Enamel staining with e-cigarettes, tobacco heating products and modern oral nicotine products compared with cigarettes and snus: An in vitro study

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ABSTRACT: Purpose: To evaluate the effect of cigarette smoke, smokeless tobacco (e.g. snus), tobacco heating products (THP), electronic cigarettes (EC), and modern oral nicotine products on tooth staining. **Methods:** In this in vitro study, staining was assessed for 86 days following exposure of bovine enamel samples to a scientific reference cigarette (1R6F), a THP (glo), an EC (ePen 3), a reference snus product (CRP1.1), and a modern oral product (LYFT). Red wine and coffee were used as positive controls and DMSO and complete artificial saliva as negative controls. Whether brushing could reduce staining levels was also assessed. Changes in staining levels were assessed using the Commission Internationale de L'éclairage L*a*b* method. **Results:** Enamel staining increased with incubation time, and cigarette smoke, snus, coffee and wine induced statistically higher staining levels. THP, EC and modern oral exposure induced minimal staining levels that were also comparable to negative control samples. At day 86, ΔE mean and SD values were 28.50 ± 3.14, 19.76 ± 1.26, 17.35 ± 3.44, 16.22 ± 2.07, 18.30 ± 3.82, 4.10 ± 1.99, 11.30 ± 2.60, 49.56 ± 2.44 for cigarette, glo, EC with blended tobacco, EC with rich tobacco, reference snus product, modern oral product, coffee or wine. The control ΔE mean and SD values at day 86 were 18.68 ± 3.89 for DMSO and 2.17± 0.78 for complete artificial saliva. The ΔE values for all DMSO extracted samples and control increased from day 1 to 86, which suggests that the DMSO used to extract the samples contributes to the enamel sample staining levels. Staining levels were reduced by brushing. (*Am J Dent* 2021;34:3-9).

CLINICAL SIGNIFICANCE: Cigarette smoke, red wine, snus and coffee stained enamel. Exposure to THP, EC or modern oral product extracts for 86 days resulted in minimal enamel staining. Further studies are required to assess the long-term impact on staining and the oral cavity following consumer exclusive use of EC, THP or modern oral products.

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Introduction

In the past 10 years, the number of cigarette smokers switching to electronic cigarettes (EC) and tobacco heating products (THP) has increased substantially. The aerosols of EC and THP have much lower levels of toxicants than cigarette smoke; and exposure of cells in vitro leads to greatly reduced or no biological response.[1-4] Likewise, smokeless tobacco products (e.g. snus, moist snuff and chewing tobacco), which are consumed without heating or combustion, have shown negative toxicology findings.[5] These products may be loose or in pouches, pasteurized or unpasteurized, but all are consumed by placing a portion under the upper or lower lip, in the lower buccal vestibule on the gums, or chewed.[5-7] Smokeless tobacco is regarded as a reduced harm tobacco product due to much lower prevalence of smoking-associated diseases in Sweden compared with other countries.[8] It may also be used as a smoking cessation aid, as 47% of Swedish regular smokeless tobacco users are former smokers.[9] Modern oral products (also known as white snus) are nicotine pouches that might include small amounts of tobacco but are often tobacco-free. With little or no tobacco, modern oral products have toxicant profiles like those of nicotine replacement therapies.[10] In gingival cells, exposure to modern oral products resulted in reduced responses compared to traditional smokeless tobacco products.[11]

In the oral cavity, cigarette smoke has been associated with gingivitis, periodontitis, tooth loss, epithelial malignancy and tooth staining.[12-15] Similarly, smokeless tobacco is thought to be linked to changes in the oral cavity, including keratoses, tooth staining, gingival and periodontal inflammation, alveolar bone damage, dental caries, dysplasia and oral cancer.[16,17] However, not all studies have controlled for potential confounding lifestyle factors, such as smoking, alcohol consumption or underlying systemic disease.[16] With regards to oral cancer, the risk was thought to be minimal with smokeless tobacco use, and significantly reduced relative to the risk associated with smoking,[16,17] however, a recent review[8] of published studies has concluded that snus is not a significant risk factor for oral cavity cancer. Studies[16,17] have confirmed that smokeless tobacco induced keratoses and tooth staining are associated with smokeless tobacco use. As modern oral products have only recently been launched, studies have yet to be published looking at the effects on oral tissues or teeth staining.

In this study, bovine enamel samples were exposed in vitro for 86 days to a scientific reference cigarette (1R6F[a]), a commercial THP (glo[b]), a commercial EC (ePen 3[b]), a scientific reference snus (CRP1.1[a]) and a modern oral product (LYFT[b]) to understand staining propensity. Changes in the level of enamel sample staining were assessed using the Commission Internationale de L'éclairage (CIE) L*a*b* method.[18]

Materials and Methods

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

Products - Products used in this study are detailed in Table 1. Scientific reference cigarettes, 1R6F were obtained from the Center for Tobacco Reference Products, University of Ken-

Table 1. Test articles used for exposure.

Brand name	Product code	Source	Consumable/product details	Product extracted in	Concentration assessed
Reference cigarette	1R6F	UoK*	9.9 TPM# mg/stick, 10.1 mg/stick carbon monoxide, 0.7 mg/stick nicotine	DMSO	24 mg/mL
glo	THP1.0	BAT, UK	Rich Tobacco Neostick	DMSO	24 mg/mL
ePen 3	EC (BT)	BAT, UK	Vype ePen Blended Tobacco (BT) 18 mg/mL nicotine	DMSO	24 mg/mL
ePen 3	EC (vRT)	BAT, UK	Vype ePen Rich Tobacco (vRT) 18 mg/mL nicotine salts	DMSO	24 mg/mL
DMSO	N/A Neat	Fisher Scientific, UK		BP231-1	N/A
Reference snus	CRP1.1	UoK*	0.8% nicotine (~8mg)	CAS	Neat
LYFT	N/A	BAT, UK	Ice cool, 10 mg nicotine	CAS	Neat
CAS	N/A	N/A	N/A	CAS	Neat
Coffee	N/A	Nestlé, UK	Nescafé Original, Lot number 908010921D	N/A	0.5% (1.5 g in 300 mL)
Wine	N/A	Sainsbury, UK	House Shiraz, 12.5% Vol, Lot number L9343	N/A	Neat

* Center for Tobacco Reference Products, University of Kentucky, Lexington, USA (https://ctrp.uky.edu/).
# Total particulate matter.

Table 2. Product used for particulate matter collection.

Product	Regime	Puff Vol (mL)	Puff duration (sec)	Intensity (sec)	Vent blocking	Puff profile
1R6F	HCI	55	2	30	100%	Bell
THP1.0	HCIm	55	2	30	No	Bell
EC (BT)	CRM81	55	3	30	No	Square
EC (vRT)	CRM81	55	3	30	No	Square

HCI = Health Canada Intense (Health Canada Official Method T-115, 1999).[22]
HCIm = HCI modified (no vent blocking).
CRM81 = CORESTA recommended method No 81 (CORESTA, No. 81, 2015).[23]

tucky, Lexington, USA (https://ctrp.uky.edu/). The THP1.0 device (glo) and tobacco consumable, Rich Tobacco Neostik,[b] are commercially available and were manufactured by British American Tobacco (BAT); both are previously described in detail.[19] Prior to use, 1R6F cigarettes were conditioned for a minimum of 48 hours and a maximum of 10 days, and THP Neostiks for a minimum of 48 hours and maximum of 5 days, at 22 ± 1°C and 58 ± 3% relative humidity, according to the International Organization for Standardization 340220. ePen 3, also manufactured by BAT, is a closed modular rechargeable EC which is used with a replaceable e-liquid cartridge. Two e-liquid cartridges were used: Blended Tobacco (BT) containing 18 mg/mL nicotine and vPro Rich Tobacco (vRT) containing 18 mg/mL nicotine salts. To differentiate the two consumables used in this study, they are named EC (BT) and EC (vRT) (Table 1). ePen 3 devices and e-liquid cartridges were stored at room temperature prior to use. Both glo and ePen 3 devices were charged daily before use.

Particulate matter preparation - 1R6F reference CS, THP1.0, EC (BT) or EC (vRT) aerosols were generated using LM20X[d] or LM20E[d] linear engines. Specific puffing regimes were used for each product as detailed in Table 2. The smoke or aerosol from each product was collected onto 44 mm Cambridge filter pads[e] and PM eluted to a concentration of 24 mg/mL with DMSO[f] as previously described.[4]

Smokeless tobacco extracts preparation - Complete artificial saliva (CAS) was prepared as previously described.[23,24] Snus and modern oral samples (Table 1) were cut to 4 mm pieces and 60 g added to 200 mL of CAS. Samples were then homogenized with an ultrasonic homogenizer, incubated at 37ºC for 2 hours and centrifuged (2,739 × g, 20 minutes at room temperature). Supernatants were filtered through 1.6 µm glass filters, followed by 0.22 µm cellulose acetate filters, aliquoted into 5 mL samples and then stored at -70ºC.

Coffee and wine - Products known to stain enamel, coffee, and wine were included in the study as positive controls. The coffee and wine were purchased at Sainsbury Supermarket, UK. The coffee test article (Nescafé Original Coffee,[g] Lot number 908010921D) was prepared by dissolving 1.5 g in 300 mL of deionized water, which was heated and mixed thoroughly. The solution was then aliquoted into 7 mL samples and stored at -20ºC. The wine (Sainsbury House Shiraz, Lot number L9343) was decanted from the bottle as required and stored at room temperature between exposure time points.

Enamel sample preparation - Bovine incisors were collected, disinfected, and stored in 0.1% thymol solution. Enamel blocks measuring 4 mm × 4 mm and 2 mm thick were prepared from the incisors, and approximately two were obtained from each specimen. Each sample was individually cast in a two-part resin/hardener (Epoxicure 2 Resin[h] and Epoxicure 2 Hardener[h]) in 11 mm diameter lubricated plastic caps. Enamel surfaces were then polished using 400-grit abrasive paper to provide a surface roughness comparable to human enamel (Ra value ~0.5 µm). To enable pellicle formation, each enamel sample was

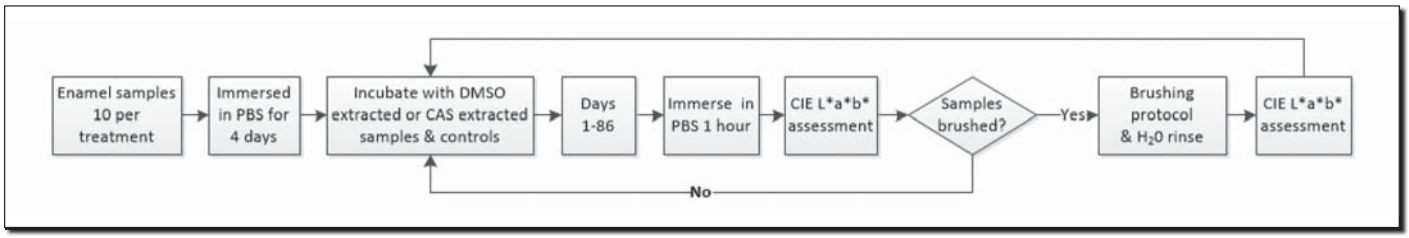

Fig. 1. Overview of experimental methods detailing sample processing, CIE L*a*b* assessment, brushing and repeat analysis.

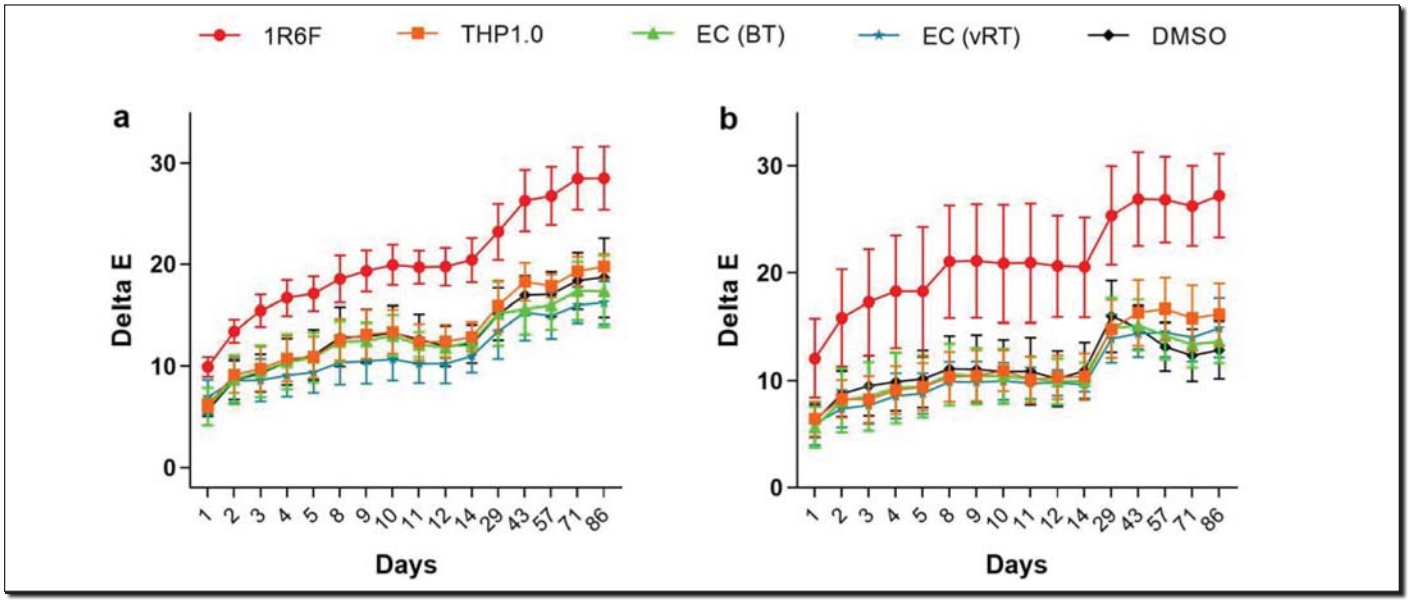

Fig. 2. ΔE values following exposure of enamel samples to DMSO extracted product aerosols or DMSO control. Values are means and standard deviations. Enamel samples were exposed to DMSO extracted particulate matter from 1R6F references cigarettes, a tobacco heating product (THP1.0), e-cigarettes with blended tobacco e-liquid (BT) or rich tobacco e-liquid (vRT) or DMSO alone as a negative control for 86 days. **a**. unbrushed samples and **b**. brushed samples.

incubated in sterile human saliva for a minimum of 1 hour at 35ºC. Samples were then stored in 0.01 M phosphate-buffered saline (PBS) for a minimum of 4 days to achieve full hydration prior to baseline color measurements being taken. Twenty enamel samples were prepared per product or control; 10 of each were brushed at each timepoint and 10 were not brushed.

Enamel sample exposure - An overview of the exposure and measurement procedure used is detailed in Figure 1 and each test article was replaced every 14 days. On day 0, baseline L*, a*, b* values were taken (see below). Enamel blocks were then immersed individually in 7 mL plastic vials containing 0.4 mL of test article and incubated at 35ºC. On each measurement day, samples were removed, immersed for 60 minutes in 0.1 M PBS at room temperature and then L*, a*, b* values assessed as detailed below. The samples were then returned to their vials for further incubation. Ten enamel samples were brushed at each timepoint. Briefly, samples were brushed for 10 seconds using an Oral B[i] rotary toothbrush using approximately 100 g force and a Colgate Total[j] toothpaste slurry. Samples were then rinsed with deionized water and L*, a*, b* values measured as described below. Following brushing, samples were placed back into their individual plastic 7 mL vial, containing their assigned test article and placed into an incubator at 35°C.

Color measurements - Staining levels were measured at baseline and at every timepoint 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.[18] Samples were individually removed from 0.1 M PBS and color readings (L*, a*, b*) measured, at four orientations on each enamel sample, using a Konica Minolta CM-700d Spectro-photometer.[k] Data was captured using a ColorCalc Excel 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 on days 1-5, 8-12, 14, 28, 42, 56, 71 and 86. DMSO or CAS control samples were measured at the same time points. Color change was determined in Excel by calculating ΔL*, Δa*, Δb* and ΔE 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 – SAS[l] version 9.4 was used for all analyses. Generalized linear models (GLMs) were used to assess the differences in ΔE values between the products that had a comparable extraction solution (DMSO or CAS) at each timepoint. For samples that were also brushed, models were created to compare only day 86 non-brushed and brushed enamel samples for each product, including a brushing effect, in order to see if brushing significantly reduces the level of difference in ΔE values. Values are compared at a significance level (α) of 0.05. Post-hoc Tukey adjustment for pairwise comparisons was also used. Three unbrushed enamel samples that were exposed to coffee developed cracks during the study; these samples were excluded from all analyses.

Table 3. Mean ΔE values following exposure to product particulate matter, snus or modern oral extracts for 86 days. ΔE mean and standard deviation values following the exposure of enamel samples to DMSO extracted particulate matter from 1R6F references cigarettes, a tobacco heating product (THP1.0), e-cigarettes with blended tobacco (BT) or rich tobacco (vRT) e-liquid, DMSO alone as a negative control, CAS extracted reference snus (CRP1.1) or modern oral product (LYFT), complete artificial saliva (CAS) as a control, coffee or wine for 86 days.

	1R6F		THP1.0		EC (BT)		EC (vRT)		DMSO		CRP1.1		LYFT		CAS		Coffee		Wine	
Days	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	9.95	0.98	6.15 a	0.66	6.03 a	1.87	6.97 a	1.69	5.65 a	0.57	6.26	2.30	0.90 d	0.75	0.59 c	0.47	4.19	1.38	18.01 c	5.23
2	13.45	1.11	9.16 b	1.79	8.69 a	2.44	8.59 a	2.17	8.65 a	1.93	8.18	3.41	0.74 c	0.58	0.46 c	0.33	4.81	1.26	23.82 c	5.05
3	15.44	1.55	9.75 a	2.18	9.51 a	2.56	8.63 a	2.09	9.34 a	1.85	8.42	2.24	0.92 c	0.74	0.51 c	0.40	5.30	1.50	27.74 c	4.93
4	16.68	1.77	10.77 a	2.25	10.46 a	2.73	9.12 a	2.12	10.46 a	2.28	8.65	2.37	0.94 c	0.43	0.71 c	0.52	5.64	1.44	29.03 c	4.82
5	17.10	1.70	10.91 a	2.05	10.86 a	2.43	9.41 a	2.04	11.12 a	2.49	9.58	2.52	1.12 c	0.55	0.83 c	0.74	6.04	1.61	31.01 c	5.10
8	18.53	2.31	12.62 a	2.00	12.42 a	2.00	10.39 a	2.18	12.88 a	2.89	9.66	2.48	1.68 c	0.68	1.10 c	0.83	6.22 d	1.44	34.64 c	4.49
9	19.32	2.04	13.15 a	2.38	12.52 a	1.77	10.53 a	2.23	12.96 a	2.63	10.18	3.11	1.61 c	1.00	0.86 c	0.76	6.25 d	1.17	36.03 c	4.75
10	19.92	1.99	13.41 a	2.13	13.01 a	1.98	10.71 a	2.12	13.31 a	2.61	9.75	2.83	1.72 c	1.13	1.03 c	0.94	6.15 d	1.30	36.83 c	4.66
11	19.71	1.63	12.39 a	1.79	12.16 a	1.24	10.28 a	1.92	12.69 a	2.41	9.73	2.88	1.80 c	1.23	1.17 c	0.91	6.38	1.44	37.37 c	4.55
12	19.75	1.86	12.46 a	1.65	11.86 a	0.94	10.27 a	1.94	12.00 a	2.00	10.12	3.07	1.90 c	1.33	1.02 c	0.60	6.47 d	1.54	38.64 c	4.46
14	20.41	2.18	12.90 a	1.45	12.47 a	0.81	11.03 a	1.64	12.21 a	1.88	10.39	2.92	2.22 c	1.53	1.12 c	0.75	6.67 d	1.39	40.40 c	4.20
29	23.20	2.77	15.91 a	2.47	15.14 a	3.12	13.49 a	2.74	15.11 a	2.54	13.35	3.06	3.17 c	1.88	1.56 c	0.61	7.95 d	1.43	44.54 c	2.48
43	26.27	3.03	18.25 a	1.88	15.60 a	2.46	15.27 a	2.72	16.95 a	1.91	15.49	3.67	3.33 c	1.90	1.80 c	0.68	9.17 c	1.42	46.19 c	2.90
57	26.75	2.89	17.85 a	1.12	15.97 a	2.31	14.94 a	2.22	17.03 a	2.18	16.55	3.69	3.72 c	1.93	1.88 c	0.73	9.42 c	2.06	47.71 c	1.96
71	28.47	3.11	19.28 a	1.44	17.39 a	2.85	15.97 a	1.74	18.37 a	2.78	17.45	3.35	3.99 c	1.98	2.03 c	0.68	10.35 c	2.07	48.23 c	1.64
86	28.50	3.14	19.76 a	1.26	17.35 a	3.44	16.22 a	2.07	18.68 a	3.89	18.30	3.82	4.10 c	1.99	2.17 c	0.78	11.30 c	2.60	49.56 c	2.44

a = Significantly different from 1R6F P< 0.0001.
b = Significantly different from 1R6F P< 0.05.
c = Significantly different from CRP1.1 P< 0.0001.
d = Significantly different from CRP1.1 P< 0.05.

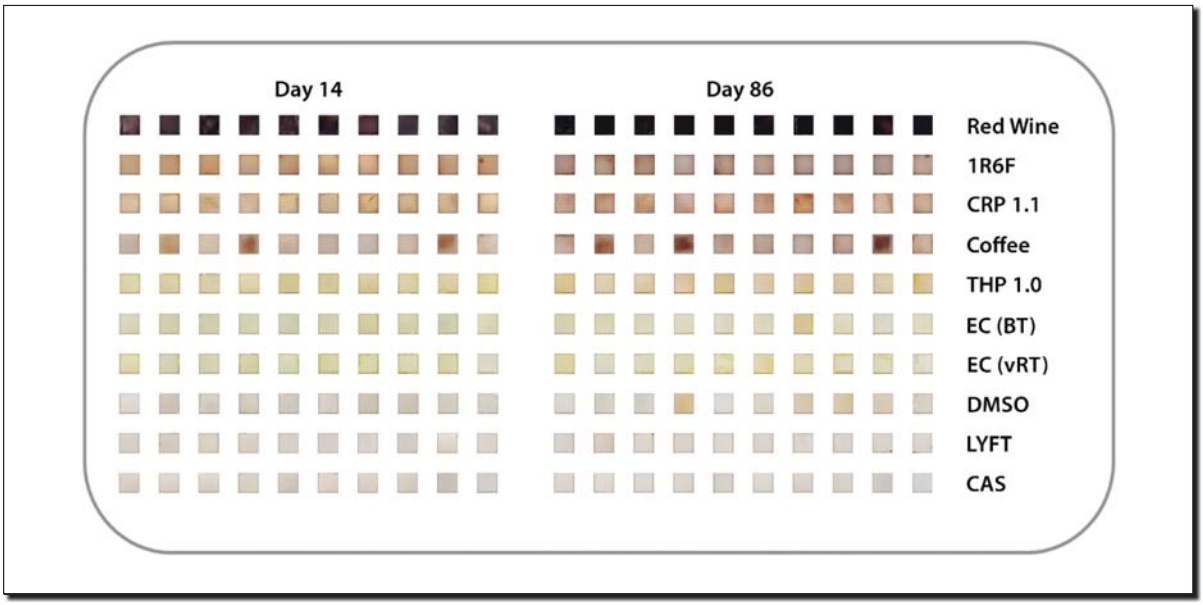

Fig. 3. Enamel sample staining levels following exposure to products and controls for 14 or 86 days. Enamel samples were exposed to red wine, a reference cigarette (1R6F), a reference snus product (CRP1.1), coffee, a tobacco heating product (THP 1.0), e-cigarettes (EC) with blended tobacco e-liquid (BT) or rich tobacco e-liquid (vRT), DMSO (as a control), modern oral product (LYFT), or complete artificial saliva (CAS, as a control).

Results

Enamel sample staining levels following exposure to product particulate matter - The ΔE, the overall color change of the enamel blocks, increased from day 1 to 86 following all treatments. This suggests that the DMSO used to extract the samples contributes to enamel sample staining and not the THP1.0 or EC (BT) and EC (vRT) aerosols. 1R6F ΔE values were significantly higher than all other products from day 1 (P≤ 0.0001). The ΔE values for THP1.0, EC (BT) and DMSO were comparable at all timepoints. EC (vRT) ΔE values were significantly lower at day 11 than DMSO ΔE values (P= 0.04). ΔE values for EC (vRT) and DMSO were comparable at all other timepoints (Fig. 2, Table 3). The significant increase in enamel block staining following 1R6F exposure and comparable results of THP1.0, EC (BT), EC (vRT) and DMSO control can be observed in Fig. 3.

Enamel sample staining levels following exposure to CAS extracted products - The ΔE, or total colour change significantly increased after 1 day following CRP1.1 exposure, compared to CAS (P< 0.0001) and LYFT (P= 0.0002). The ΔE values were also significantly increased following coffee exposure compared to CAS and LYFT after day 2 (P= 0.0474). LYFT and CAS ΔE values were minimal and comparable at all time points (Table 3, Fig. 4).

Along with coffee, red wine was used as a positive control. Due to the higher levels of staining, the data was not included in Fig. 2 or 4 but data is presented in Tables 3 and 4 and Fig. 3.

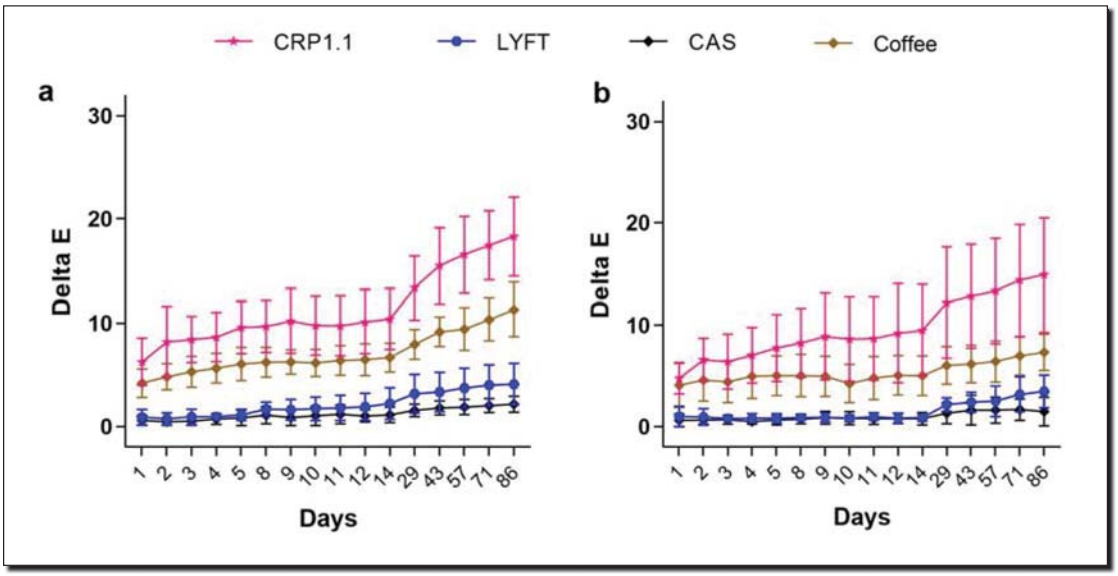

Fig. 4. ΔE values following exposure of enamel samples to CAS extracted products. Values are means and standard deviations. ΔE mean and standard deviation values following the exposure of enamel samples to complete artificial saliva (CAS) extracted reference snus (CRP1.1), modern oral product (LYFT), coffee or to CAS alone as a negative control for 86 days. **a.** unbrushed samples and **b.** brushed samples.

Table 4. Mean ΔE values following exposure to product particulate matter, snus or modern oral extracts for 86 days when samples were also brushed. ΔE mean and standard deviation values following the exposure of enamel samples to DMSO extracted particulate matter from 1R6F references cigarettes, a tobacco heating product (THP1.0), e-cigarettes with blended tobacco (BT) or rich tobacco (vRT) e-liquid, DMSO alone as a negative control, CAS extracted reference snus (CRP1.1) or modern oral product (LYFT), complete artificial saliva (CAS) as a control, coffee or wine for 86 days. Brushing was performed for 10 seconds at each timepoint, followed by L*a*b* value measurement.

	1R6F		THP1.0		EC (BT)		EC (vRT)		DMSO		CRP1.1		LYFT		CAS		Coffee		Wine	
Days	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	12.08	3.66	6.44 a	1.62	5.66 a	1.90	6.00 a	2.00	6.25 a	1.53	4.79	1.55	1.02c	0.99	0.64 c	0.64	4.08	2.17	13.14 c	2.30
2	15.85	4.50	8.32 a	1.75	8.15 a	2.98	7.39 a	1.75	8.79 a	2.14	6.59	2.12	0.96 c	0.82	0.65 c	0.40	4.61	2.09	16.70 c	2.21
3	17.29	4.92	8.26 a	2.18	8.54 a	3.22	7.73 a	1.77	9.53 a	2.80	6.43	2.71	0.80 c	0.44	0.69 c	0.42	4.43	2.03	18.38 c	3.04
4	18.27	5.22	9.14 a	2.24	9.35 a	3.33	8.59 a	2.11	9.92 a	2.73	7.06	2.73	0.84 c	0.45	0.53 c	0.28	4.97	2.17	20.60 c	3.13
5	18.26	6.03	9.44 a	2.23	9.47 a	2.92	8.81 a	1.89	10.19 a	2.67	7.76	3.31	0.84 c	0.47	0.65 c	0.47	5.05	1.96	22.19 c	3.46
8	21.06	5.25	10.37 a	2.33	10.59 a	2.87	9.91 a	1.86	11.12 a	3.06	8.26	3.40	0.89 c	0.24	0.76 c	0.47	5.05 d	2.09	24.87 c	3.53
9	21.13	5.28	10.48 a	2.36	10.48 a	2.60	9.89 a	1.92	11.06 a	3.22	8.90	4.26	0.91 c	0.43	0.90 c	0.63	4.99 d	1.99	25.49 c	3.66
10	20.87	5.48	10.94 a	1.90	10.43 a	2.54	9.96 a	1.73	10.87 a	2.96	8.64	4.15	0.83 c	0.32	0.85 c	0.64	4.27 d	1.89	24.54 c	3.96
11	20.94	5.53	10.12 a	2.16	10.21 a	2.00	9.79 a	1.47	10.89 a	3.14	8.70	4.09	0.98 c	0.36	0.80 c	0.57	4.81 d	2.14	25.40 c	4.10
12	20.63	4.71	10.32 a	2.05	9.94 a	2.12	9.82 a	1.20	10.19 a	2.59	9.20	4.86	0.84 c	0.50	0.80 c	0.49	5.08 d	1.95	26.18 c	3.96
14	20.55	4.64	10.38 a	2.18	9.98 a	1.73	9.65 a	0.65	10.96 a	2.62	9.49	4.47	0.89 c	0.33	0.80 c	0.62	5.04 d	1.95	27.86 c	4.19
29	25.36	4.61	14.82 a	2.65	14.91 a	2.76	13.94 a	2.24	15.98 a	3.31	12.21	5.43	2.18 c	0.66	1.32 c	1.04	6.05 d	1.86	33.25 c	3.60
43	26.89	4.38	16.32 a	3.03	15.18 a	2.33	14.43 a	2.20	14.96 a	2.01	12.83	5.08	2.41 c	1.00	1.64 c	1.46	6.18 c	1.84	35.81 c	2.86
57	26.84	4.02	16.66 a	2.89	14.25 a	2.25	14.53 a	2.31	13.17 a	2.25	13.33	5.16	2.52 c	1.52	1.64 c	1.28	6.44 c	2.03	36.77 c	3.10
71	26.25	3.75	15.82 a	3.00	13.37 a	2.13	14.05 a	2.23	12.38 a	2.44	14.37	5.50	3.21 c	1.70	1.69 c	1.05	7.00 c	1.91	38.74 c	2.15
86	27.23	3.91	16.16 a,f	2.85	13.71 a,f	2.06	14.92 a	2.72	12.87 a,f	2.68	14.90	5.60	3.49 c	1.61	1.51 c	1.41	7.34 c,f	1.79	39.08 c,e	2.32

a = Significantly different from 1R6F P< 0.0001.
b = Significantly different from 1R6F P< 0.05.
c = Significantly different from CRP1.1 P< 0.0001.
d = Significantly different from CRP1.1 P< 0.05.
e = Significantly different than unbrushed sample at same timepoint P< 0.0001.
f = Significantly different than unbrushed sample at same timepoint P< 0.05.

Red wine ΔE values were significantly different for all products and controls, as well as coffee. Figure 3 also details the differences in staining levels; the highest staining level was recorded with red wine followed by 1R6F, CRP1.1, coffee, THP 1.0, EC (BT), EC (vRT), DMSO, LYFT, and then CAS with the lowest level of staining.

The effect of brushing - The mean data of unbrushed samples and brushed samples (Figs. 2,4) show similar trends. To understand the effect of brushing, sample staining levels were measured directly after staining with no brushing throughout the study and compared to samples that were brushed at each timepoint throughout the study. Statistical analysis was performed only on day 86 samples. DMSO extracted products are detailed in Table 4 and Fig. 2 and CAS extracted samples in Table 4 and Fig. 4. ΔE* values were significantly reduced for DMSO (P= 0.0011), EC (BT) (P= 0.0101) and THP1.0 (P= 0.0018) samples that were brushed, signifying a reduction in staining with brushing.

For CAS extracted samples that were brushed (Table 4), ΔE values for coffee (P= 0.002) and red wine (P< 0.0001) were significantly reduced signifying that brushing reduced staining. There were no significant differences of ΔE values for LYFT exposed samples following brushing, which is possibly due to the low values recorded at all timepoints and the fact that all values were comparable to the CAS control values.

Discussion

In this study, bovine enamel samples were exposed to cigarette, THP, EC, snus or modern oral product extracts. Compared with cigarette, snus, wine or coffee, staining levels were minimal or comparable to the controls for THP, EC or modern oral product. DMSO extracted samples and the DMSO control had higher staining levels than CAS extracted samples, which suggests that DMSO contributes to the staining observed. Aerosol exposure is more aligned to consumer use of a cigarette, EC or THP and more appropriate method for testing these products.[15,29] In the case of snus and modern oral products, as they are used in the mouth, CAS is an appropriate material to extract the samples.

Coffee, red wine and cigarette smoke are well known to stain enamel and have been used to test the efficiency of whitening toothpastes or bleaching agents.[25,26] By including coffee and red wine as positive controls in this study along with the negative controls, we were able to place staining levels in a spectrum of high to low. Red wine induced the highest level of staining followed by the reference cigarette, reference snus, coffee, THP, EC, DMSO, modern oral and then CAS.

To align the study to a consumer's lifestyle, brushing was applied to a subset of samples. Brushing removed some surface staining, indicating that the staining was on the enamel surface only and was not due to product extracts or controls penetrating the cut edge of the enamel samples. In a human mouth, if cracks appear in the enamel due to age or damage, stains can penetrate the enamel and increase the level of staining.[27] In this study three enamel samples that were exposed to coffee did have cracks and coffee penetrated into the enamel during exposure. Cracks may have developed during enamel preparation or could have been present before sampling. These samples were excluded from analysis, however, to enable the increased levels of staining to be visualized in enamel samples with cracks they are included in Fig. 3.

Other studies[26,28,29] have confirmed that brushing can reduce the level of cigarette smoke, THP and EC aerosol staining on bovine enamel samples and on human enamel. The present study differs from those studies as staining levels on samples were assessed at each timepoint with and without brushing, allowing assessment of cumulative staining and brushing reductions. The absence of changes in staining levels following brushing of modern oral samples was probably due to the low associated levels of staining without brushing, even at 86 days.

The data generated in this study suggests that switching to THP, EC or modern oral products could have cosmetic benefits for cigarette smokers and traditional snus consumers. However, data is from a laboratory assessment, a long-term clinical study where smokers or snus users switch for several months to the exclusive use of a THP, EC or modern oral product, could provide information on consumer's teeth staining levels.

In conclusion, for the first time, the staining potential of THP, EC and modern oral products have been assessed for up to 86 days in vitro. Compared to cigarette, snus, wine and coffee, this study confirms significant reductions in THP, EC and modern oral product staining levels, and that THP, EC and modern oral product staining levels after 86 days exposure are comparable to controls.

a. University of Kentucky, Lexington, KY, USA.
b. British American Tobacco, Southampton, Hampshire, UK.
c. Sigma Aldrich, Gillingham, UK or St. Louis MO, USA.
d. Borgwaldt-KC, Hamburg, Germany.
e. Whatman, Maidstone, UK.
f. Fisher Scientific, Loughborough, UK.
g. Nestlé, Gatwick, UK.
h. Buehler Esslingen, Warwick , UK,
i. Procter & Gamble, Weybridge, UK.
j. Colgate-Palmolive Company, Guildford, UK.
k. Konica Minolta Sensing, Warrington, UK.
l. SAS, Marlow, UK.

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