the different storing conditions. The median (interquartile range; IQR) values were respectively 11.9 (11.7–11.9) of TPM for the control group (group 0), 12.1 (12.05–12.18) µg/mg of TPM for group 1, 11.25 (10.83–11.55) µg/mg of TPM for group 2, 11.75 (11.5–12.1) µg/mg of TPM for group 3 and 12.1 (11.6–12.4) µg/mg of TPM for group 4. **(B)** Comparison of TPM-normalized nicotine on CFPs exposed to Vype eStick among the different storing conditions. The median (IQR) values were respectively 9.1 (9.05–9.1) µg/mg of TPM for the control group (group 0), 8.9 (8.65–9.33) µg/mg of TPM for group 1, 8.5 (8.25–8.83) µg/mg of TPM for group 2, 9.25 (9.18–9.35) µg/mg of TPM for group 3 and 9.15 (8.9–9.43) µg/mg of TPM for group 4. Boxplots represent "minimum", first quartile (Q1), median, third quartile (Q3), and "maximum" of TPM-normalized nicotine for each storage condition group.

test showed significant difference among the storing conditions and the control group, with an overall p value of 0.01226 (Fig. 5A). Post-hoc Dunn's test showed a significant decrease of TPM-normalized nicotine only in group 2 compared to the control group ($p = 0.0127$).

The distributions of TPM-normalized nicotine data for CFPs exposed to 1R6F following HCI regime, were normal in the control group ($p = 0.3322$), and the groups 1 ($p = 0.4892$), 2 ($p = 0.1829$) and 4 ($p = 0.2882$). Conversely, the normality in group 3 was not verified ($p = 0.03522$). The mean values of each storing condition group were not significantly different from the mean value of the control group ($p = 0.7179$ for group 1; $p = 0.2368$ for group 2; $p = 0.9837$ for group 3; $p = 1$ for group 4). Likewise, the comparison of TPM-normalized nicotine concentration on CFPs exposed to 1R6F under HCI regime by Kruskal-Wallis test did not show significant difference among the storing conditions and the control group ($p = 0.7156$) (Fig. 5B).

4. Discussion

Nicotine dosimetry, for the purpose of comparing exposure from different products (cigarettes, e-cig, THP), is a common and often used practice in studies that aim to evaluate the different impact of cigarette smoke and vapors produced by alternative products on different cell systems (Adamson et al, 2016, 2017; Behrsing et al., 2017; Bode et al., 2017; Wieczorek et al., 2017). The nicotine retention and its stability on the CFPs over time is a fundamental validation step for smoke particulate phase science. The use of CFP to collect and analyze TPM constituents was first referenced approximately 50 years ago (Wartman et al., 1959). Previously, the stability of TPM retained on CFPs was evaluated by assessing its ability to induce genotoxicity and cytotoxicity *in vitro* without carrying out proper chemical analysis of its components, including nicotine (Crooks et al., 2013; Roemer et al., 2002; Wartman et al., 1959).

The CORESTA *in vitro* Toxicology Task Force (CORESTA In Vitro Toxicology Task Force, 2004) recommend to store TPM extracts and CFP at −70 °C within 1 h of extraction for up to 4 years and that extracts should not be refrozen once thawed. However, none of these reports specify whether the nicotine content on CFPs is maintained consistently stable during this time.

A standardized and reliable nicotine dosimetry test applied to various electronic devices and combustible cigarettes is therefore of great importance when evaluating the accuracy of the exposures carried out in *in vitro* assays and its impact on cellular response when using the same exposure conditions (Adamson et al., 2017).

Furthermore, since the smoker who is switching to electronic devices gets satisfaction only upon reaching the same blood concentration of nicotine acquired with combustion cigarette (Marsot and Simon, 2016), it is necessary to compare toxicity of ENDs and tobacco products at the same dose of released nicotine.

Such practice is also of particular importance in interlaboratory comparative studies where it is necessary to perform nicotine dosimetry before *in vitro* assays in order to obtain reliable and comparable results among all partners. Indeed, there is no certainty that the exposure performed in one partner's laboratory will be comparable to the ones performed in other satellite laboratories if standards are not in place. For this reason, it also needs to be considered that nicotine stability during shipment is an intrinsic part of Nicotine dosimetry analysis. In an interlaboratory study these tests are usually conducted by one leading lab who receives the samples by its international partners (Garner et al., 2015). Time, temperature, humidity, light and air exposure are factors to consider during storage and transit of test samples to be analyzed.

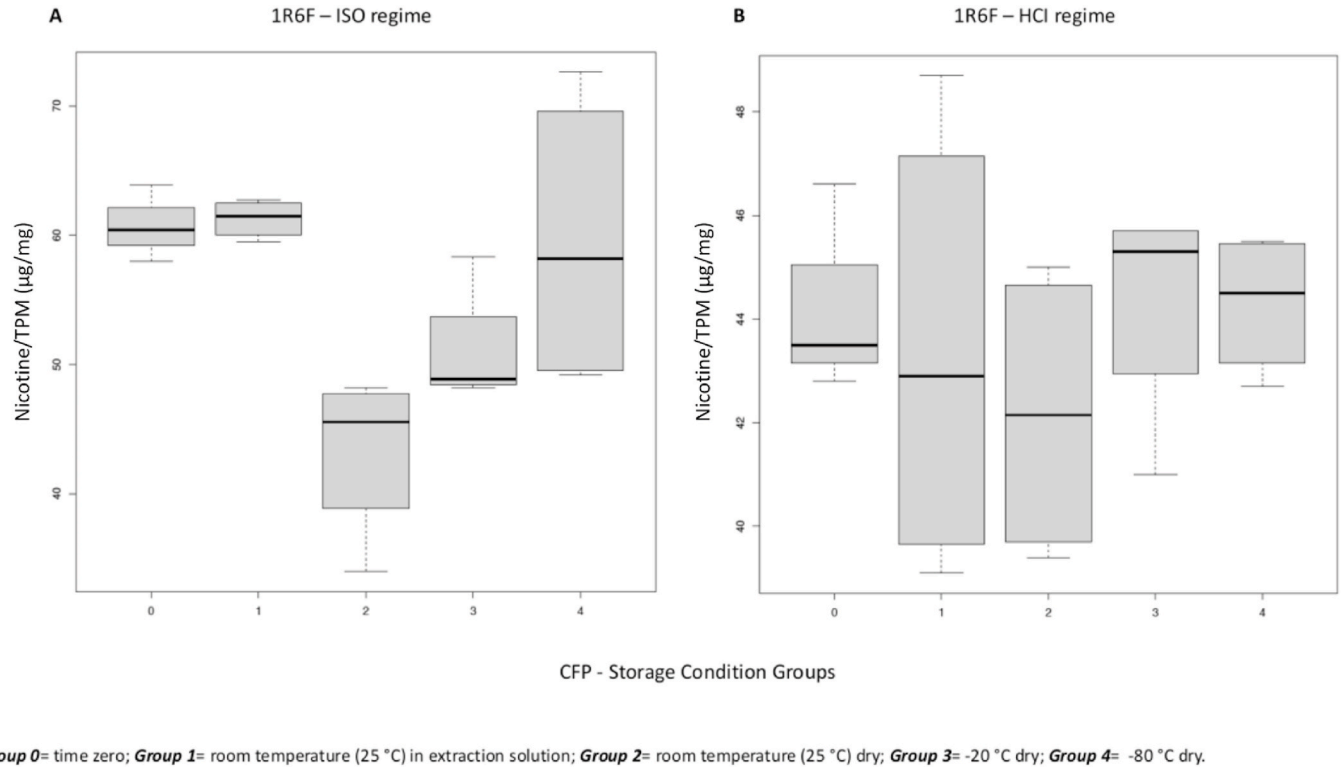

Fig. 5. **(A)** Comparison of TPM-normalized nicotine on CFPs exposed to 1R6F following ISO regime among the storing conditions. The median (interquartile range; IQR) values were respectively, 60.4 (59.2–62.15) µg/mg of TPM for the control group (group 0), 61.45 (60.33–62.4) µg/mg of TPM for group 1, 45.55 (41.35–47.53) µg/mg of TPM for group 2, 48.85 (48.5–51.4) µg/mg of TPM for group 3 and 58.2 (49.7–68.03) µg/mg of TPM for group 4. **(B)** Comparison of TPM-normalized nicotine on CFPs exposed to 1R6F following HCI regime among the different storing conditions. The median (IQR) values were respectively 43.5 (43.15–45.05) µg/mg of TPM for the control group (group 0), 42.9 (39.93–46.38) µg/mg of TPM for group 1, 42.15 (39.85–44.48) µg/mg of TPM for group 2, 45.3 (43.93–45.7) µg/mg of TPM for group 3 and 44.5 (43.38–45.43) µg/mg of TPM for group 4. Boxplots represent "minimum", first quartile (Q1), median, third quartile (Q3), and "maximum" of TPM-normalized nicotine for each storage condition group.

To this regard, no protocols on nicotine samples storage (i.e. filter pads), shipping temperature or extraction solvent are so far available. To assess these conditions, we used nicotine as a reference since it is a major substance released from both smoke and vapor from all test products. Hence, we measured nicotine on CFPs to evaluate whether the same exposure with the same number of puffs, at different regimes, produces the same quantity of nicotine and warrant the uniform performance of the system. This is an important step to harmonize protocols prior to conducting any *in vitro* assays. Many studies did not address issues relating to storage and stability of the CFPs post exposure. Since Watson and colleagues noted the semi volatility and degradability of nicotine (Watson et al., 2004), data on both the loss rate for nicotine and the stability under different storage conditions, such as room temperature, refrigerated (4 °C), frozen (−20 °C), and ultralow freezing (−70 °C) would be desirable.

Based on the assessment of nicotine post smoke and aerosol exposure where nicotine may interact with other constituents under diverse thermal conditions, our results showed no significant differences of nicotine stability when using Vype eStick or reference cigarette under HCI regime ($p > 0.05$). Interestingly, when using HCI regime, we observed no significant difference in nicotine amount in all the experimental groups when compared to control, whereas when using ISO regime, we observed a significant difference of mean value in CFP group stored at room temperature and at −20 °C ($p < 0.05$). Moreover, a significant difference of variance in CFP group stored at room temperature was also shown.

Such difference in nicotine stability between the two smoking regimes may be dependent on filter ventilation (1R6F), the difference in intensity, and changes in retention due to the different ratio of smoke constituents and possible interaction with nicotine.

Regarding the exposure by ePen, a significant difference ($p < 0.05$) was shown for the mean value of the CFPs group stored in solvent solution, higher than the mean value of CFPs of the control group. The longer contact time of the CFPs with the extraction solvent could have significantly improved the extraction efficiency and increased the nicotine amounts compared to the extemporaneous extraction of the samples.

However, although it was not always statistically significant, the nicotine amounts found on CFPs stored at room temperature was always lower than the filters of the control group and the other groups. This denotes a sensitive degradation of nicotine even at temperatures below 30 °C.

Instead, the preservation of the samples at −80 °C has always proved effective in maintaining the nicotine content on CFPs.

The Principal Component Analysis showed a high correlation between nicotine and TPM values. This means that the delivery of nicotine is directly proportional to that of the particulate matter. In terms of RSD %, the exposure under ISO regime showed a high inaccuracy in the release of TPM (15.4%) and the number of puffs (14.6%), as well as of the nicotine amounts found on CFPs (17.4%). The normalization of the nicotine amounts on the CFPs to the amount of TPM released during the exposure allowed to reduce the variability of the study due to the precision and accuracy of the exposure systems. After assessing and characterizing the systems under different conditions, we were able to provide reliable data generated by each exposure. In particular, the nicotine values on the CFPs were homogeneous at the time of exposure and any change was due to the method and the time of storage of the samples.

5. Conclusion

This study highlights that different exposure regimes and different products can affect the preservation of nicotine titer on CFPs. However, refrigerating the samples at minus 80 °C up to 30 days before analysis prevents the oxidative and thermal degradation effects on this substance. This storing procedure of CFPs is strongly recommended for the standardization of protocol and it should be required for inter-laboratory studies on tobacco and nicotine containing products for regulatory purposes. Also, our results showed as normalization of nicotine for TPM is a crucial point to correctly assess repeatability of exposure test. This allowed us to compare the nicotine amount trapped in CFPs and the storage conditions, identifying key output variables during the smoking/vaping process and increasing robustness of the data. Without normalization, the amount of nicotine in CFPs did would supply not reliable information to perform the storage condition comparison.

In future studies we intend to test another critical check point in the exposure system -impinger trap- and investigate optional storing conditions for bubbled media used for interlaboratory comparative studies.

Funding

This work was supported by the Foundation for a Smoke-Free World [REPLICA project] (PIs Prof. Giovanni Li Volti and Massimo Caruso).

CRediT authorship contribution statement

Pietro Zuccarello: Methodology, Formal analysis, Investigation, Writing – original draft. **Sonja Rust:** Project administration, Review manuscript. **Massimo Caruso:** Conceptualization, Validation, Supervision, Writing – review & editing. **Rosalia Emma:** Data curation, Writing – original draft, Formal analysis. **Roberta Pulvirenti:** Investigation, Resources. **Claudia Favara:** Investigation. **Riccardo Polosa:** Funding acquisition, Supervision. **Giovanni Li Volti:** Conceptualization, Writing – review & editing, Supervision. **Margherita Ferrante:** Conceptualization, Validation, Formal analysis.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: In relation to his work in the area of tobacco control and respiratory diseases, Riccardo Polosa has received lecture fees and research funding from Pfizer, Inc., GlaxoSmithKline plc, CV Therapeutics, NeuroSearch A/S, Sandoz, MSD, Boehringer Ingelheim, Novartis, Duska Therapeutics, and Forest Laboratories. He has also served as a consultant for Pfizer, Inc., Global Health Alliance for treatment of tobacco dependence, CV Therapeutics, NeuroSearch A/S, Boehringer Ingelheim, Duska Therapeutics, Forest Laboratories, ECITA (Electronic Cigarette Industry Trade Association, in the UK), and Health Diplomat (consulting company that delivers solutions to global health problems with special emphasis on harm minimization). Lecture fees from a number of European EC industry and trade associations (including Fédération Interprofessionnelle de la VAPE in France and Federazione Italiana Esercenti Svapo Elettronico in Italy) were directly donated to vaper advocacy no-profit organizations. He is currently Head of the European Technical Committee for standardization on "Requirements and test methods for emissions of electronic cigarettes" (CEN/TC 437; WG4). He is also founder of the Center of Excellence for the acceleration of Harm Reduction at the University of Catania (CoEHAR), which has received a grant from the Foundation for a Smoke Free World to support 8 independent investigator-initiated research projects on tobacco harm reduction, and scientific advisor for LIAF, Lega Italiana Anti Fumo (Italian acronym for Italian Anti- Smoking League). Giovanni Li Volti is currently elected Director of the Center of Excellence for the acceleration of HArm Reduction. All the other authors declare no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2021.104917.

Funding body information

This research was supported by a grant of the Foundation for a Smoke-Free World (FSFW). Grant was provided to start the activities of the multicenter research project “Replica”, that aims to replicate researches from tobacco industries and other independent researches providing the most relevant data in the toxicology of cigarette smoke and aerosol “smoke-free” alternative products.

Here, I declare that the FSFW have not had any role in the conduction of the research and/or preparation of the article and in study design. FSWF had no role in the collection, analysis and interpretation of data nor in the writing of the report and in the decision to submit the article for publication.

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