Assessment of enamel discoloration in vitro following exposure to cigarette smoke and emissions from novel vapor and tobacco heating products

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Abstract: Purpose: To evaluate in vitro enamel sample discoloration following exposure to a scientific reference cigarette (3R4F) or emissions from next generation tobacco and nicotine products (NGPs) such as electronic cigarettes (EC) and tobacco heating products (THP). **Methods:** Bovine enamel blocks (6.5 × 6.5 mm) were prepared and pre-incubated with human or artificial saliva, to form a pellicle layer before exposure to either particulate matter (PM) or whole aerosols. PM was prepared by capturing 3R4F cigarette smoke (CS), a commercial THP (THP1.0) or a novel vapor product (NVP)/next generation e-cigarette aerosols on Cambridge filter pads followed by elution with dimethyl sulfoxide (DMSO). Ten enamel samples were exposed to each PM for 14 days. For aerosol exposure, 12 enamel samples were exposed (200 puffs per day, for 5 consecutive days) to 3R4F CS or THP1.0 and NVP aerosols. Control samples were incubated with DMSO (PM study) or phosphate buffered saline (PBS, aerosol study). Individual enamel sample color readings (L*, a*, b*) were measured at baseline and on each exposure day. Mean ΔL*, Δa*, Δb* and ΔE values were calculated for each product or control. A one-way ANOVA was used to assess the differences between the products and controls. The Tukey procedure for pairwise comparisons was also used. **Results:** At all timepoints, 3R4F PM and CS induced enamel discoloration that was statistically significant ($P < 0.0001$) when compared to THP1.0 or NVP. After 14-day PM exposure, mean ΔE values were 29.4 ± 3.6, 10.5 ± 2.3, 10.7 ± 2.6 and 12.6 ± 2.0 for 3R4F, THP1.0, NVP and DMSO control respectively. After 5-day CS or aerosol exposure, mean ΔE values were 26.2 ± 3.2, 3.6 ± 1.9, 3.4 ± 1.3, 5.3 ± 0.8 for 3R4F CS, THP1.0, NVP or PBS control, respectively. Both exposure methods demonstrated that THP1.0 and NVP induced minimal staining, mean ΔL*, Δa*, Δb* and ΔE values were comparable to DMSO or PBS controls. (*Am J Dent* 2018;31:227-233).

CLINICAL SIGNIFICANCE: For the first time, diverse NGPs across the risk continuum were assessed in vitro for their impact on enamel staining. CS exposure significantly increased the level of bovine enamel sample discoloration, whereas THP1.0 or NVP exposure resulted in values comparable to the controls.

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Introduction

There is a growing consensus that next generation tobacco and nicotine products (NGPs) such as electronic cigarettes (EC) and heat-not-burn/tobacco heating products (THP) hold great potential for reducing the risks associated with cigarette smoking[1,2] and should be promoted as smoking substitutes.[3] The use of EC is increasing in popularity globally.[4-6] THPs are newer to the market; however, the data published recently demonstrate that, like ECs, they produce aerosols with significantly reduced toxicant levels compared to cigarettes[7-9] and therefore like ECs have reduced risk potential.

Numerous ECs are commercially available, that vary in size, shape and power, and can be used with a multitude of e-liquids with varying flavors and nicotine concentrations.[4-6] Regardless of design, ECs are relatively simple devices, consisting of battery, microprocessor, e-liquid tank and heating coil. E-liquids in general consist of propylene glycol, vegetable glycerol, water, flavors, and can be purchased with or without nicotine. THPs are designed to heat a tobacco consumable to temperatures sufficient to vaporize volatile compounds including nicotine into an inhalable aerosol (< 350°C), but not high enough to burn the tobacco.[7-9]

Cigarette smoke (CS) contains over 7,000 chemicals and is composed of two phases, the particulate, also known as tar, and the vapor phase.[10] In comparison, the aerosols produced by EC and THP are relatively simple, with very few components; the majority of these components are present within a distinct particle (droplet) phase.[7,8,11,12] Differences between CS and NGP aerosols can easily be observed when CS, THP or EC aerosols are captured on Cambridge filter pads (Fig. 1). Recent publications have demonstrated the reduced toxicity of ECs and THP in vitro[7,13-21] and in clinical studies.[22-27] To date, the effect on oral health of EC and THP consumers has not been assessed in detail. However, reduced responses, when compared to CS, have been observed in vitro in human oral tissues exposed to a THP aerosol[16,21] and also in a study where dental composites were exposed to CS or a THP aerosol for 12 days.[28]

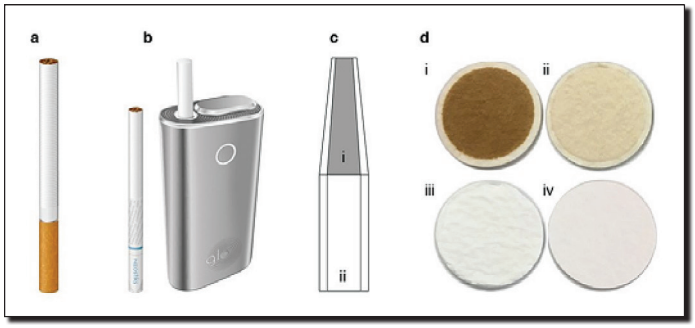

Fig. 1. Products assessed for enamel discoloration in vitro. (a) 3R4F Kentucky scientific reference cigarette; (b) THP1.0 (glo) with tobacco consumable (Bright Tobacco Neostick); (c) Schematic of BAT novel vapor product (NVP), i: consumable (containing e-liquid with 5 mg/mL nicotine and flavors), ii: battery, (d) representative Cambridge filter pads with collected particulate matter/aerosol collected from: i: 3R4F, ii: THP1.0, iii: NVP and iv: blank.

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Table 1. Exposure regimes used for particulate matter (PM) and aerosol generation.

Product	Regime	Puff Vol (mL)	Puff duration (sec)	Intensity (sec)	Vent blocking	Puff profile	Puffs per product/cartridge
3R4F	HCI	55	2	30	100%	Bell	10
THP1.0	HCIm	55	2	30	No	Bell	8
NVP	CRM81	55	3	30	No	Square	200

HCI = Health Canada Intense.[44]
HCIm = HCI modified (no vent blocking).
CRM81 = CORESTA recommended method No 81.[45]

In the oral cavity, CS has been associated with gingivitis, periodontitis, tooth loss, epithelial malignancy and tooth staining.[29-32] The particulate fraction of CS is postulated to deposit on the surface of teeth and dental restorations, resulting in yellowing and darkening.[31,33,34] CS is also thought to penetrate cracks within teeth resulting in further staining,[33] the level of discoloration is also proposed to be proportional to the number of cigarettes smoked per day.[34] CS staining cannot be easily removed; a recent study that used nicotine as a marker of particulate deposition, highlighted that brushing removed only 36% of the deposited nicotine from teeth.[35]

Enamel shade/color can be measured in the clinic using commercially available spectrophotometers[36] or by trained personnel using recognized staining indexes such as the modified Lobene Stain Index.[37] In the laboratory, color is also measured spectrophotometrically and differences before and after treatment, the ΔE value, can be determined using the Commission Internationale de L'éclairage (CIE) L*a*b* method. L* is a measure of the lightness, whereas a* and b* are measures of the green-red and blue-yellow color components respectively.[38] Enamel blocks or dental composites are routinely used to assess staining or the performance of a toothpaste or whitening/bleaching agents,[39,40] where CS is often used as a control to stain samples. A recent study has investigated the staining of dental composites by a commercial THP,[28] however the effect on teeth/enamel samples or the staining induced by an EC are unknown.

In this study, tooth enamel discoloration was assessed in vitro following exposure to emissions from a scientific reference cigarette (3R4F), a commercial THP (THP1.0) or a novel vapor product (NVP)/next generation EC. Two exposure methods were used, particulate matter (PM) and whole aerosol exposure, similar to other recently published in vitro studies.[13] Both methods were assessed, as the particulate fraction is postulated to deposit on the surface of teeth, and whole aerosol is more aligned to consumer use. Changes in the level of enamel sample discoloration following exposures were subsequently assessed using the CIE L*a*b* method.

Materials and Methods

Chemicals and reagents - All chemicals and reagents were obtained from Sigma-Aldrich[a] unless otherwise stated.

Test articles - Scientific reference cigarettes, 3R4F (Fig. 1a), were obtained from the Center for Tobacco Reference Products, University of Kentucky, Lexington, KY, USA (https://ctrp.uky.edu/). The THP1.0 device (glo[b]) and tobacco consumable, Bright Tobacco Neostick[b] (Fig. 1b) are commercially available and were manufactured by British American Tobacco (BAT), both of which are previously described in detail.[41] The THP1.0 device heats the Neostick to around 240°C, significantly lower than a lit cigarette which is between 350 to 900°C. Prior to use, 3R4F cigarettes were conditioned for a minimum of 48 hours and a maximum of 10 days, and THP Neosticks for a maximum of 5 days, at 22 ± 1°C and 58 ± 3% relative humidity, according to International Organization for Standardization 3402.[42] The NVP, manufactured by BAT R&D (Fig. 1c), is a closed modular rechargeable device and has a replaceable e-liquid cartridge. The e-liquid used was Twilight Tobacco, which contains 5 mg/mL nicotine. The NVP device and e-liquid cartridges were stored at room temperature prior to use. Both THP1.0 and NVP devices were charged daily.

Enamel sample preparation - Bovine incisors were collected, disinfected and stored in 0.1% thymol solution. Enamel blocks measuring 6.5 mm × 6.5 mm and 2 mm thick were prepared from the incisors, approximately two from each specimen. Enamel surfaces were polished using 400-grit abrasive paper to provide a surface roughness comparable to human enamel (Ra value ~0.5 μm). The underlying dentin surfaces and all exposed dentin at the sides were protected as detailed below.

Particulate matter preparation and enamel sample exposure - 3R4F reference CS, THP1.0 and NVP aerosols were generated and collected using LM20X[c] linear engines; representative Cambridge filter pads[d] are detailed in Fig. 1d. Specific puffing regimes were used for each product as detailed in Table 1. Equal weights of CS, THP1.0 or NVP or aerosols were collected onto 44 mm Cambridge filter pads and PM eluted with DMSO as described before.[13,43]

For each product and control, 10 enamel samples were prepared. For ease of handling, the dentin underside of each sample was bonded to the end of a roughened glass rod using Fuji II[e] light-cured glass-ionomer cement. To prevent ingress of DMSO/stain, the enamel edges and exposed dentin were etched with phosphoric acid and coated with dentin bonding agent (Scotchbond 1XT[f]) followed by non-viscous composite (Filtek Supreme XTE[f]); both light-cured for 45 seconds. To enable pellicle formation, each enamel sample was incubated in sterile human saliva for a minimum of 1 hour at 35°C. Samples were stored in phosphate-buffered saline (PBS) until use. For exposure, enamel blocks were immersed individually in amber vials containing 0.5 mL of PM and incubated at 35°C. On each working day (Monday to Friday), samples were removed, immersed for 60 minutes in PBS at room temperature and then the level of color was assessed as detailed below. After the reading, the samples were returned to their vials for further incubation.

Enamel sample aerosol exposure - LM20X linear engines were used to generate smoke from 3R4F or aerosol from THP1.0 and

Table 2. Aerosol exposure per day.

Product	Puffs per product/NVP cartridge	Machine ports/products per exposure run	Puffs per exposure run	Number of exposure runs per day	Total puffs per day
3R4F	10	5	50	4	200
THP1.0	8	5	40	5	200
NVP	10	5	50	4	200

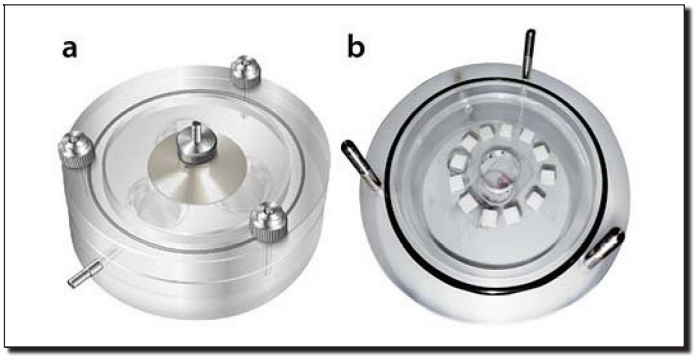

Fig. 2. Aerosol exposure chamber. (a) Aerosol exposure chamber (Patent publication number WO 03/100417 A1); (b) Enamel blocks on Perspex.

NVP. The machines were adapted to enable five ports to deliver five puffs concurrently to a BAT designed exposure chamber (Fig. 2a, Patent publication number WO 03/100417 A1). This setup enabled 275 mL to be delivered to each chamber every 30 seconds. Specific puffing regimes were used for each product as detailed in Table 1.

Prior to exposure, the underlying dentin surfaces and all exposed dentin at the sides were protected by clear nitrocellulose varnish (Superstay[g] 7 days clear). To enable pellicle formation, each enamel sample was incubated in sterile human artificial saliva for a minimum of 1 hour at 35°C. Samples were stored in PBS until use. For each product, 12 enamel samples were mounted onto a 9.4 cm diameter Perspex disc that was inserted into an exposure chamber (Fig. 2b). The enamel blocks were exposed daily to 200 puffs of aerosol, for 5 consecutive days (Table 2). 200 puffs were selected as an approximation of one pack of cigarettes per day. 200 puffs of the NVP and THP1.0 were selected to match the number of puffs of CS, but also to represent a consumer switching from smoking to using NVP or THPs. In the case of 3R4F and the NVP, 200 puffs were delivered in 50 puff batches over four exposure runs. For THP1.0, which delivers eight puffs per tobacco consumable, 200 puffs were delivered in 40 puff batches over five exposure runs (Table 2). A settling time of 5 minutes was used between each exposure run to allow for deposition of the aerosol within the chamber. Following exposure to 200 puffs, the Perspex discs were removed from the exposure chambers, enamel samples detached and stored independently at room temperature in PBS for a minimum of 30 minutes prior to color analysis as detailed below. Exposed enamel samples were stored overnight in PBS and then reattached to a Perspex disc each day, prior to exposure. Control enamel samples were stored in PBS for the duration of the study.

Color measurements - To ensure consistent measurement, samples were immersed for at least 30 minutes in PBS to rehydrate before analysis. Samples were then individually removed from PBS and color readings (L*, a*, b*) measured, at four orientations on each enamel sample, using a Konica Minolta CM-700d[h] spectrophotometer. Data was captured using a ColourCalc Excel[i] data capture spreadsheet (Chameleon color services). The CM-700d was calibrated daily using a white reference tile and a 3-mm aperture in SCI mode. Each enamel sample was measured at baseline and following daily exposure to PM or aerosol. DMSO or PBS control samples were measured at the same time points. In the case of aerosol exposed samples, enamel blocks were also analyzed following 1-month storage in PBS. Color change was determined in Excel by calculating ΔL*, a*, b* and E values between baseline and each treatment day using the following equation:

$$\Delta E = \sqrt{((\Delta L^*)^2+(\Delta a^*)^2+(\Delta b^*)^2)}$$

Statistical methods - A one-way ANOVA was used to assess the differences in ΔL*, Δa*, Δb* and ΔE between the three products and controls. The Tukey procedure for pairwise comparisons was also used.

Results

Particulate matter enamel sample exposure - Exposure to 3R4F PM resulted in discoloration of the enamel samples; dose dependent changes were also observed over the 14 days of exposure. After 1-day exposure, 3R4F ΔL*, Δa*, Δb* and ΔE values were significantly different ($P< 0.0001$) from THP1.0 and NVP values (Table 3). The ΔL* values decreased from Days 1-14, demonstrating that the enamel blocks darkened following the exposure to all products and DMSO. From Days 2-14, the ΔL* for THP1.0, the NVP and DMSO were comparable, which suggests that the DMSO used to extract the sample contributes to the darkening of the enamel and not the THP1.0 or NVP. The ΔL* values for 3R4F from Days 1-14 were significantly lower than THP1.0, NVP and DMSO demonstrating that 3R4F treatment significantly darkens the enamel. At Day 14, 3R4F ΔL* values were 2.8 times greater than THP1.0 and NVP. The Δa* values, which measure the green to red space, increased following 3R4F exposure at Days 1-7 and then decreased slightly from Days 8-14. The Δb* values, the blue to yellow space, following 3R4F exposure also increased from Days 1-3 and then decreased. The Δa* and Δb* values suggest that 3R4F exposed enamel blocks initially have a yellow color and then a red-brown color. The Δa* and Δb* values for THP1.0, the NVP and DMSO were also minimal and within the normal range of untreated samples. The ΔE, the overall color change of the enamel blocks, increased from Days 1 to 14 following all treatments. However, the values for THP1.0, the NVP and DMSO were comparable, which suggests that the DMSO used to extract the PM sample contributed to the discoloration of the enamel samples. The ΔE values for 3R4F exposure were 2.75 times higher than the THP1.0 and NVP. The significant increase in enamel block discoloration following 14-day exposure to 3R4F PM and

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Table 3. Mean ΔL*, Δa*, Δb* and ΔE values following exposure to product particulate matter (PM) extracts for 14 days. ΔL*, Δa*, Δb* and ΔE mean and standard deviation values following the exposure of enamel samples for 1-14 days to 3R4F, THP1.0 or NVP PM and DMSO solvent control.

Product	Day	1	2	3	4	7	8	9	10	11	14
ΔL*											
3R4F	Mean (SD)	-6.3 (2.7)	-10.7 (3.0)	-14.1 (3.5)	-16.1 (3.8)	-20.1 (3.9)	-22.1 (3.7)	-23.7 (3.9)	-24.8 (3.8)	-26.0 (4.3)	-28.3 (3.7)
THP1.0	Mean (SD)	-2.0 (2.6)	-3.2 (2.5)	-3.9 (2.7)	-4.9 (2.8)	-7.0 (3.4)	-7.7 (3.2)	-8.4 (3.0)	-8.5 (3.0)	-9.2 (2.9)	-10.0 (2.6)
NVP	Mean (SD)	-2.1 (2.7)	-4.0 (2.4)	-5.1 (2.6)	-6.1 (2.6)	-7.5 (2.4)	-8.0 (2.2)	-8.3 (2.3)	-8.5 (2.2)	-8.8 (2.2)	-10.0 (2.3)
DMSO Control	Mean (SD)	-0.6 (1.7)	-2.4 (2.6)	-4.1 (3.0)	-5.7 (3.1)	-8.9 (2.7)	-9.2 (2.6)	-9.9 (2.5)	-9.9 (2.0)	-10.8 (1.7)	-12.0 (1.9)
Δa*											
3R4F	Mean (SD)	2.8 (1.0)	4.0 (1.3)	4.9 (1.6)	5.2 (1.9)	5.8 (2.0)	5.6 (1.7)	5.5 (1.5)	5.4 (1.5)	5.4 (1.4)	5.1 (1.6)
THP1.0	Mean (SD)	0.2 (0.3)	0.0 (0.3)	0.0 (0.3)	0.1 (0.3)	-0.1 (0.5)	-0.1 (0.4)	-0.1 (0.4)	-0.1 (0.5)	-0.2 (0.5)	-0.2 (0.5)
NVP	Mean (SD)	0.1 (0.3)	-0.1 (0.3)	-0.2 (0.3)	-0.2 (0.4)	-0.4 (0.4)	-0.4 (0.4)	-0.4 (0.4)	-0.4 (0.3)	-0.5 (0.4)	-0.6 (0.4)
DMSO Control	Mean (SD)	0.3 (0.3)	0.1 (0.3)	0.0 (0.2)	-0.1 (0.2)	-0.2 (0.2)	-0.2 (0.3)	-0.2 (0.3)	-0.2 (0.3)	-0.2 (0.3)	-0.4 (0.3)
Δb*											
3R4F	Mean (SD)	11.0 (2.6)	12.3 (3.7)	12.7 (3.8)	11.9 (3.9)	10.0 (4.3)	8.6 (3.2)	7.8 (2.9)	7.1 (3.0)	6.8 (3.0)	5.3 (3.2)
THP1.0	Mean (SD)	2.8 (2.2)	2.3 (2.2)	2.1 (2.3)	1.9 (2.5)	1.0 (3.0)	0.8 (2.8)	0.7 (3.1)	0.7 (3.0)	0.4 (3.0)	0.5 (2.8)
NVP	Mean (SD)	2.0 (2.2)	0.7 (2.3)	-0.1 (2.3)	-0.7 (2.4)	-1.5 (2.3)	-1.9 (2.2)	-1.9 (2.2)	-2.1 (2.1)	-2.4 (2.2)	-3.1 (2.3)
DMSO Control	Mean (SD)	3.1 (1.3)	2.0 (1.9)	1.0 (2.1)	0.2 (2.4)	-1.5 (2.4)	-1.81 (2.4)	-2.1 (2.6)	-2.1 (2.2)	-2.6 (2.1)	-3.2 (1.9)
ΔE											
3R4F	Mean (SD)	13.3 (1.8)	17.2 (2.6)	20.0 (3.0)	21.2 (3.0)	23.7 (3.1)	24.6 (3.4)	25.7 (3.6)	26.6 (3.7)	27.6 (4.2)	29.4 (3.6)
THP1.0	Mean (SD)	4.6 (1.2)	5.0 (1.2)	5.4 (1.4)	6.2 (1.4)	8.0 (2.3)	8.4 (2.7)	9.1 (2.4)	9.1 (2.5)	9.7 (2.5)	10.5 (2.3)
NVP	Mean (SD)	4.1 (1.7)	4.7 (2.2)	5.5 (2.7)	6.4 (2.9)	7.9 (2.7)	8.4 (2.4)	8.7 (2.6)	9.0 (2.4)	9.3 (2.5)	10.7 (2.6)
DMSO Control	Mean (SD)	3.7 (0.8)	4.3 (1.0)	5.2 (1.6)	6.5 (1.9)	9.3 (2.6)	9.7 (2.5)	10.4 (2.6)	10.4 (2.2)	11.3 (1.8)	12.6 (2.0)

Fig. 3. Enamel discoloration following exposure to product particulate matter (PM) extracts for 14 days. Enamel blocks were exposed to PM generated from, top to bottom, 3R4F cigarettes, NVP, THP1.0 and DMSO control.

comparable results of THP1.0, the NVP and DMSO control are shown in Fig. 3.

Aerosol exposure - Exposure to 3R4F CS resulted in discoloration of the enamel samples; dose dependent changes were also observed over the 5-day exposure. After 1-day exposure to 3R4F (200 puffs), ΔL*, Δa*, Δb* and ΔE values were significantly different (P< 0.0001) from THP1.0 and NVP values (Table 4). The ΔL* values following 3R4F exposure changed from Days 1-5, demonstrating that the enamel blocks darken in a dose dependent manner. At Day 5 the 3R4F samples were six times darker than the THP1.0 and the NVP samples; the ΔL* values for these products were minimal across the 5 days, did not increase in a dose dependent manner and were within the normal range of untreated samples. The Δa* and Δb* values also increased following 3R4F exposure, indicating a reddening and yellowing of the enamel blocks. At Day 5 the Δa* and Δb* values were 20 or nine times higher, respectively, than THP1.0 or NVP. The Δa* and Δb* values following THP1.0 and NVP exposure again were minimal, were within the normal range of untreated samples and comparable to PBS control values. Similar to the PM study, the Δb* values increased from Days 1-3 and then slightly decreased on Days 4 and 5. The ΔE, total color change, increased following 3R4F exposure; at Day 5 the ΔE value was over 7 times more than the THP1.0 and NVP. The level of discoloration following exposure to THP1.0 or NVP was minimal, did not increase in a dose dependent manner and was comparable to the PBS control samples. Figure 4 shows the color difference of the enamel blocks after 5-day exposure to the three products. After 30-day storage of enamel samples in PBS, ΔL*, Δa*, Δb* and ΔE values (Table 4) were remeasured. For most of the values, 30-day PBS storage resulted in approximately a 50% reduction in the mean values, whereas the 3R4F ΔL* value had a 10% reduction and 3R4F ΔE value had a 30% reduction.

Discussion

In the oral cavity, CS has been associated with changes to the soft tissues, tooth loss, and tooth staining.[29-32] EC and THP are relatively new products and their full effect on the oral cavity and tooth staining is unknown. In this study, the repeated in vitro exposure of bovine enamel samples to 3R4F CS, THP1.0 or NVP particulate fraction/PM and aerosols were assessed. The data presented confirms that CS discolored enamel samples, however exposure to THP1.0 or NVP induced limited staining; the values obtained for these products were comparable to DMSO or PBS controls.

Two methods were used for enamel sample exposure: extracts of captured aerosol particulate fraction or the whole aerosol as used by the consumer. The particulate fraction/PM is often used for tobacco product in vitro testing.[13,18,43] PM was also selected for this study, as the particulate fraction of CS, and

Table 4. Mean ΔL*, Δa*, Δb* and ΔE values following exposure to product aerosols for 5 days. Mean ΔL*, Δa*, Δb* and ΔE mean and standard deviation values following the exposure of enamel samples for 5 days to 3R4F, THP1.0 or NVP aerosol and the control samples that were stored in PBS. Enamel blocks were also analyzed post 5 days exposure following the storage in PBS for 30 days.

Product	Day	1	2	3	4	5	30
ΔL*							
3R4F	Mean (SD)	-5.6 (1.6)	-9.4 (1.6)	-12.2 (2.0)	-14.3 (2.2)	-16.0 (2.3)	-14.5 (2.3)
THP1.0	Mean (SD)	2.4 (1.3)	2.9 (1.8)	2.8 (1.7)	2.4 (1.7)	2.4 (2.0)	-1.1 (0.9)
NVP	Mean (SD)	2.2 (0.9)	2.7 (1.2)	2.8 (1.3)	2.7 (1.2)	2.6 (1.2)	-1.2 (0.9)
PBS	Mean (SD)	-1.8 (0.5)	-3.2 (0.7)	-3.9 (0.7)	-4.0 (0.7)	-4.0 (0.7)	-4.1 (0.8)
Δa*							
3R4F	Mean (SD)	2.9 (1.1)	4.7 (1.2)	5.9 (1.1)	6.2 (1.2)	6.6 (1.3)	3.9 (0.6)
THP1.0	Mean (SD)	0.4 (0.3)	0.4 (0.3)	0.4 (0.3)	0.3 (0.3)	0.3 (0.3)	-0.3 (0.1)
NVP	Mean (SD)	0.4 (0.2)	0.4 (0.2)	0.4 (0.2)	0.4 (0.2)	0.3 (0.2)	-0.3 (0.2)
PBS	Mean (SD)	-0.3 (0.1)	-0.5 (0.2)	-0.6 (0.2)	-0.5 (0.2)	-0.6 (0.2)	-0.6 (0.2)
Δb*							
3R4F	Mean (SD)	15.8 (3.5)	18.6 (3.1)	20.4 (2.6)	19.9 (2.6)	19.7 (2.6)	9.7 (2.8)
THP1.0	Mean (SD)	2.0 (1.2)	2.3 (1.5)	2.2 (1.5)	1.8 (1.5)	1.9 (1.8)	-1.0 (1.0)
NVP	Mean (SD)	1.8 (0.6)	2.3 (1.0)	2.3 (1.1)	2.2 (1.1)	2.1 (1.0)	-1.1 (1.1)
PBS	Mean (SD)	-1.6 (0.4)	-2.9 (0.5)	-3.4 (0.5)	-3.4 (0.5)	-3.5 (0.5)	-3.6 (0.6)
ΔE							
3R4F	Mean (SD)	17.1 (3.8)	21.4 (3.2)	24.5 (3.1)	25.3 (3.2)	26.2 (3.2)	18.1 (2.9)
THP1.0	Mean (SD)	3.3 (1.7)	3.9 (2.0)	3.9 (1.8)	3.4 (1.6)	3.6 (1.9)	1.6 (1.3)
NVP	Mean (SD)	2.9 (0.9)	3.6 (1.4)	3.8 (1.4)	3.6 (1.4)	3.4 (1.3)	1.7 (1.4)
PBS	Mean (SD)	2.5 (0.7)	4.4 (0.8)	5.2 (0.8)	5.3 (0.8)	5.3 (0.8)	5.5 (1.0)

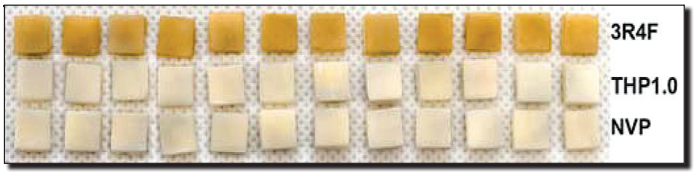

Fig. 4. Enamel discoloration following exposure to product aerosols for 5 days. Enamel blocks were exposed to 200 puffs per day, for 5 consecutive days, to aerosols generated, from top to bottom, 3R4F cigarettes, THP1.0 or NVP.

not the vapor phase, is thought to deposit on the tooth surface causing yellow or brown/black stains.[31,33,34] Similar to CS, THP and EC aerosols produce a particle (droplet) phase. The particles produced are similar in diameter to CS, resulting in comparable deposition behavior, which occurs principally by sedimentation.[46] Due to aerosol physical similarities, a matched mass approach was selected. In this study, repeated 3R4F PM exposure resulted in a dose related increase in enamel discoloration, confirming that CS particulate fraction contributes to enamel staining. Limited discoloration was observed following exposure to THP1.0 or NVP, which could be due to the differences in the aerosol produced, CS contains over 7,000 chemicals,[10] whereas the aerosols produced by EC and THP are simple and have significantly less chemical constituents.[7,8,11,12]

Aerosol studies were also performed to ensure the study was relevant to consumer use of the products, where teeth are exposed directly to CS or NGP aerosols and not the isolated particulate fraction. The puff number used per day, 200, was selected as this approximates a consumer's use of one pack of cigarettes per day. In the case of 3R4F CS exposure, there was a dose related increase in enamel block discoloration over the 5 days of aerosol exposure. Others[34] have proposed that the number of cigarettes smoked per day influences the level of tooth discoloration. Conversely, repeated THP1.0 or NVP exposure had no effect on the level of staining and a dose response was not observed following 5-day exposure. When the two exposure methods are compared, the 3R4F mean ΔE values at Days 5 and 14 are similar, confirming that particulate matter could be used as a surrogate for cigarette aerosol testing in enamel discoloration studies.

A recent study[28] investigated the discoloration of dental composites by 3R4F CS and a THP, described as Tobacco Heating System 2.2 (THS2.2), aerosol for 12 days. The mean ΔE values reported following 12 days 3R4F exposure[28] was comparable to the ΔE value obtained in the current study for 5-day exposure, confirming that the in vitro exposure time can be reduced using the presented novel method. Zhao et al[28] also observed that THS2.2 aerosol exposure results in significantly less discoloration when compared to 3R4F CS. However, Zhao et al[28] observed a dose dependent increase in discoloration following exposure to the THS2.2 aerosol over the 12-day timeframe. In the present study, discoloration was not observed when enamel samples were exposed to THP1.0 or the NVP, values obtained were comparable to controls. Differences could be due to the study time (5 vs 12 days), dental composite used or in aerosol produced by the THS2.2. The THP1.0 used in the current study heats the tobacco consumable at a lower temperature, which could result in less pigments or compounds being released into the aerosol.

When assessing any consumer product, it is important to assess the product as recommended by the manufacturer and as used by the consumer. Internationally recognized methods can be used, if available. In this study, CS, THP1.0 or NVP; aerosols were generated using commercially available equipment that was manufactured for the testing of cigarettes and adapted by the manufacturer for the testing of ECs or THPs. Specific puffing regimes were also used for aerosol generation; Health Canada Intense (HCI)[44] for the THP and the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) recommended method No. 81 (CRM 81)[45] for the NVP. Standard methods enable data from different studies to be compared but also ensures that the EC or THP are analyzed in the laboratory using appropriate testing methods. When the 3R4F aerosol data generated in this study is compared with Zhao et al,[28] which also used standard methods and the HCI puffing regime, the mean ΔE values on the final day of testing are comparable, highlighting that if standard methods are used, data from independent studies can be easily compared.

The present study did not account for the effect of saliva,

food or beverages and daily brushing which might add or reduce enamel discoloration. This study was designed to compare the level of discoloration of CS and NGPs particulate matter extracts and aerosols. However, a 50% reduction in some of the measured color parameters was observed following the storage of the enamel blocks in PBS for a month, which suggests brushing could reduce the level of deposited stain. The reduction observed following PBS storage is similar to a study[35] where CS exposed enamel samples were brushed post exposure; 36% of the deposited nicotine was removed by brushing. A clinical study could assess the impact of eating and drinking to enamel discoloration and stain removal by daily brushing and also the long-term impact on tooth discoloration and the oral cavity of consumers either switching from cigarettes to NGPs, or solely using these reduced exposure products. The data presented clearly shows the differences in the level of discoloration between CS and the NGPs, THP1.0 and NVP exposure induced limited discoloration, that was also comparable to controls.

Further studies are needed to assess the long-term impact on tooth staining and the oral cavity following consumers switching to different types of NGPs.

a. Sigma-Aldrich, Gillingham, UK.
b. British American Tobacco, Southampton, UK.
c. Borgwaldt-KC, Hamburg, Germany.
d. Whatman, Maidstone, UK.
e. GC, Tokyo, Japan.
f. 3M ESPE, St. Paul, MN, USA.
g. Maybelline, New York City, NY, USA.
h. Konica Minolta Sensing Europe B.V., Nieuwegein, The Netherlands.
i. Chameleon Color Services, Kettering, UK.

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Author contributions: Annette Dalrymple, David Thorne, Marianna Gaça, Steven Coburn and Christopher Proctor designed the aerosol studies, approved all the data and prepared the manuscript. Annette Dalrymple and Anya Terry produced the particulate matter samples and performed the enamel block aerosol exposures. Mark Barber supported aerosol exposures. Thomas Badrock prepared the enamel samples, designed the particulate matter studies and performed all the color analysis. Peter Hall managed all enamel sample preparation, designed the particulate matter studies and approved all data prepared by Intertek. All authors approved the final manuscript.

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