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## Evaluation of the Tobacco Heating System 2.2. Part 2: Chemical composition, genotoxicity, cytotoxicity, and physical properties of the aerosol



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## ABSTRACT

The chemical composition, *in vitro* genotoxicity, and cytotoxicity of the mainstream aerosol from the Tobacco Heating System 2.2 (THS2.2) were compared with those of the mainstream smoke from the 3R4F reference cigarette. In contrast to the 3R4F, the tobacco plug in the THS2.2 is not burnt. The low operating temperature of THS2.2 caused distinct shifts in the aerosol composition compared with 3R4F. This resulted in a reduction of more than 90% for the majority of the analyzed harmful and potentially harmful constituents (HPHCs), while the mass median aerodynamic diameter of the aerosol remained similar. A reduction of about 90% was also observed when comparing the cytotoxicity determined by the neutral red uptake assay and the mutagenic potency in the mouse lymphoma assay. The THS2.2 aerosol was not mutagenic in the Ames assay. The chemical composition of the THS2.2 aerosol was also evaluated under extreme climatic and puffing conditions. When generating the THS2.2 aerosol under “desert” or “tropical” conditions, the generation of HPHCs was not significantly modified. When using puffing regimens that were more intense than the standard Health Canada Intense (HCI) machine-smoking conditions, the HPHC yields remained lower than when smoking the 3R4F reference cigarette with the HCI regimen.

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### 1. Introduction

The U.S. Family Smoking Prevention and Tobacco Control Act (FSPTCA) defines a Modified Risk Tobacco Product (MRTP) as “any tobacco product that is sold or distributed for use to reduce harm or the risk of tobacco related disease associated with commercially marketed tobacco products” (Family Smoking Prevention and Tobacco Control Act, 2009). This publication is part of a series of nine publications describing the nonclinical and part of the clinical assessment of a candidate MRTP, THS2.2 regular and a mentholated version (THS2.2M). The series of publications provides part

of the overall scientific program to assess the potential for THS2.2 to be a reduced risk product. The first publication in this series describes THS2.2 and the assessment program for MRTPs (Smith et al., 2016). This is followed by six publications, including this one, that describe the nonclinical assessment of THS2.2 regular and THS2.2M (Kogel et al., 2016; Oviedo et al., 2016; Schaller et al., 2016a; Schaller et al., 2016b; Sewer et al., 2016; Wong et al., 2016). The eighth publication in the series describes a clinical study to assess whether the reduced formation of Harmful and Potentially Harmful Constituents (HPHCs) for THS2.2 regular also leads to reduced exposure to HPHCs when the product is used in a clinical setting (Haziza et al., 2016). A final publication utilizes data gathered from the reduced exposure clinical study on THS2.2 regular to determine if a systems pharmacology approach can identify exposure response markers in peripheral blood of

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**Abbreviations**

(NNK)	4-( <i>N</i> -nitrosomethylamino)-1-(3-pyridyl)-1-butanone	(MMAD)	mass median aerodynamic diameter
(EHCS)	Electrically Heated Cigarette Smoking System	(MRTP)	Modified Risk Tobacco Product
(FID)	flame ionization detection	(MLA)	mouse lymphoma assay
(GC/MS)	gas chromatograph-mass spectrometer	(MF)	mutation frequency
(GC)	gas chromatography	(NRU)	neutral red uptake
(GVP)	gas-vapor phase	(NFDPM)	nicotine-free dry particulate matter
(GEF)	global evaluation factor	(NO <sub>x</sub> )	nitrogen oxides
(HPHC)	harmful and potentially harmful constituent	(NAB)	<i>N</i> -nitrosoanabasine
(HCI)	Health Canada Intense	(NAT)	<i>N</i> -nitrosoanatabine
(ICP-MS)	inductively coupled plasma mass spectrometry	(NNN)	<i>N</i> -nitrososonornicotine
(IARC)	International Agency for Research on Cancer	(PBS)	phosphate-buffered saline
(LOQ)	limit of quantification	(PDSP)	programmable dual syringe pump
(LC-MS/MS)	liquid chromatograph tandem mass spectrometer	(RH)	relative humidity
(LC)	liquid chromatography	(RTG)	relative total growth
(LB)	lower boundaries	(IP)	Intermediate Precision
(LOGEL)	lowest observed genotoxic effect level	(THS2.2)	Tobacco Heating System 2.2
		(TPM)	total particulate matter
		(UB)	upper boundaries

smokers switching to THS2.2 (Martin et al., 2016). This publication is the second of the series and presents the chemical analyses, the physical characterization, and the *in vitro* genotoxicity and cytotoxicity assessments of the mainstream aerosol of the THS2.2.

The smoke produced by the combustion of tobacco in a combustible cigarette (CC) is a complex and dynamic chemical mixture which contains more than 8000 identified chemical compounds (Rodgman and Perfetti, 2013). Tobacco smoke from CC consists of an aerosol containing liquid droplets ('particulate phase') suspended in a carrier gas and surrounded by its own gas-vapor phase. It is generated by complex and overlapping burning, pyrolysis, pyrosynthesis, distillation, sublimation, and condensation processes (Borgerding and Klus, 2005). With minor exceptions, both pyrogenesis and pyrosynthesis of HPHCs result from the thermal decomposition of organic compounds present in tobacco occurring at temperatures up to 900 °C observed in cigarettes (Torikai et al., 2005; Baker, 2006); thus, a reduction of these toxicants may be achieved by heating rather than burning tobacco to produce an aerosol. The development of *heat-not-burn* tobacco products is not new and earlier efforts to develop such products (notably Premier and Eclipse products from The R.J. Reynolds Tobacco Company and Accord from Philip Morris) have been reviewed by Baker (Baker, 2006). The Electrically Heated Cigarette Smoking System (EHCS) was the first generation of tobacco heated products commercialized by Philip Morris. The EHCS series-E has been subject to extensive analytical and toxicological evaluation, demonstrating simplified smoke chemistry compared with the 1R4F reference cigarette of the University of Kentucky (Patskan and Reininghaus, 2003). Notably there was a significant reduction in carbon monoxide (CO) and an increased yield of formaldehyde in EHCS-E mainstream smoke, compared with the 1R4F cigarette. On a per-milligram total particulate matter (TPM) basis, the concentration of formaldehyde was increased approximately sevenfold (Stabbert et al., 2003). In later developments of EHCS (series-JLI and series-K), in order to reduce these excessive levels of formaldehyde, ammonium magnesium phosphate (AMP) was used in the cigarette paper to replace calcium carbonate. It was anticipated that ammonia released during the pyrolysis of AMP would condense with formaldehyde to form hexamethylenetetramine (HMT) (Schorp

et al., 2012). Chemical analysis of smoke from the EHCS-JLI and EHCS-K cigarettes containing AMP showed lower yields of formaldehyde and several HPHCs, a further decrease in CO yield, and increased yields of ammonia and HMT (Roemer et al., 2008; Werley et al., 2008; Zenzen et al., 2012). The THS2.2 is the latest generation of *heat-not-burn* products from Philip Morris International. It produces an aerosol by carefully heating the tobacco with a heater blade reaching a maximum temperature of 350 °C. This system enables a careful control of the energy applied to the tobacco plug (Smith et al., 2016) and limits the thermal physico-chemical processes while producing an aerosol capable of satisfying adult smokers enabling them to switch from cigarettes.

Although the causal relationship between smoking and several diseases is well established (Doll et al., 2004), there is still very little understanding of the underlying mechanisms by which smoking causes disease. Among the more than 8000 chemical compounds that have been identified in cigarette tobacco smoke (Rodgman and Perfetti, 2013), public health authorities and others have proposed some 100 HPHCs as possible causes of smoking-related diseases such as lung cancer, heart disease, and emphysema (Health Canada, 2000; World Health Organisation, 2008; U.S. Food and Drug Administration, 2012). For the US Food and Drug Administration, the notion of "harmful and potentially harmful constituent" includes any chemical or chemical compound in a tobacco product or in tobacco smoke that is, or potentially is, inhaled, ingested, or absorbed into the body, including as an aerosol (vapor) or any other emission; and causes or has the potential to cause direct or indirect harm to users or non-users of tobacco products (U.S. Food and Drug Administration, 2016). However, there is no consensus, that lowering or eliminating any single compound (or even a combination of compounds) in smoke would have a significant impact on risk. The current approach, which eliminates direct tobacco combustion and limits tobacco pyrolysis by heating at significantly lower temperatures than encountered in CC, has the potential to reduce a broad range of HPHCs in the THS2.2 aerosol. Consequently, criteria were established to develop a list of relevant analytes, including HPHCs to assess their reductions in the THS2.2 aerosol, compared to the mainstream smoke of the University of Kentucky reference cigarette 3R4F, as follows:

- **Criterion 1:** Smoke constituents determined by International Organization for Standardization (ISO) methods. This list includes total particulate matter (TPM) (International Organisation for Standardization, 2011), water in TPM (International Organisation for Standardization, 2011), nicotine (International Organisation for Standardization, 2013), nicotine-free dry particulate matter (NFDPM) (International Organisation for Standardization, 2011); carbon monoxide (CO) (International Organisation for Standardization, 2010b) and benzo[a]pyrene (International Organisation for Standardization, 2012).
- **Criterion 2:** Priority toxicants in tobacco smoke selected from the lists issued by regulatory bodies, or proposed by cognizant authorities (Health Canada, 2000; World Health Organisation, 2008; U.S. Food and Drug Administration, 2012). This list includes the analytes recommended by ISO under Criterion 1. In addition, the following HPHCs are also included: 1-aminonaphthalene, 2-aminonaphthalene, 3-aminobiphenyl, 4-aminobiphenyl, acetaldehyde, acetone, acrolein, butyraldehyde, crotonaldehyde, formaldehyde, methyl ethyl ketone, propionaldehyde, acrylonitrile, 1,3-butadiene, benzene, isoprene, pyridine, quinoline, styrene, toluene, catechol, *m*-cresol, *p*-cresol, *o*-cresol, hydroquinone, phenol, resorcinol, *N*-nitrosoanabasine (NAB), *N*-nitrosoanatabine (NAT), 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK), *N*-nitrosornicotine (NNN), ammonia, hydrogen cyanide, nitric oxide (NO), nitrogen oxides (NO<sub>x</sub>), arsenic, cadmium, chromium, lead, mercury, nickel, and selenium.
- **Criterion 3:** Toxicants with an established biomarker of exposure, i.e., for use in a clinical study to determine exposure to the parent toxicant (Haziza et al., 2016). The toxicants include some analytes already listed under “Criterion 1” and “Criterion 2”: CO (biomarker: blood carboxyhemoglobin (COHb) (Pojer et al., 1984), nicotine (biomarker: serum cotinine (Benowitz and Iii, 1984) or total nicotine equivalents in urine (Benowitz and Jacob, 1994)), 2-aminonaphthalene, 4-aminobiphenyl and *o*-toluidine (biomarker: parent amines in urine (Riedel et al., 2006)), acrolein (biomarker: 3-hydroxypropylmercapturic acid (3-HPMA) in urine (Mascher et al., 2001)), crotonaldehyde (biomarker: 3-hydroxy-2-methylpropyl mercapturic acid (HMPMA) in urine (Scherer et al., 2007)), acrylonitrile (biomarker: 2-cyanoethylmercapturic acid (CEMA) in urine (Minet et al., 2011)), acrylamide (biomarker: acrylamide mercapturic acid (AAMA) in urine (Urban et al., 2006)), 1,3-butadiene (biomarker: 1-hydroxy-2-(*N*-acetyl-cysteineyl)-3-butene (MHBMA) in urine (van Sittert et al., 2000)), benzene (biomarker: *S*-phenylmercapturic acid (*S*-PMA) in urine (Medeiros et al., 1997)), NNK (biomarker: total 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in urine (Carmella et al., 2003)), NNN (total NNN in urine (Kavvadias et al., 2009)), benzo[a]pyrene and pyrene (biomarker: total 1-hydroxypyrene (1-OHP) in urine (Strickland et al., 1996)).
- **Criterion 4:** Toxicants which are predominantly formed below 400 °C, and which are not included under “Criterion 2”: acrylamide (possibly formed from asparagine and reducing sugars through a Maillard type of reaction occurring between 120 and 200 °C (Stadler et al., 2002; Blank et al., 2005; Becalski et al., 2011)), acetamide (possibly formed from the pyrolysis of Amadori compounds (formed by the reaction of amino acids and sugars) and from the decomposition of ammonium acetate at around 250 °C (Moldoveanu, 2010)), propylene oxide (possibly formed by dehydration of propylene glycol which is used as a humectant in CC and for application of flavors to tobacco (Diekmann et al., 2006; Laino et al., 2012)), nitrobenzene, ethylene oxide and vinyl chloride (the source of the 3 last compounds is less clear but ethylene oxide and vinyl chloride

are both classified by the International Agency for Research on Cancer (IARC) as Group 1 (carcinogenic to humans), and nitrobenzene as Group 2B (possibly carcinogenic to humans).

- **Criterion 5:** Toxicants which are predominantly formed above 400 °C, and which are not included under “Criterion 1” and “Criterion 2”: dibenz[*a,h*]anthracene and benz[*a*]anthracene (McGrath et al., 2007).

In addition, glycerin (a humectant used during CC and Tobacco stick manufacturing) and menthol (for mentholated products only) were also quantified. This results in a total of 59 analytes for THS2.2 regular products (60 for mentholated products) that were quantified to perform the chemical assessment of the THS2.2 aerosol. Among them, 54 are HPHCs targets for reduction compared to 3R4F when developing *heat-not-burn* products: carbon monoxide, benzo[*a*]pyrene, 1-aminonaphthalene, 2-aminonaphthalene, 3-aminobiphenyl, 4-aminobiphenyl, acetaldehyde, acetone, acrolein, butyraldehyde, crotonaldehyde, formaldehyde, methyl ethyl ketone, propionaldehyde, acrylonitrile, 1,3-butadiene, benzene, isoprene, pyridine, quinoline, styrene, toluene, catechol, *o*-cresol, *m*-cresol, *p*-cresol, hydroquinone, phenol, resorcinol, NAB, NAT, NNK, NNN, ammonia, hydrogen cyanide, nitric oxide, nitrogen oxides, arsenic, cadmium, chromium, lead, mercury, nickel, selenium, pyrene, *o*-toluidine, acetamide, acrylamide, ethylene oxide, nitrobenzene, propylene oxide, vinyl chloride, benz[*a*]anthracene and dibenz[*a,h*]anthracene. As previously mentioned, this list was mainly based on analytes proposed by public health authorities and it covers a large range of potential toxicants identified in cigarette smoke. However, the scientific literature continues to describe new compounds with a potential toxicity mainly in aerosols from new products. For instance, only recently has glycidol been identified as a potential toxic compound in aerosols from electronic cigarettes (Sleiman et al., 2016). The present list of HPHCs was based on knowledge available at the time of designing the studies and glycidol, which was not included in the FDA list (U.S. Food and Drug Administration, 2012), was not identified at the time as a potential target to be included.

In addition to the 54 HPHCs listed above, TPM, water, nicotine, NFDPM, glycerin and menthol (for mentholated products only) are used to assess product performance but are not targets for reduction compared to the 3R4F. These analytes are major aerosol constituents for which the level has to be maintained in order to provide satisfactory sensory properties. For instance, nicotine is addictive and has toxic properties. However, at the levels nicotine is consumed from tobacco products, it is not considered to be a primary cause of smoking related disease (The Royal College of Physicians, 2016). As noted by the Royal College of Physicians (The Royal College of Physicians, 2016) “The ideal harm-reduction device should therefore deliver nicotine in a manner as similar as possible to cigarettes, while at the same time maximizing palatability and nicotine delivery to approximate the experience of cigarette smoking more closely.” Following this logic, the nicotine content in the delivered aerosol was carefully monitored and the nicotine level was by design maintained at an appropriate level. It is important to note that, although TPM and NFDPM are analytes that were originally defined in the context of CC smoke, they can also be used in the context of aerosol produced by heating tobacco, as they characterize the mass of aerosol delivered. However, TPM and NFDPM yields obtained from heated products should not be directly compared to the values obtained for cigarettes, as the smoke produced by combustion and the aerosol produced by heating tobacco have substantially different chemical compositions.

The exposure to HPHCs may also be affected by the physical properties of the inhaled aerosol. The particle/droplet size

distribution determines the fraction of aerosol or smoke that is able to pass through the upper respiratory tract to reach the lungs and the fraction that is retained in the respiratory system (Robinson and Yu, 2001; Bernstein, 2004; Kane et al., 2010). An aerosol is considered respirable when the mass median aerodynamic diameter (MMAD) calculated from the measured size distribution is below 2.5  $\mu\text{m}$  (Hinds, 1999). In the past decades, several instruments have been used to determine the size distributions of CC mainstream smoke, and more recently, of e-cigarette aerosols as well (Singh et al., 2006; Ingebrethsen et al., 2012; Fuoco et al., 2014; Geiss et al., 2015). These analytical techniques have been shown to have both advantages and drawbacks in measuring the aerosol physical parameters. Past studies revealed that different methodologies led to size distributions ranging from 0.2 to 0.9  $\mu\text{m}$  for CC smoke (Ishizu et al., 1978; Davies, 1988). This wide range of droplet sizes obtained for CC was presumably due to particular aerosol sampling and dilution methodologies. The methodologies enabling a real-time aerosol physical characterization used the measured aerosol number size distribution to calculate the related mass concentration distribution and the MMAD, assuming spherical particles or droplets with a chosen arbitrary density. Consequently, the conversion from number to mass distribution could lead to the overestimation or underestimation of the MMAD. To minimize these problems, the selected physical characterization of the aerosol was based on multistage cascade impactor technology, allowing a determination of the MMAD. The impactor technique enabled the gravimetric classification of aerosol droplets in distinct size classes, and was often associated with the related aerosol deposition behavior in lungs (Hinds, 1999). Additionally, the technique allowed the determination of the MMAD assuming equivalent unit density and spherical droplet shapes.

The development of a new product such as the THS2.2 should be accompanied not only by the analysis of its chemical composition but also by *in vitro* and *in vivo* toxicological assays, because current knowledge is insufficient for predicting the effect of complex mixtures based solely on the chemical composition (Carchman, 1988; Institute of Medicine, 2001; Tewes et al., 2003). To further extend the characterization of the THS2.2 aerosol, both the cytotoxic and mutagenic activity of the aerosol were compared to the activities of mainstream smoke of the reference cigarette 3R4F. Furthermore, two 90-day rodent inhalation studies are also reported in this issue (Sewer et al., 2016; Wong et al., 2016).

The current studies report the aerosol characterization from 4 different versions of the THS2.2 Tobacco Sticks: two THS2.2 Regular (THS2.2 FR1 and THS2.2 D2) and two THS2.2 Menthol (THS2.2 FR1 M and THS2.2 D1 M). These studies include the cytotoxic and genotoxic activities of the mainstream THS2.2 aerosol compared to mainstream smoke of the reference cigarette 3R4F and a comprehensive physical and chemical characterization of the produced aerosols. For both types of aerosols, the cytotoxicity of the gas-vapor phase (GVP) and the particulate phase (TPM) was assessed using the neutral red uptake (NRU) assay (Borenfreund and Puerfer, 1985) and the genotoxicity was assessed using both the *Salmonella typhimurium* reverse mutation assay (TPM only) (Ames (Ames et al., 1973)) and the mouse lymphoma assay (MLA) (Clive et al., 1972). The physicochemical characterization was based on the comparison of the droplet diameter of both aerosols and the presence of 54 HPHCs in each aerosol using the Health Canada Intense (HCI) machine-smoking protocol (Health Canada, 2000). In addition, the THS2.2 aerosol chemical composition was assessed under different climatic conditions (temperature and relative humidity) and with different machine-smoking regimens to simulate different use than described in the HCI machine-smoking protocol.

## 2. Materials and methods

### 2.1. Reference cigarette

The 3R4F reference cigarette was obtained from the University of Kentucky (Lexington, KY, USA; <http://www.ca.uky.edu/refcig/>).

### 2.2. THS2.2 tobacco stick

The tobacco stick was designed to be used with the THS2.2 holder (Smith et al., 2016). Its construction is shown in Fig. 1.

A tobacco stick is constructed by the sequential assembly of the following components:

1. Tobacco plug wrapped in a paper over-wrap.
2. Hollow acetate tube wrapped in a paper over-wrap.
3. Polymer-film filter, wrapped in a paper over-wrap.
4. Mouthpiece filter wrapped in a paper over-wrap.

All these elements are wrapped in an outer paper, and a tipping paper is added on the mouth end (Fig. 1).

Unlike CC, the THS tobacco stick does not burn when used, and thus its length remains unchanged. The tobacco plug is made of reconstituted cast leaf tobacco containing various tobacco types from different origins, as well as binders and humectants. The humectants were added to prevent the cast leaves becoming too brittle. Heating the humectants caused them to evaporate and recondense to form small droplets, generating a visible aerosol. Four different tobacco stick variants were used for aerosol characterization: two versions of the THS2.2 Regular (THS2.2 FR1 and THS2.2 D2) and two versions of the THS2.2 Menthol (THS2.2 FR1 M and THS2.2 D1 M). These four THS2.2 tobacco sticks contain flavor ingredients. THS2.2 FR1 M and THS2.2 D1 M contain natural *l*-menthol applied to a cellulose acetate yarn included in the polymer-film filter and to the inner liner paper included in the tobacco stick pack. FR1, D2 and D1 are different tobacco blends but the tobacco sticks were of the same design.

### 2.3. Cambridge glass-fiber filter pad, cigarette, and tobacco stick conditioning

The reference cigarette 3R4F and THS2.2 tobacco sticks were stored at  $5 \pm 3$  °C with uncontrolled humidity conditions in the original packaging, before conditioning. For the ISO/Health Canada conditioning, test articles were conditioned for at least 48 h at  $22 \pm 1$  °C and  $60 \pm 2\%$  relative humidity (RH), according to the International Organization for Standardization (ISO) method 3402 (International Organisation for Standardization, 2010a). Cambridge glass-fiber filter pads were conditioned under the same conditions.

Prior to the analyses performed in “tropical” and “desert” conditions, test articles were either conditioned for at least 48 h at  $30 \pm 1$  °C and  $75 \pm 2\%$  RH (“tropical” conditions), or at  $30 \pm 1$  °C and  $35 \pm 2\%$  RH (“desert” conditions). The corresponding Cambridge glass-fiber filter pads were conditioned under the same climatic conditions.

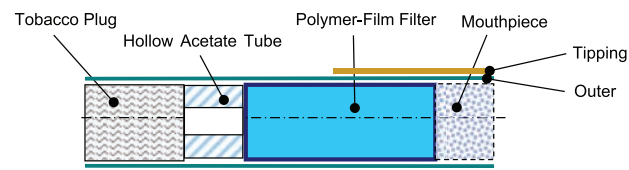


Fig. 1. THS2.2 tobacco stick.

#### 2.4. Generation of THS2.2 aerosol and mainstream smoke of 3R4F according to the HCI machine-smoking regimen

The mainstream aerosol of THS2.2 and the smoke of the reference cigarette 3R4F were generated on a Borgwaldt linear smoking machine type LM20X (Borgwaldt KC GmbH, Hamburg, Germany) for the determination of all analytes (except elements) according to the HCI machine-smoking regimen (Health Canada, 2000). For the elements, the mainstream aerosol of THS2.2 and the smoke of the reference cigarette 3R4F were generated from a Burghart rotary smoking machine type RMB 20 (Burghart Tabaktechnik GmbH, Wedel, Germany) with the same smoking regimen.

For the *in vitro* biological assays test battery (Ames, MLA, and NRU), the 3R4F mainstream smoke and THS2.2 aerosols were generated using a Burghart rotary smoking machine type RMB 20 (Burghart Tabaktechnik GmbH, Wedel, Germany) according to the Health Canada Intense (HCI) smoking regimen (Health Canada, 2000). The generated aerosol and smoke were trapped to analyze the aerosols. After trapping, the samples were analyzed and processed (section 2.7). Processing describes the conversion from a primary result, e.g., a peak area or counts to a value per cigarette or per stick, taking the number of accumulations, the trapping or extraction volume or dilution into account. For *in vitro* assessments, the aerosol was fractionated into two parts, TPM and GVP, during the same aerosol collection (except for the Ames assay, where only TPM was tested). At the end of the aerosol generation, the collected TPM aerosol fraction on the Cambridge glass-fiber filter pad was solubilized in dimethyl sulfoxide (DMSO), and the water-soluble GVP fraction was immobilized into an impinger of ice-cold  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ -free phosphate-buffered saline (PBS) solution.

The reference cigarette 3R4F was smoked to a butt length of 35 mm using a bell-shaped puff profile and 100% blocking of ventilation holes. THS2.2 Regular and Menthol tobacco sticks were 'smoked' using a bell-shaped puff profile to a defined puff count of 12 puffs. The limitation to 12 puffs is based on the fixed settings of the THS2.2 system, which is programmed to finish heating after a maximum period of 6 min, and the puff interval of the HCI regimen (30 s).

#### 2.5. Generation of THS 2.2 aerosol under different climatic conditions

THS2.2 aerosol was generated under different ambient temperature and RH conditions of  $22 \pm 2$  °C and  $60 \pm 5\%$  RH,  $30 \pm 2$  °C and  $75 \pm 5\%$  RH, and  $30 \pm 2$  °C and  $35 \pm 5\%$  RH to simulate "Mediterranean", "Tropical" and "Desert" climates, respectively (Table 1), using a linear smoking machine prototype SM405XR (Cerulean Molins PLC, Milton Keynes, UK) and the HCI machine-smoking regimen (Health Canada, 2000). The smoking machine was housed in a conditioned air cabinet (temperature range: 10 °C–35 °C; humidity range: 10%–80% RH) fitted with a Delta 335 air conditioning unit (Design Environmental Ltd, Ebbw Vale, UK). The atmosphere of the cabinet was constantly refreshed with conditioned air. The temperature and RH in the cabinet was

**Table 1**  
Climatic conditions.

Conditions	Temperature [°C]	Relative Humidity [% RH]
Mediterranean	$22 \pm 2$	$60 \pm 5\%$
Tropical	$30 \pm 2$	$75 \pm 5\%$
Desert	$30 \pm 2$	$35 \pm 5\%$

monitored using a TH1 datalogger (ELPRO-Buchs AG, Buchs, Switzerland).

#### 2.6. Generation of THS2.2 aerosol under alternative puffing regimens

THS2.2 aerosol was generated according to the alternative puffing regimen presented in Table 2. A Cerulean SM450RH smoking machine (Cerulean Molins PLC, Milton Keynes, UK) was used to generate aerosols for the analysis of all analytes except nitric oxide (NO) and nitrogen oxides ( $\text{NO}_x$ ). The NO and  $\text{NO}_x$  measurements were performed on a Borgwaldt linear smoking machine type LM20X (Borgwaldt KC GmbH, Hamburg, Germany). Since this smoking machine is limited to puffs of 100 ml, NO and  $\text{NO}_x$  measurement were not performed for the LR-3 regimen (Table 2).

The alternative puffing regimens (SR-1, SR-4, SR-5, SR-6 and LR-3) were selected according to human puffing behavior observed with THS2.2 users (Campelos et al., 2016).

#### 2.7. Chemical analyses

All analytes were determined using 15 separate aerosol and smoke generations. The 15 separate analyte groups and corresponding individual analytes are shown in Table 3.

The description of the analytical methods used to quantify the analytes in the THS2.2 aerosol and in the smoke of the 3R4F reference cigarette are presented in the supplementary material section.

#### 2.8. Physical measurements

The droplet size distribution measurements were conducted using a PIXE multistage cascade impactor (PIXE International Corp., Tallahassee, FL USA) using a sampling flow rate of 1 l/min. During this study, the PIXE cascade impactor was composed of nine impactor stages. For both 3R4F and THS2.2 Tobacco Sticks, the average mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD) were estimated from 10 replicate aerosol samples. The average GSD was determined as the square root of the average  $\text{GSD}^2$ . The test items were connected to the inlet of a programmable dual syringe pump (PDSP) (Burghart Messtechnik GmbH, Wedel, Germany). The outlet of the PDSP was connected to a glass T-junction that allowed aerosol transfer before it entered the PIXE cascade impactor. The outlet of the PIXE cascade impactor was connected to a pump (Vacuubrand GmbH + CO KG, Wertheim, Germany) (Fig. 2).

**Table 2**  
Smoking machine settings for the generation of THS2.2 aerosol generation under alternative puffing regimens.

Regimen	Puff volume [ml]	Puff duration [s]	Puff interval [s]	Number of puffs <sup>a</sup> [n]
ISO	35	2.0	60	6
SR-1	40	2.4	30	8
SR-5	80	2.4	30	8
HCI	55	2.0	30	12
SR-4	60	2.4	25	14
SR-6	80	2.4	25	14
LR-3	110	4.5	22	14

<sup>a</sup> The number of puffs results from puff intervals of the different smoking regimens and the fixed settings of the THS2.2 system, which is programmed to finish heating after a maximum period of 6 min and allows up to 14 puffs to be taken during that time.

**Table 3**

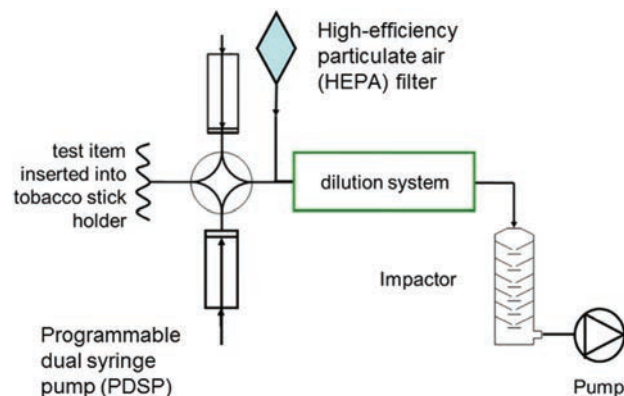
Analyte groups and corresponding individual analytes.

Analyte Group	Individual Analytes
ISO parameters and product-specific constituents	Total particulate matter (TPM), water, nicotine, nicotine-free dry particulate matter (NFDPM), carbon monoxide (CO), glycerol
Volatiles- and semi-volatiles	1,3-butadiene, isoprene, benzene, toluene, styrene, pyridine, quinoline, acrylonitrile
Carbonyls	acetaldehyde, acetone, acrolein, butyraldehyde, crotonaldehyde, formaldehyde, methyl ethyl ketone, propionaldehyde
Aromatic amines	1-aminonaphthalene, 2-aminonaphthalene, 3-aminobiphenyl, 4-aminobiphenyl, o-toluidine
Nitrogen oxides	nitric oxide (NO), oxides of nitrogen (NO <sub>x</sub> )
Hydrogen cyanide	hydrogen cyanide
Ammonia	Ammonia
Epoxides and vinyl chloride	ethylene oxide, propylene oxide, vinyl chloride
Tobacco-specific nitrosamines	<i>N</i> -nitrosoanabasine (NAB), <i>N</i> -nitrosoanatabine (NAT), <i>N</i> -nitrososnicotine (NNN), 4-( <i>N</i> -nitrosomethylamino)-1-(3-pyridyl)-1-butanone (NNK)
Phenols and acid derivatives	catechol, <i>o</i> -cresol, <i>m</i> -cresol, <i>p</i> -cresol, hydroquinone, phenol, resorcinol, acetamide, acrylamide
Polycyclic aromatic hydrocarbons	benzo[ <i>a</i> ]pyrene, benz[ <i>a</i> ]anthracene, dibenz[ <i>a,h</i> ]anthracene, pyrene
Nitrobenzene	Nitrobenzene
Elements (except mercury)	arsenic, cadmium, chromium, lead, nickel, selenium
Mercury	Mercury
Menthol	Menthol

The calculation of MMAD and GSD was done separately for each measurement. The following steps were performed:

1. Calculation of the net weight for each impactor stage of the cascade impactor:  $\Delta M_i = [\text{Weight loaded (g)}]_i - [\text{Weight empty (g)}]_i$  ( $i = 1, \dots, i$ ).
2. Calculation of the total mass by summing up all of the  $i$  net weights:  $TM = \sum \Delta M_i$ .
3. Calculation of the mass fraction for each stage:  $Mf_i = \Delta M_i/TM$  ( $i = 1, \dots, i$ ).
4. Normalization of the mass fraction for each stage by the width of successive cutoff diameter [ $\Delta D50\%$ ]: The cutoff diameter represents the smaller size that can be captured on the related stage, whereas the maximum size collected on that stage is related to the next larger cutoff diameter.  
 $nMf_i = Mf_i/[\Delta D50\%]_i$  ( $i = 1, \dots, i$ )  
 The values for the cutoff diameters [ $\Delta D50\%$ ] <sub>$i$</sub>  ( $i = 1, \dots, i$ ) are given in Table 4.
5. The calculation of the MMAD and GSD was performed using Igor Pro version 6.3.2.3 (WaveMetrics, Inc., Lake Oswego, OR, USA) using a lognormal mono-modal fitting distribution (Equation (1)) of the normalized mass fraction  $nMf_i$  vs. the respective mid-point diameter  $D_i$

$$f(d_d) = \frac{1}{d_d \ln(GSD) \sqrt{2\pi}} e^{-\frac{(\ln(d_d) - \ln(MMAD))^2}{2(\ln(GSD))^2}} \quad (1)$$

**Fig. 2.** Experimental setup for aerosol physical measurement.

where  $d_d$  is the size diameter in micrometers, GSD is the geometric standard deviation, MMAD is the mass median aerodynamic diameter, and  $f(d_d)$  represents the normalized mass fraction data.

The mass of each impactor stage was recorded prior to and after each aerosol collection. Subsequently, the mass fraction deposited on the different impactor stages was calculated, whereas the size bins were normalized by dividing the mass fractions with their respective widths. This process permitted the transformation of discrete data points into density functions that could be fitted with a continuous lognormal distribution function, from which both the MMAD and the GSD were calculated. The negative values were not replaced by zero. The lower boundaries (LB) and upper boundaries (UB) were calculated at the 95% confidence interval using the following equation:

$$LB = MMAD/GSD^2; \quad UB = MMAD \times GSD^2$$

## 2.9. *In vitro* toxicology

All *in vitro* studies were performed in full accordance with the principles of Good Laboratory Practice.

### 2.9.1. Neutral RED uptake (NRU) assay

The mouse embryonic fibroblast cell line Balb/c 3T3 (clone A31) was obtained from the European Collection of Authenticated Cell Cultures (Salisbury, UK), and was used to perform the NRU assay according to INVITTOX protocol 3a (INVITTOX, 1990), with some modifications (Borenfreund and Puermer, 1985). Sodium dodecyl

**Table 4**

Cutoff diameters and mid-point diameters for each stage of the PIXE cascade impactor.

Stage	Cutoff diameter ( $\mu\text{m}$ ) $\Delta D50\%$	Mid-point ( $\mu\text{m}$ ) $D_i$
7	16	16
6	8	12
5	4	6
4	2	3
3	1	1.5
2	0.5	0.75
1	0.25	0.375
L2	0.12	0.185
L1	0.06	0.09

sulfate was used as the positive control.

In brief, 20–28 h prior to aerosol fraction generation, cells were trypsinized and resuspended in Dulbecco's modified Eagle's medium supplemented with heat-inactivated fetal bovine serum (10% v/v) (Thermo Fisher Scientific, Waltham, MA, USA), 4 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Subsequently,  $4.75\text{--}5.25 \times 10^3$  viable cells were seeded in each well of a 96-well plate and cultured at 37 °C (5% CO<sub>2</sub> and 70% RH). Cells were exposed to eight concentrations of each test substance for  $23 \pm 1$  h in 96-well plates with six wells used per concentration. The exposure plates were sealed with a CO<sub>2</sub>-permeable plastic film to prevent potential carry-over of volatile substances. Following the exposure phase, the cell culture medium was replaced with cell culture medium containing neutral red dye at 50 µg/ml, and incubated at 37 °C (5% CO<sub>2</sub> and 70% RH) for an additional  $3 \pm 0.5$  h. Subsequently, the cells were washed with PBS, and the neutral red dye taken up by cells was extracted by the addition of destaining solution (ethanol, water, and acetic acid, mixed in a 50:49:1 ratio). The plates were mechanically shaken using a vibrating platform shaker (Titramax 1000, Heidolph Instruments, Schwabach, Germany) for 10 min at approximately 450 strokes/min. Neutral red absorbance was measured at 540 nm with a microplate reader (Safire 2, Tecan GmbH, Grödig, Austria). The measured absorbance for each concentration was normalized against the appropriate solvent control and converted to a percentage value. Cytotoxicity was expressed as  $1/EC_{50}$ , and expressed as a function of the mass of TPM trapped on the Cambridge glass-fiber filter pads (TPM basis) and on a per-mg nicotine basis. For consistency and to compare GVP fractions, data were calculated and expressed on a per-mg TPM basis and on a per-mg nicotine basis (Roemer et al., 2014). The  $EC_{50}$  endpoint measurement corresponds to the concentration of test substance for which a decrease of 50% in the uptake of the neutral red dye is observed, and was determined with the SAS<sup>®</sup> Enterprise guide<sup>®</sup> 4.3 (SAS 9.2) software program (SAS, Cary, NC, USA).

The relationship between the concentration of the substance and the decrease in the uptake of neutral red dye has a sigmoid shape and is described by the Hill function, which is a four parameter non-linear function. Statistical analysis was performed using SAS. Unless mentioned otherwise in the text, all reagents were obtained from Sigma–Aldrich (St. Louis, MO, USA).

### 2.9.2. Ames assay

Mutagenic activity was evaluated by using the *Salmonella typhimurium* tester strains TA98, TA100, TA102, TA1535, and TA1537 with and without an S9 enzymatic metabolizing fraction, by following a pre-incubation method (Maron and Ames, 1983) and the OECD 471 test guideline (Organisation for Economic Co-operation and Development, 1997). The S9 enzymatic metabolizing fraction was obtained from Aroclor 1254-induced male Sprague–Dawley rat liver (Moltox, NC, USA). The TPM mainstream smoke fraction from the 3R4F reference cigarette was generated and tested in parallel with the THS2.2 aerosol fraction. The strains were grown overnight in a shaking incubator at 37 °C for approximately 10 h in Oxoid Nutrient Broth No. 2 (Fisher Scientific, Reinach, Switzerland). To determine mutagenic activity, seven different concentrations of THS2.2 TPM diluted in DMSO were tested. The bacteria (approximately  $1 \times 10^9$  in 100 µl) were combined with 50 µl of either the test item, the solvent, or the positive control, and 500 µl of the cofactor buffer (pH 7.4) supplemented with S9 as appropriate and pre-incubated at 37 °C for 20 min prior to adding 2 ml of histidine (50 µM final concentration) supplemented soft top agar and plating the entire mixture onto histidine-deficient 90 mm minimal glucose agar base plates for 2 days at 37 °C. Revertant colonies were counted using an automatic colony counter (Sorcerer, Perceptive Instruments, Bury Saint Edmunds,

UK). All experiments were performed in triplicate. Toxicity was detected as either a reduction in the number of histidine revertants or as a thinning of the auxotrophic background lawn. The mutagens used as positive controls, i.e. substances known to induce a mutagenic response to demonstrate the assay is working efficiently, in experiments without the S9 mix were 4-nitrophenylenediamine (10 µg/plate) for TA98 and TA100, sodium azide (1.25 µg/plate) for TA1535 and TA1537, and cumene hydroperoxide (3 µg/plate) for TA102. In the experiments that included the S9 fraction, benzo[a]pyrene (1 µg/plate) was used for TA98, and 2-aminoanthracene (2.5 µg/plate) was used for TA100, TA102, TA1535, and TA1537. DMSO (50 µl/plate) served as the solvent control. All positive control chemicals were obtained from either Sigma–Aldrich (St. Louis, MO, USA) or Moltox (Boone, NC, USA). The biological activity after 1 mg of TPM exposure is reported as a means to permit a rapid assessment of the impact of the 3R4F aerosol vs. the THS2.2 aerosol. One milligram was the maximum dose tested of the 3R4F aerosol (for toxicity reasons) and thus comparison at 5 mg or higher concentrations was not technically possible.

### 2.9.3. Mouse lymphoma assay (MLA)

The L5178Y *tk*<sup>±</sup> cell line (sub-clone 3.7.2C (IVGT), Public Health England, UK) was used in the MLA. Spontaneously-occurring *tk*<sup>-/-</sup> mutants were purged from working stocks using methotrexate to select against *tk*-deficient cells and thymidine, hypoxanthine, and guanine to ensure optimal growth of *tk*-proficient cells as previously described (Chen and Moore, 2004). Cells were maintained in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with heat-inactivated horse serum (10% v/v) (Thermo Fisher Scientific, Waltham, MA, USA), penicillin (200 U/ml), streptomycin (200 µg/ml), L-glutamine (2 mM), sodium pyruvate (1 mM), and pluronic acid F68 (0.1% v/v) (Sigma–Aldrich, St. Louis, MO, USA). Short-term treatments (4 h) were carried out in reduced serum medium (3% v/v), while 10% v/v-containing medium was utilized for longer-term exposures (24 h). Cloning was carried out in 20% v/v-containing serum medium in the absence of pluronic acid F68. Aroclor 1254-induced male Sprague–Dawley rat liver S9 in 0.15 M KCl (Moltox, USA), in combination with a cofactor mix of glucose-6-phosphate and nicotinamide adenine dinucleotide phosphate (both from Roche Applied Science, Basel, Switzerland), were used as the exogenous metabolic activation system in the assay. The final concentration of S9 in cell cultures was 0.95 mg protein/ml (2% v/v). The controls used were methyl methanesulfonate (15 and 20 µg/ml) for the 4 h S9- test arm, methyl methanesulfonate (5 and 7.5 µg/ml) for the 24 h S9- arm, and 7,12-dimethylbenz[a]anthracene (1 and 1.5 µg/ml) for the 4 h S9+ arm.

The microwell version of the MLA was performed according to the OECD TG 490 guideline (Organisation for Economic Co-operation and Development, 2015). Briefly, on two independent test occasions, L5178Y cells in single replicate cultures were seeded at a density of  $5 \times 10^5$  or  $2 \times 10^5$  cells/ml (at least  $6 \times 10^6$  cells), and exposed to 14 concentrations of TPM and GVP derived from THS2.2 and the 3R4F reference cigarette for 4 h in the presence (+S9) and absence (-S9) of metabolic activation and 24 h in the absence of metabolic activation (-S9) treatment conditions, respectively. For each treatment condition, cells were exposed for 4 or 24 h at 37 °C in the presence of 5% CO<sub>2</sub> and RH  $\geq$  65%. Following treatment, cells were washed and sub-cultured at a maximum density of  $2 \times 10^5$  cells/ml (at most  $6 \times 10^6$  cells) for two further days to allow phenotypic expression of the *tk* gene prior to mutant selection. Cells at 8 cells/ml or  $1 \times 10^4$  cells/ml were then distributed into 96-microwell plates (200 µl per well) to determine final levels of TPM- and GVP-induced cytotoxicity and mutagenicity, respectively. Cytotoxicity was determined from the relative total growth (RTG) of

the cell cultures following treatment and sub-culture periods in non-selective growth medium (typically 10–11 days). TPM- and GVP-treated cell cultures which underwent excessive cytotoxicity were discarded through the assay procedure as mutagenicity data derived from these cells are difficult to interpret because of their questionable biological relevance. *Tk* mutants were detected following culture in trifluorothymidine (TFT)-containing growth medium (Sigma–Aldrich, St. Louis, MO, USA) for typically 14 days. Mutant colonies were enumerated visually; colonies with a size less than a quarter of the microwell's surface area were defined as small colonies, while ones covering more than a quarter of the microwell's surface area were defined as large colonies. Mutation frequencies were calculated according to published method (Clements, 2000). The controls used were methyl methanesulfonate (15 and 20 µg/ml) for the 4 h S9- test arm, methyl methanesulfonate (5 and 7.5 µg/ml) for the 24 h S9- arm and finally 7,12-dimethylbenz[*a*]anthracene (1 and 1.5 µg/ml) for the 4 h S9+ arm.

The data generated from solvent-treated and positive controls in each treatment condition on the separate test occasions were evaluated for acceptability according to OECD TG 490 guideline (Organisation for Economic Co-operation and Development, 2015) and the laboratory's historical control database. Furthermore, a response to an aerosol fraction was considered positive, i.e. mutagenic, in the MLA if there was a concentration-related increase in mutation frequency (MF) with a corresponding RTG not lower than 10%, and if an MF exceeded the sum of the microwell global evaluation factor (GEF) of 126 plus the mean MF of the solvent-treated controls (Moore et al., 2006, 2007). The microwell GEF of 126 mutants per 10<sup>6</sup> viable cells was previously defined as the mean of the negative/solvent control MF distribution plus one standard deviation in a multi-laboratory microwell MLA study (Moore et al., 2006). Mutagenic potencies were evaluated using the lowest observed genotoxic effect level (LOGEL) (Guo et al., 2015). A LOGEL in this study was defined as the lowest concentration of TPM or GVP (expressed on a per-mg nicotine basis) tested that induced a mutagenic response which exceeded the GEF.

## 2.10. Statistical analyses

The results of the analyte quantifications are expressed in two different ways: On a per-Tobacco Stick/cigarette basis, and on a per-mg nicotine basis. For all analyte, the number of values (N), the arithmetic mean (M), and the confidence interval of the mean at 95% (CI<sub>95%</sub>) are given. For groups including at least one measured value below the limit of quantification (LOQ) of the analytical method, only the median or the LOQ was given, depending on whether the median was above or below the LOQ. The mean on a per-mg nicotine basis and the respective confidence interval of the mean were calculated as follows:

$$M = \frac{M(A)}{M(B)}$$

where A denotes the value of an analyte on a cigarette basis, and B denotes the mean value for nicotine on a cigarette basis.

and

$$\pm CI_{95\%} \left( \frac{M(A)}{M(B)} \right) = \pm t(0.975, N - 1) * \frac{\frac{M(A)}{M(B)} * \sqrt{\frac{S_{M(A)}^2}{M(A)^2} + \frac{S_{M(B)}^2}{M(B)^2}}}{\sqrt{N}}$$

where A denotes the mean of an analyte on a cigarette basis, B denotes the mean for nicotine on a cigarette basis, S is the standard deviation, N is the number of measurements, t(p,df) is the

percentile of Student's distribution, and df is degrees of freedom.

For the NRU assay, the cytotoxicity (1/EC<sub>50</sub>) of the 3R4F reference cigarette and the THS2.2 FR1 and THS2.2 FR1 M Tobacco Sticks, expressed on a per-TPM basis and on a per-mg nicotine basis, was compared using one-way analysis of variance followed by Dunnett's multiple comparison procedure, with the 3R4F reference cigarette as the reference group. This statistical approach was used because 2 THS2.2 test items were compared with 3R4F within one study. However, the cytotoxicity of the THS2.2 D2 and THS2.2 D1 M tobacco sticks was compared against the 3R4F reference cigarette using Student's *t*-test. In this case the *t*-test was used because 2 studies were performed, each with one test item and the reference cigarette 3R4F.

## 3. Results

### 3.1. Chemical composition of the THS2.2 aerosol, and comparison with the mainstream smoke from the 3R4F reference cigarette

Two versions of the THS2.2 Regular (THS2.2 FR1 and THS2.2 D2) and two versions of the THS2.2 Menthol (THS2.2 FR1 M and THS2.2 D1 M) were compared with the 3R4F reference cigarette under HCl machine-smoking conditions. Fifty-nine analytes (60 for menthol products) were determined, covering various chemical classes present in different aerosol phases (Table 5 and Table 6). Fifteen analytes in THS2.2 FR1 (benzo[*a*]pyrene, 3- and 4-aminobiphenyl, quinoline, NAT, pyrene, nitrobenzene, vinyl chloride, dibenz[*a,h*]anthracene, and all the elements except mercury) and 11 analytes in THS2.2 D2 (2-aminonaphthalene, 3- and 4-aminobiphenyl, vinyl chloride, dibenz[*a,h*]anthracene, and all the elements except mercury) were below the LOQ. In THS2.2 FR1 M, the yields of 11 analytes (2-aminonaphthalene, 4-aminobiphenyl, quinoline, NAT, all the elements except mercury and selenium, vinyl chloride, and dibenz[*a,h*]anthracene and 8 analytes in the THS2.2 D1 M (2-aminonaphthalene, 3-aminobiphenyl, arsenic, cadmium, lead, selenium, vinyl chloride, and dibenz[*a,h*]anthracene) were below the LOQ. The 54 HPHCs which were targets for reduction (Section 1) were lower in the THS2.2 aerosol than in the 3R4F smoke. The yields of analytes expressed on a per-mg nicotine basis are presented in Table A and in Table B of the supplementary material.

The pie charts in Fig. 3 illustrates the differences in TPM composition between the 3R4F and the THS2.2 FR1. While the THS2.2 FR1 delivered about the same TPM yield as the 3R4F, the THS2.2 aerosol composition was qualitatively and quantitatively different from that of 3R4F. The quantities of water and glycerol relative to total TPM were considerably higher for the THS2.2, whereas the amount of nicotine was approximately 30% lower for the THS2.2. Therefore, the relative yields of the 'other' aerosol constituents were noticeably lower in the THS2.2 TPM. This is also evident when comparing the color of the Cambridge glass-fiber filter pads after collection of the same amount of aerosol mass and TPM from THS2.2 tobacco sticks and 3R4F cigarettes, respectively (Fig. 3). Previous *heat-not-burn* products such as Premier (deBethizy et al., 1990) and Eclipse (Borgerding et al., 1998) also produce TPM compositions that contained mainly water and glycerol. Visually, the collected aerosol from THS2.2 on a Cambridge glass-fiber filter pad appears similar to that seen for both Premier and Eclipse.

### 3.2. Chemistry of the THS2.2 aerosol generated under different climatic conditions

The three climatic conditions, "Mediterranean", "desert", and "tropical", were selected according to ICH (International Council

**Table 5**

Analyte yields from THS2.2 FR1, THS2.2 FR1 M, and 3R4F obtained under HCl machine-smoking conditions and expressed on a per-cigarette/tobacco stick basis.

Parameter	Unit	THS2.2 FR1		THS2.2 FR1 M		3R4F	
		Mean ± CI <sub>95%</sub>	N	Mean ± CI <sub>95%</sub>	N	Mean ± CI <sub>95%</sub>	N
TPM	mg/stick	48.2 ± 2.4	4	43.5 ± 1.5	4	49.0 ± 4.8	4
Water	mg/stick	36.5 ± 3.1	4	29.7 ± 3.6	4	15.8 ± 2.9	4
Nicotine	mg/stick	1.32 ± 0.16	4	1.21 ± 0.09	4	1.89 ± 0.16	4
NFDPM	mg/stick	10.3 ± 0.9	4	12.6 ± 2.2	4	31.2 ± 1.8	4
Carbon monoxide	mg/stick	0.531 ± 0.068	4	0.594 ± 0.110	4	32.8 ± 2.4	4
Benzo[a]pyrene	ng/stick	<1.00	4	1.29 ± 0.10	4	14.2 ± 0.3	4
Puff Count	/stick	12 ± 0	4	12 ± 0	4	10.6 ± 0.4	4
Menthol	mg/stick	n.a.		2.62 ± 0.1	4	n.a.	
Glycerin	mg/stick	4.63 ± 0.83	4	3.94 ± 0.87	4	2.42 ± 0.14	4
1-aminonaphthalene	ng/stick	0.077	4	0.086	4	20.8 ± 1.3	4
2-aminonaphthalene	ng/stick	0.046 ± 0.008	4	<0.035	4	11.0 ± 0.6	4
3-aminobiphenyl	ng/stick	<0.032	4	0.032	4	3.77 ± 0.47	4
4-aminobiphenyl	ng/stick	<0.051	4	<0.051	4	3.26 ± 0.12	4
Acetaldehyde	µg/stick	219 ± 31	4	205 ± 12	4	1555 ± 184	4
Acetone	µg/stick	40.7 ± 6.2	4	39.4 ± 2.3	4	736 ± 129	4
Acrolein	µg/stick	11.30 ± 2.36	4	9.15 ± 0.43	4	154 ± 20	4
Butyraldehyde	µg/stick	26.1 ± 2.3	4	26.7 ± 2	4	88.4 ± 10.7	4
Crotonaldehyde	µg/stick	4.14 ± 0.23	4	3.24 ± 0.21	4	68.8 ± 14.4	4
Formaldehyde	µg/stick	5.53 ± 0.69	4	4.55 ± 0.25	4	56.5 ± 12.1	4
Methyl ethyl ketone	µg/stick	7.18 ± 1.19	4	6.93 ± 0.64	4	187 ± 30	4
Propionaldehyde	µg/stick	14.5 ± 2.4	4	13.9 ± 0.7	4	125 ± 16	4
Acrylonitrile	µg/stick	0.258 ± 0.041	4	0.220 ± 0.014	4	31.9 ± 1.8	4
1,3-butadiene	µg/stick	0.294 ± 0.042	4	0.265 ± 0.024	4	63.8 ± 3.5	4
Benzene	µg/stick	0.649 ± 0.074	4	0.640 ± 0.040	4	97.6 ± 4.7	4
Isoprene	µg/stick	2.35 ± 0.39	4	2.11 ± 0.18	4	798 ± 49	4
Pyridine	µg/stick	7.54 ± 0.26	4	7.21 ± 0.25	4	36.1 ± 2.2	4
Quinoline	µg/stick	<0.012	4	<0.012	4	0.513 ± 0.023	4
Styrene	µg/stick	0.608 ± 0.058	4	0.561 ± 0.033	4	24.5 ± 1.2	4
Toluene	µg/stick	2.59 ± 0.43	4	2.39 ± 0.16	4	188 ± 11	4
Catechol	µg/stick	16.3 ± 1.5	4	17.1 ± 1.1	4	91.4 ± 5.6	4
<i>o</i> -cresol	µg/stick	0.069 ± 0.008	4	0.095 ± 0.025	4	4.47 ± 0.16	4
<i>m</i> -cresol	µg/stick	0.029 ± 0.004	4	0.033 ± 0.006	4	3.03 ± 0.08	4
<i>p</i> -cresol	µg/stick	0.072 ± 0.008	4	0.083 ± 0.010	4	9.17 ± 0.44	4
Hydroquinone	µg/stick	8.10 ± 0.48	4	8.98 ± 1.02	4	83.1 ± 5.5	4
Phenol	µg/stick	1.16 ± 0.12	4	1.60 ± 0.4	4	13.6 ± 0.9	4
Resorcinol	µg/stick	0.041 ± 0.003	4	0.048 ± 0.004	4	1.85 ± 0.08	4
NAB	ng/stick	<3.15	4	<3.15	4	33.7 ± 8.5	4
NAT	ng/stick	20.5 ± 0.5	4	19.7 ± 3.6	4	318 ± 74	4
NNK	ng/stick	6.7 ± 0.6	4	5.9 ± 0.4	4	266 ± 15	4
NNN	ng/stick	17.2 ± 1.25	4	13.7 ± 1.21	4	309 ± 41	4
Ammonia	µg/stick	14.2 ± 1.1	4	13.8 ± 0.7	4	39.3 ± 3.2	4
Hydrogen cyanide	µg/stick	4.81 ± 0.35	4	5.14 ± 0.70	4	493 ± 78	4
Nitric oxide	µg/stick	16.8 ± 2.3	4	12.3 ± 1.7	4	491 ± 38	4
Nitrogen oxides	µg/stick	17.3 ± 2.6	4	12.6 ± 1.7	4	537 ± 43	4
Arsenic	ng/stick	<1.13	4	<1.13	4	8.51 ± 0.34	4
Cadmium	ng/stick	<0.350	4	<0.350	4	161 ± 4	4
Chromium	ng/stick	<0.55	4	<0.55	4	<0.55	4
Lead	ng/stick	<3.35	4	<3.35	4	37.0 ± 0.7	4
Mercury	ng/stick	1.17 ± 0.05	4	1.34 ± 0.18	4	4.80 ± 0.13	4
Nickel	ng/stick	<0.55	4	<0.55	4	<0.55	4
Selenium	ng/stick	<0.550	2	0.780	4	1.62 ± 0.32	4
Pyrene	ng/stick	<5.00	4	9.06 ± 0.68	4	87.3 ± 2.5	4
<i>o</i> -toluidine	ng/stick	1.260 ± 0.187	4	0.777 ± 0.287	4	85.5 ± 2.7	4
Acetamide	µg/stick	4.02 ± 0.18	4	4.30 ± 0.24	4	13.9 ± 0.5	4
Acrylamide	µg/stick	1.73 ± 0.12	4	1.91 ± 0.16	4	4.8 ± 0.3	4
Ethylene oxide	µg/stick	0.201 ± 0.014	4	0.202 ± 0.013	4	29.4 ± 2.0	4
Nitrobenzene	ng/stick	<0.188	4	0.335 ± 0.164	4	8.62 ± 1.10	4
Propylene oxide	µg/stick	0.148 ± 0.018	4	0.149 ± 0.017	4	1.32 ± 0.12	4
Vinyl chloride	ng/stick	<3.54	4	<3.54	4	96.7 ± 2.0	4
Benz[a]anthracene	ng/stick	1.45 ± 0.14	4	2.49 ± 0.17	4	28.0 ± 0.6	4
Dibenz[a,h]anthracene	ng/stick	<0.100	4	<0.100	4	1.70 ± 0.11	4

N is the number of determinations, CI is the confidence interval of the mean, n.a.: not analyzed.

&lt;: median lower than the limit of quantitation, in this case LOQ is given.

If at least one value is below the LOQ, the median is given and the CI is not mentioned.

TPM: Total particulate matter, NFDPM: Nicotine-free dry particulate matter, NAB: *N*-nitrosoanabasine, NAT: *N*-nitrosoanatabine, NNK: 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone, NNN: *N*-nitrososornicotine.

**Table 6**

Analytes yields from THS2.2 D2, THS2.2 D1 M, and 3R4F obtained under HCl machine-smoking conditions and expressed on a per-cigarette/tobacco stick basis.

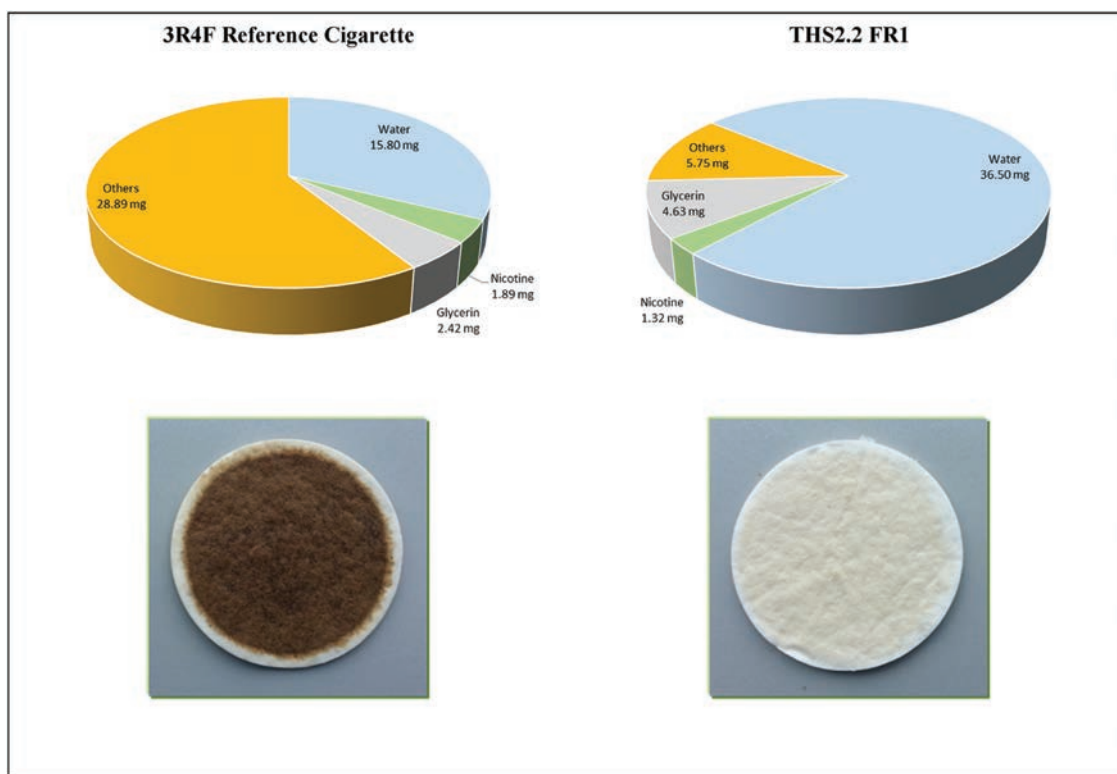
Parameter	Unit	THS2.2 D2		THS2.2 D1 M		3R4F	
		mean $\pm$ CI <sub>95%</sub>	N	mean $\pm$ CI <sub>95%</sub>	N	mean $\pm$ CI <sub>95%</sub>	N
TPM	mg/stick	54.1 $\pm$ 2.4	4	53.8 $\pm$ 3.6	4	46.3 $\pm$ 2.9	4
Water	mg/stick	39.4 $\pm$ 4.6	4	39.1 $\pm$ 3.6	4	13.3 $\pm$ 1.6	4
Nicotine	mg/stick	1.26 $\pm$ 0.24	4	1.32 $\pm$ 0.11	4	2.09 $\pm$ 0.14	4
NFDPM	mg/stick	13.4 $\pm$ 2.8	4	13.4 $\pm$ 0.6	4	30.9 $\pm$ 1.9	4
Carbon monoxide	mg/stick	0.598 $\pm$ 0.072	4	0.620 $\pm$ 0	4	30.7 $\pm$ 3.0	4
Benzo[a]pyrene	ng/stick	1.19 $\pm$ 0.08	4	1.08 $\pm$ 0.09	3	13.7 $\pm$ 0.8	4
Puff Count	/stick	12 $\pm$ 0	4	12 $\pm$ 0	4	10.7 $\pm$ 0.7	4
Menthol	mg/stick	n.a.		2.98 $\pm$ 0.21	4	n.a.	
Glycerin	mg/stick	4.1 $\pm$ 1.07	4	4.59 $\pm$ 0.47	4	2.39 $\pm$ 0.15	4
1-aminonaphthalene	ng/stick	0.063 $\pm$ 0.006	4	<0.061	4	19.7 $\pm$ 1.6	4
2-aminonaphthalene	ng/stick	<0.035	4	<0.035	4	14.8 $\pm$ 1.9	4
3-aminobiphenyl	ng/stick	<0.013	4	<0.013	4	3.90 $\pm$ 0.42	4
4-aminobiphenyl	ng/stick	<0.021	4	n.a.		3.13 $\pm$ 0.60	4
Acetaldehyde	$\mu$ g/stick	213 $\pm$ 19	4	220 $\pm$ 22	4	1589 $\pm$ 76	4
Acetone	$\mu$ g/stick	33.8 $\pm$ 6.4	4	42.6 $\pm$ 8.1	4	729 $\pm$ 36	4
Acrolein	$\mu$ g/stick	9.44 $\pm$ 0.87	4	10.91 $\pm$ 2.98	4	193 $\pm$ 21	4
Butyraldehyde	$\mu$ g/stick	25.3 $\pm$ 2.7	4	26.4 $\pm$ 0.9	4	103.9 $\pm$ 8.3	4
Crotonaldehyde	$\mu$ g/stick	3.75 $\pm$ 0.34	4	4.15 $\pm$ 0.64	4	92.1 $\pm$ 13.2	4
Formaldehyde	$\mu$ g/stick	5.22 $\pm$ 0.24	4	6.19 $\pm$ 2.00	4	68.7 $\pm$ 7.8	4
Methyl ethyl ketone	$\mu$ g/stick	7.94 $\pm$ 0.75	4	10.19 $\pm$ 2.23	4	241 $\pm$ 16	4
Propionaldehyde	$\mu$ g/stick	13.6 $\pm$ 1.5	4	15.9 $\pm$ 2.2	4	147 $\pm$ 8	4
Acrylonitrile	$\mu$ g/stick	0.186 $\pm$ 0.028	4	0.196 $\pm$ 0.016	4	31.6 $\pm$ 2.3	4
1,3-butadiene	$\mu$ g/stick	0.319 $\pm$ 0.073	4	0.411 $\pm$ 0.093	4	91.8 $\pm$ 11.0	4
Benzene	$\mu$ g/stick	0.575 $\pm$ 0.072	4	0.628 $\pm$ 0.073	4	100.4 $\pm$ 2.8	4
Isoprene	$\mu$ g/stick	2.44 $\pm$ 0.50	4	2.63 $\pm$ 0.60	4	869 $\pm$ 50	4
Pyridine	$\mu$ g/stick	9.38 $\pm$ 0.95	4	10.08 $\pm$ 0.46	4	51.8 $\pm$ 7.5	4
Quinoline	$\mu$ g/stick	0.014 $\pm$ 0.002	4	0.010 $\pm$ 0.003	4	0.390 $\pm$ 0.101	4
Styrene	$\mu$ g/stick	0.672 $\pm$ 0.063	4	0.632 $\pm$ 0.079	4	28.9 $\pm$ 2.2	4
Toluene	$\mu$ g/stick	1.61 $\pm$ 0.17	4	1.67 $\pm$ 0.37	4	198.8 $\pm$ 10.9	4
Catechol	$\mu$ g/stick	16.4 $\pm$ 0.6	4	12.8 $\pm$ 1.3	4	88.7 $\pm$ 2.6	4
<i>o</i> -cresol	$\mu$ g/stick	0.105 $\pm$ 0.017	4	0.059 $\pm$ 0.007	4	4.86 $\pm$ 0.50	4
<i>m</i> -cresol	$\mu$ g/stick	0.042 $\pm$ 0.006	4	0.032 $\pm$ 0.005	4	3.71 $\pm$ 0.34	4
<i>p</i> -cresol	$\mu$ g/stick	0.073 $\pm$ 0.009	4	0.042 $\pm$ 0.007	4	8.50 $\pm$ 0.78	4
Hydroquinone	$\mu$ g/stick	7.86 $\pm$ 0.63	4	6.21 $\pm$ 0.86	4	84.1 $\pm$ 3.3	4
Phenol	$\mu$ g/stick	1.51 $\pm$ 0.23	4	1.00 $\pm$ 0.17	4	13.2 $\pm$ 0.9	4
Resorcinol	$\mu$ g/stick	0.055 $\pm$ 0.013	4	0.036 $\pm$ 0.005	4	1.95 $\pm$ 0.55	4
NAB	ng/stick	3.52 $\pm$ 0.48	4	3.27 $\pm$ 0.15	4	34.1 $\pm$ 3.0	4
NAT	ng/stick	22.3 $\pm$ 1.6	4	18.6 $\pm$ 2.9	4	300 $\pm$ 53	4
NNK	ng/stick	10.1 $\pm$ 0.4	4	7.9 $\pm$ 1.1	4	257 $\pm$ 39	4
NNN	ng/stick	10.3 $\pm$ 0.4	4	7.7 $\pm$ 1.0	4	268 $\pm$ 50	4
Ammonia	$\mu$ g/stick	15.6 $\pm$ 1.1	4	13.9 $\pm$ 1.1	4	39.2 $\pm$ 4.1	4
Hydrogen cyanide	$\mu$ g/stick	3.78 $\pm$ 0.44	4	5.57 $\pm$ 0.35	4	451 $\pm$ 47	4
Nitric oxide	$\mu$ g/stick	21.0 $\pm$ 8.1	3	18.4 $\pm$ 3.6	4	501 $\pm$ 33	3
Nitrogen oxides	$\mu$ g/stick	22.6 $\pm$ 8.8	3	19.4 $\pm$ 4.0	4	541 $\pm$ 74	3
Arsenic	ng/stick	<1.13	4	<1.13	4	6.56 $\pm$ 0.46	4
Cadmium	ng/stick	<0.350	4	<0.350	4	122 $\pm$ 12	4
Chromium	ng/stick	<0.17	4	0.44	4	2.70 <sup>a</sup>	2
Lead	ng/stick	<3.35	4	<3.35	4	25.1 $\pm$ 2.1	4
Mercury	ng/stick	1.02 $\pm$ 0.05	4	1.12 $\pm$ 0.19	4	4.17 $\pm$ 0.74	4
Nickel	ng/stick	<0.55	4	0.88	4	1.30 <sup>a</sup>	2
Selenium	ng/stick	<0.550	4	<0.550	4	1.43 $\pm$ 0.15	4
Pyrene	ng/stick	7.93 $\pm$ 0.78	4	7.71 $\pm$ 0.63	3	87.3 $\pm$ 4.1	4
<i>o</i> -toluidine	ng/stick	1.204 $\pm$ 0.149	4	0.868 $\pm$ 0.087	4	90.5 $\pm$ 3.1	4
Acetamide	$\mu$ g/stick	4.13 $\pm$ 0.21	4	3.43 $\pm$ 0.17	4	13.7 $\pm$ 0.7	4
Acrylamide	$\mu$ g/stick	2.27 $\pm$ 0.28	4	1.90 $\pm$ 0.12	4	5.3 $\pm$ 0.4	4
Ethylene oxide	$\mu$ g/stick	0.314 $\pm$ 0.011	4	0.273 $\pm$ 0.036	4	34.2 $\pm$ 3.6	4
Nitrobenzene	ng/stick	0.092 $\pm$ 0.008	4	0.155 $\pm$ 0.004	8	0.55 $\pm$ 0.04	4
Propylene oxide	$\mu$ g/stick	0.175 $\pm$ 0.03	4	0.14 $\pm$ 0.019	4	1.72 $\pm$ 0.16	4
Vinyl chloride	ng/stick	<3.47	4	<3.47	4	95.3 $\pm$ 12.3	4
Benz[a]anthracene	ng/stick	2.58 $\pm$ 0.17	4	2.50 $\pm$ 0.06	3	26.6 $\pm$ 1.7	4
Dibenz[a,h]anthracene	ng/stick	<0.100	4	<0.100	4	1.79 $\pm$ 0.14	4

N is the number of determinations, CI is the confidence interval of the mean, n.a.: not analyzed.

&lt;: median lower than the limit of quantitation, in this case LOQ is given.

If at least one value is below the LOQ, the median is given and the CI is not mentioned.

TPM: Total particulate matter, NFDPM: Nicotine-free dry particulate matter, NAB: *N*-nitrosoanabasine, NAT: *N*-nitrosoanatabine, NNK: 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone, NNN: *N*-nitrosomethylamine.<sup>a</sup> CI not calculated.



**Fig. 3.** Cigarette total particulate matter (TPM) compared with THS2.2 aerosol composition generated according to the HCl machine-smoking conditions for measuring emissions. The photographs of the Cambridge glass-fiber filter pads after the collection of cigarette smoke (left) and THS2.2 aerosol (right) are also shown.

for Harmonisation, 2003) and WHO (World Health Organisation Technical Report, 2015) guidelines for stability testing. Since the generation of HPHCs either through pyrosynthesis or distillation should be enhanced when increasing the temperature, lower temperatures were not considered for this comparison. The impact of temperature and relative humidity (RH) on the deliveries of the different analytes in the THS2.2 FR1 aerosol is presented in Table 7. The data expressed on a per-mg nicotine basis are presented in Table C of the supplementary material.

### 3.3. Aerosol chemistry of the THS2.2 FR1 generated with different machine-smoking regimens

The range of machine-smoking regimens used during this test was quite broad, and ranged from a total puff volume of 210 ml for the ISO conditions to 1120 ml for the LR-3 regimen (see Table 2). Despite these substantial differences in puff volumes, benzo[a]pyrene, 1-aminonaphthalene, 2-aminonaphthalene, 3-aminobiphenyl, 4-aminobiphenyl, dibenz[a,h]anthracene, and vinyl chloride remained below the LOQ for all machine-smoking regimens. For the other analytes, ISO and SR-1 delivered the lowest yields of HPHCs, while machine-smoking regimens SR-4, SR-6, and LR-3 delivered the highest HPHC yields. A summary of the obtained results is presented in Table 8 together with the yields obtained for the 3R4F reference cigarette smoked in the HCl conditions. All the individual results are presented in Tables D and E of the supplementary material.

The LR-3 smoking regimen could not be performed with the Borgwaldt linear smoking machine type used to quantify NO and NO<sub>x</sub>. Therefore, this value was not reported. For TPM and carbonyls,

the breakthrough was specifically tested with the most intense smoking regimens to ensure that losses were negligible and did not affect the accuracy of the measurements.

### 3.4. Physical measurement of the aerosol

Ten series of measurement were performed on the 3R4F reference cigarette and on the THS2.2 FR1. All the calculated MMAD, GSD, and boundary values are presented in Table 9. The upper boundaries (UB) were below 2.5 μm. Consequently, the smoke generated from the 3R4F and the aerosol generated from the THS2.2 were respirable for all replicates with a margin of error of 5% and, according to Hinds (Hinds, 1999), more than 85% of the aerosol droplets could reach the alveoli in the lung.

### 3.5. Neutral red uptake (NRU) assay

Two versions of the THS2.2 Regular (THS2.2 FR1 and THS2.2 D2) and two versions of the THS2.2 Menthol (THS2.2 FR1 M and THS2.2 D1 M) were tested and compared with the 3R4F under HCl machine-smoking conditions in the NRU assay in independent studies. The cytotoxic activity of both the TPM and GVP fractions from the THS2.2 and the 3R4F reference cigarette was determined. A clear concentration-dependent decrease in the number of viable cells was observed for the aerosol fractions generated from the THS2.2 Regular (FR1 and D2) and THS2.2. Menthol (FR1 and D1) and the smoke fractions generated from the 3R4F reference cigarette. The cytotoxicity levels induced by both products covered a range spanning from no or low to high cytotoxicity. The 1/EC<sub>50</sub> values (expressed on a per-mg TPM basis and on a per-mg nicotine

**Table 7**

Yields on tobacco stick basis from THS2.2 FR1 obtained under three climatic conditions.

Parameter	Unit	Mediterranean 22 °C, 60% RH		Desert 30 °C, 35% RH		Tropical 30 °C, 75% RH		Range <sup>a</sup>
		mean ± CI <sub>95%</sub>	N	mean ± CI <sub>95%</sub>	N	mean ± CI <sub>95%</sub>	N	
TPM	mg/stick	47.1 ± 2.0	5	27.9 ± 1.4	5	65.1 ± 4.5	5	37.2
Water	mg/stick	33.5 ± 1.5	5	15.1 ± 0.4	5	50.6 ± 3.7	5	35.5
Nicotine	mg/stick	1.42 ± 0.05	5	1.46 ± 0.05	5	1.21 ± 0.02	5	0.25 <sup>b</sup>
NFDPM	mg/stick	12.2 ± 1.1	5	11.3 ± 1.4	5	12.6 ± 1.5	5	1.3
Carbon monoxide	mg/stick	0.612 ± 0.020	5	0.454 ± 0.047	5	0.468 ± 0.024	5	0.158
Benzo[a]pyrene	ng/stick	<1.00	5	1.03	5	<1.00	5	
Glycerin	mg/stick	4.68 ± 0.36	5	4.25 ± 0.25	5	4.23 ± 0.09	5	0.45 <sup>b</sup>
1-aminonaphthalene	ng/stick	<0.069	5	<0.069	5	<0.069	5	
2-aminonaphthalene	ng/stick	<0.035	5	<0.035	5	0.088 ± 0.173	5	
3-aminobiphenyl	ng/stick	<0.032	5	<0.032	5	<0.032	5	
4-aminobiphenyl	ng/stick	<0.051	5	<0.051	5	0.073 ± 0.022	5	
Acetaldehyde	µg/stick	193 ± 2	5	179 ± 11	5	229 ± 5	5	50
Acetone	µg/stick	37.7 ± 1.7	5	37.0 ± 5.5	5	43.0 ± 2.9	5	6.0
Acrolein	µg/stick	9.76 ± 0.91	5	8.87 ± 1.81	5	11.54 ± 0.81	5	2.67
Butyraldehyde	µg/stick	27.3 ± 0.7	5	26.0 ± 0.5	5	29.9 ± 2	5	3.9
Crotonaldehyde	µg/stick	4.13 ± 0.55	5	3.69 ± 0.51	5	4.64 ± 0.41	5	0.95
Formaldehyde	µg/stick	3.52 ± 0.3	5	3.65 ± 0.36	5	3.57 ± 0.3	5	0.13 <sup>b</sup>
Methyl ethyl ketone	µg/stick	7.58 ± 0.71	5	6.75 ± 0.84	5	8.68 ± 0.57	5	1.93 <sup>b</sup>
Propionaldehyde	µg/stick	14.4 ± 0.6	5	13.5 ± 2.1	5	18.1 ± 0.7	5	4.6
Acrylonitrile	µg/stick	0.167 ± 0.036	5	0.178 ± 0.021	5	0.189 ± 0.035	5	0.022 <sup>b</sup>
1,3-butadiene	µg/stick	0.277 ± 0.035	5	0.248 ± 0.016	5	0.318 ± 0.027	5	0.070 <sup>b</sup>
Benzene	µg/stick	0.603 ± 0.042	5	0.591 ± 0.031	5	0.613 ± 0.030	5	0.022 <sup>b</sup>
Isoprene	µg/stick	2.19 ± 0.21	5	1.83 ± 0.17	5	2.60 ± 0.25	5	0.77
Pyridine	µg/stick	7.47 ± 0.31	5	5.76 ± 0.42	5	7.26 ± 0.29	5	1.71
Quinoline	µg/stick	<0.012	5	<0.012	5	<0.012	5	
Styrene	µg/stick	0.640 ± 0.035	5	0.619 ± 0.041	5	0.695 ± 0.038	5	0.076 <sup>b</sup>
Toluene	µg/stick	2.11 ± 0.2	5	1.85 ± 0.15	5	2.25 ± 0.19	5	0.4 <sup>b</sup>
Catechol	µg/stick	16.9 ± 1.2	5	15.0 ± 2.0	5	15.2 ± 1.4	5	1.9
<i>o</i> -cresol	µg/stick	0.109 ± 0.006	4	0.123 ± 0.021	5	0.135 ± 0.017	5	0.026 <sup>b</sup>
<i>m</i> -cresol	µg/stick	0.031 ± 0.003	4	0.061 ± 0.01	5	0.071 ± 0.029	5	0.040
<i>p</i> -cresol	µg/stick	0.070 ± 0.006	4	0.099 ± 0.019	5	0.099 ± 0.012	5	0.029 <sup>b</sup>
Hydroquinone	µg/stick	8.51 ± 0.63	5	7.11 ± 1.17	5	8.21 ± 0.25	5	1.40
Phenol	µg/stick	1.66 ± 0.36	5	2.49 ± 0.45	5	2.23 ± 0.26	5	0.83
Resorcinol	µg/stick	0.054 ± 0.004	5	0.047 ± 0.007	5	0.044 ± 0.005	5	0.01
NAB	ng/stick	3.37 ± 0.21	4	<3.15	5	<3.15	5	
NAT	ng/stick	21.7 ± 1.4	4	20.1 ± 1.7	5	17.0 ± 0.6	5	4.7
NNK	ng/stick	9.2 ± 0.3	4	8.3 ± 1	5	7.7 ± 1.2	5	1.5
NNN	ng/stick	16.4 ± 1.3	4	14.6 ± 1.6	5	13.9 ± 0.8	5	2.5 <sup>b</sup>
Ammonia	µg/stick	14.6 ± 0.4	5	15.4 ± 0.2	5	15.3 ± 0.9	5	0.8 <sup>b</sup>
Hydrogen cyanide	µg/stick	4.84 ± 0.29	4	4.14 ± 0.32	5	4.39 ± 0.68	5	0.70 <sup>b</sup>
Nitric oxide	µg/stick	18.3 ± 1.2	5	16.6 ± 0.3	4	18.2 ± 0.2	4	1.7
Nitrogen oxides	µg/stick	19.5 ± 1.5	5	17.6 ± 0.2	4	19.0 ± 0.6	4	1.9
Pyrene	ng/stick	5.66 ± 0.61	5	7.27 ± 1.63	5	5.85 ± 1.27	5	1.61
<i>o</i> -toluidine	ng/stick	1.144 ± 0.113	5	0.649 ± 0.318	5	0.62 ± 0.59	5	0.524
Acetamide	µg/stick	4.24 ± 0.13	4	4.28 ± 0.55	5	4.19 ± 0.16	5	0.09 <sup>b</sup>
Acrylamide	µg/stick	2.31 ± 0.12	5	2.30 ± 0.31	5	1.94 ± 0.05	5	0.37
Ethylene oxide	µg/stick	0.267 ± 0.017	5	0.269 ± 0.012	5	0.355 ± 0.032	5	0.088
Nitrobenzene	ng/stick	0.138 ± 0.003	4	0.087 ± 0.005	5	0.087 ± 0.009	5	0.051
Propylene oxide	µg/stick	0.144 ± 0.01	5	0.101 ± 0.004	5	0.113 ± 0.009	5	0.043
Vinyl chloride	ng/stick	<3.54	5	<3.54	5	<3.54	5	
Benz[a]anthracene	ng/stick	1.39 ± 0.1	5	1.51 ± 0.35	5	1.39 ± 0.2	5	0.12
Dibenz[a,h]anthracene	ng/stick	<0.100	5	<0.100	5	<0.100	5	

N is the number of determinations, CI is the confidence interval of the mean.

&lt;: median lower than the limit of quantitation, in this case LOQ is given.

If at least 1 value is below LOQ, the median is given and CI is not mentioned.

TPM: Total particulate matter, NFDPM: Nicotine-free dry particulate matter, NAB: *N*-nitrosoanabasine, NAT: *N*-nitrosoanatabine, NNK: 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone, NNN: *N*-nitrososnormicotine.<sup>a</sup> Range: largest mean obtained among the three climatic conditions minus the smallest mean obtained among the three climatic conditions.<sup>b</sup> Range smaller than the Intermediate Precision (IP) (International Council for Harmonisation, 1996) of the analytical method.

basis) were used to compare the relative cytotoxicity of the THS2.2 and the 3R4F reference cigarette. The results showed that the relative *in vitro* cytotoxicity of the THS2.2 Regular (FR1 and D2) and Menthol (FR1 and D1) aerosol fractions was reduced by approximately 95% when expressed on a per-mg TPM basis, compared with

the 3R4F reference cigarette (Tables F and G of the supplementary material). The *in vitro* cytotoxicity of THS2.2 Regular (FR1 and D2) and THS2.2 Menthol (FR1 and D1) aerosol fractions, expressed on a per-mg nicotine basis, was reduced by 85–90% compared with the 3R4F reference cigarette (Table 10 and Table 11).

**Table 8**

Summary of the THS2.2 FR1 yields from extreme puffing regimens; comparison with the Health Canada machine-smoking regimen and the 3R4F reference cigarette.

Parameter	Unit	THS2.2 FR1		THS2.2 FR1		THS2.2 FR1		3R4F
		Extreme Regimen Maximum Yields		Extreme Regimen Minimum Yields		HCI Regimen	Ratio <sup>b</sup>	HCI regimen
		mean	puf. reg.	mean	puf. reg.	mean	Max/HCI	mean
TPM	mg/stick	59.0	SR-4	26.9	ISO	56.8	1.0	49.0
Water	mg/stick	45.1	SR-4	21.4	ISO	44.6	1.0	15.8
Nicotine	mg/stick	2.19	LR-3	0.49	ISO	1.36	1.6	1.89
NFDPM	mg/stick	17.4	LR-3	5.1	ISO	10.8	1.6	31.2
Carbon monoxide	mg/stick	0.660	SR-6	0.238	ISO	0.532	1.2	32.8
Benzo[a]pyrene	ng/stick	<1.00		<1.00		<1.00		14.2
Glycerin	mg/stick	5.66	LR-3	1.91	ISO	4.59	1.2	2.42
1-aminonaphthalene	ng/stick	<0.069		<0.069		<0.069		20.8
2-aminonaphthalene	ng/stick	<0.035		<0.035		<0.035		11.0
3-aminobiphenyl	ng/stick	<0.032		<0.032		<0.032		3.77
4-aminobiphenyl	ng/stick	<0.051		<0.051		<0.051		3.26
Acetaldehyde	µg/stick	205	SR-4	145	SR-1	196	1.0	1555
Acetone	µg/stick	40.7	SR-4	22.0	SR-1	37.9	1.1	736
Acrolein	µg/stick	12.9	LR-3	4.89	ISO	8.83	1.5	154
Butyraldehyde	µg/stick	26.7	SR-6	16.7	ISO	22.0	1.2	88.4
Crotonaldehyde	µg/stick	4.90	LR-3	1.88	ISO	3.04	1.6	68.8
Formaldehyde	µg/stick	7.73	LR-3	1.85	ISO	3.77	2.1	56.5
Methyl ethyl ketone	µg/stick	7.39	SR-4	3.78	SR-1	7.28	1.0	187
Propionaldehyde	µg/stick	14.4	SR-4	8.5	SR-1	13.5	1.1	125
Acrylonitrile	µg/stick	0.228	LR-3	<0.111	SR-1 <sup>a</sup>	0.163	1.4	31.9
1,3-butadiene	µg/stick	0.357	SR-4	<0.236	ISO <sup>a</sup>	0.295	1.2	63.8
Benzene	µg/stick	0.708	SR-4	0.298	SR-1	0.597	1.2	97.6
Isoprene	µg/stick	2.82	SR-5	1.37	SR-1	2.56	1.1	798
Pyridine	µg/stick	8.00	SR-4	4.21	ISO	7.36	1.1	36.1
Quinoline	µg/stick	0.051	LR-3	<0.011	ISO <sup>a</sup>	0.016	3.2	0.513
Styrene	µg/stick	0.686	SR-6	0.314	SR-1	0.619	1.1	24.5
Toluene	µg/stick	2.15	LR-3	0.97	ISO	2.02	1.1	188
Catechol	µg/stick	17.9	SR-4	5.9	ISO	16.8	1.1	91.4
<i>o</i> -cresol	µg/stick	0.438	LR-3	0.021	ISO	0.108	4.1	4.47
<i>m</i> -cresol	µg/stick	0.212	LR-3	<0.010	ISO <sup>a</sup>	0.046	4.6	3.03
<i>p</i> -cresol	µg/stick	0.399	LR-3	<0.010	SR-1	0.100	4.0	9.17
Hydroquinone	µg/stick	9.99	SR-6	3.66	SR-1	8.76	1.1	83.1
Phenol	µg/stick	10.87	LR-3	0.06	ISO	1.93	5.6	13.6
Resorcinol	µg/stick	0.056	SR-6	0.020	ISO	0.047	1.2	1.85
NAB	ng/stick	4.31	LR-3	<3.15	ISO <sup>a</sup>	3.46	1.2	33.7
NAT	ng/stick	26.8	SR-6	8.5	ISO	22.4	1.2	318
NNK	ng/stick	10.2	SR-6	4.1	ISO	8.7	1.2	266
NNN	ng/stick	19.1	LR-3	6.5	ISO	16.1	1.2	309
Ammonia	µg/stick	31	LR-3	4.1	ISO	15	2.1	39.3
Nitric oxide	µg/stick	19.4	SR-4	11	ISO	18	1.1	491
Nitrogen oxides	µg/stick	20.3	SR-4	11.2	ISO	19	1.1	537
Pyrene	ng/stick	6.50	SR-4	<5.00	ISO <sup>a</sup>	<5.00		87.3
<i>o</i> -toluidine	ng/stick	2.146	SR-6	0.489	SR-1	1.195	1.8	85.5
Acetamide	µg/stick	6.62	LR-3	1.32	ISO	4.18	1.6	13.9
Acrylamide	µg/stick	4.23	LR-3	0.69	ISO	2.33	1.8	4.8
Ethylene oxide	µg/stick	0.323	LR-3	0.157	SR-1	0.242	1.3	29.4
Vinyl chloride	ng/stick	<3.54		<3.54		<3.54		96.7
Benz[a]anthracene	ng/stick	1.61	SR-4	<1.00	ISO <sup>a</sup>	<1.00		28.0
Dibenz[a,h]anthracene	ng/stick	<0.100		<0.100		<0.100		1.7

<: median lower than the limit of quantitation, in this case LOQ is given.

TPM: Total particulate matter, NFDPM: Nicotine-free dry particulate matter, NAB: *N*-nitrosoanabasine, NAT: *N*-nitrosoanatabine, NNK: 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone, NNN: *N*-nitrosoornicotine.

<sup>a</sup> At least one other smoking regimen was also below LOQ; see Tables D a in the supplementary material.

<sup>b</sup> When the, minimum value was inferior to the LOQ, the LOQ value was used to calculate the ratios.

### 3.6. Ames assay

The TPM from the THS2.2 D2, THS2.2 D1 M, and the 3R4F was tested with the *S. typhimurium* strains TA98, TA100, TA102, TA1535, and TA1537 in both the presence and absence of S9 (Table 12).

A positive response in the Ames test for the TPM from the 3R4F reference cigarette was detected in 3 of the 5 *S. typhimurium* tester strains in the presence of the S9 fraction, namely TA98, TA100, and

TA1537 (see Table 12). Nevertheless, despite testing up to 10 mg of TPM for THS2.2 D2 and 5 mg/per plate for THS2.2 D1 M, no mutagenicity in any of the tester strains was detected under the conditions of this assay.

### 3.7. Mouse lymphoma assay (MLA)

The *in vitro* MLA was used to assess the mutagenicity of both

**Table 9**

MMAD and GSD results from 3R4F and THS2.2 FR1.

Repetition	3R4F				THS2.2 FR1			
	MMAD	GSD <sup>2</sup>	LB	UB	MMAD	GSD <sup>2</sup>	LB	UB
	[μm]		[μm]	[μm]	[μm]		[μm]	[μm]
1	0.9	2.1	0.4	1.8	0.8	2.6	0.3	2.1
2	0.8	1.9	0.4	1.5	0.7	2.3	0.3	1.7
3	0.8	1.9	0.4	1.5	0.7	2.1	0.3	1.4
4	0.9	1.7	0.5	1.5	0.7	1.9	0.4	1.3
5	0.9	1.6	0.5	1.4	0.7	2.0	0.3	1.3
6	0.8	1.9	0.4	1.5	0.7	2.2	0.3	1.6
7	0.9	1.9	0.5	1.6	0.8	2.5	0.3	1.9
8	0.6	1.4	0.5	0.9	0.6	2.3	0.3	1.3
9	0.8	1.8	0.5	1.5	0.6	2.3	0.3	1.5
10	0.7	1.8	0.4	1.3	0.7	3.2	0.2	2.3
Mean	0.8	1.8			0.7	2.3		
Mean GSD		1.3				1.5		

MMAD: Mass median aerodynamic diameter; GSD: Geometric standard deviation. LB: Lower boundaries with a 95% confidence interval; UB: Upper boundaries with a 95% confidence interval.

TPM and GVP derived from THS2.2 D2, THS2.2 D1 M, and 3R4F. In both tests under the three treatment conditions, TPM and GVP derived from THS2.2 D2 and 3R4F induced concentration-dependent increases in cytotoxicity and mutagenicity. In the presence of S9, the mutagenic responses reproducibly surpassed the GEF threshold for mutagenicity at or just above the cytotoxicity limit of the assay, i.e. RTG 10%–20%. Increases in both large and small colonies were observed for TPM and GVP derived from both test articles. For THS2.2 D2, LOGELs for the TPM fraction were markedly higher (on average 17-fold) than for 3R4F-derived TPM (Fig. 4A and Table 13). A similar mutagenicity profile was observed for the GVP (Fig. 4B). In both treatment conditions (4 h and 24 h) conducted in the absence of S9, mutagenicity which exceeded the GEF threshold was also observed, however, this finding was not always reproducible between the tests. When it was the case, LOGELs for THS2.2 D2 -derived TPM were again markedly higher (on average, at least 14-fold) than for 3R4F TPM (exemplar responses illustrated in Fig. 5A and Table 13). Moreover, in these treatment conditions, the LOGELs for TPM always occurred at the RTG 10%–20% cytotoxicity level. Similar mutagenicity profiles were also observed for GVP under the same treatment conditions. Similar results were obtained for the mentholated version of THS2.2 (exemplar responses illustrated in Figs. 5B and 6, and Table 13). Mutagenicity data expressed on a per-mg TPM basis are presented as part of the supplementary material (Supplementary Figures A–C and Supplementary Table H).

#### 4. Discussion

The objective of this study was to assess the potential for reduced exposure to HPHCs from THS2.2 compared with 3R4F

**Table 10**

Cytotoxicity of TPM and GVP, expressed as 1/EC50 (ml/mg nicotine).

	THS2.2 D2		3R4F analyzed during the THS2.2 D2 study		THS2.2 D1 M		3R4F analyzed during the THS2.2 D1 M study	
	TPM	GVP	TPM	GVP	TPM	GVP	TPM	GVP
Mean	17.34	28.40	208.55		19.73	26.07	239.51	276.21
SEM	0.52	1.20	6.92		0.87	1.78	6.07	22.51
N	3	3	3		3	3	3	3
Relative cytotoxicity (%)	8.3	9.8	100		8.2	9.4	100	100

SEM: Standard error of the mean.

TPM: Total particulate matter, GVP: Gas-vapor phase.

Relative cytotoxicity (%) = (cytotoxicity of THS2.2/3R4F) × 100.

**Table 11**

Cytotoxicity of TPM and GVP, expressed as 1/EC50 (ml/mg nicotine).

	THS2.2 FR1		THS2.2 FR1 M		3R4F	
	TPM	GVP	TPM	GVP	TPM	GVP
	Mean	21.33	30.86	27.43	28.61	186.78
SEM	1.57	2.87	1.6	2.53	7.58	14.06
N	3	3	3	3	3	3
Relative cytotoxicity (%)	11.4	12.8	14.7	11.8	100	100

SEM: Standard error of the mean.

TPM: Total particulate matter, GVP: Gas-vapor phase.

Relative cytotoxicity (%) = (cytotoxicity of THS2.2 / 3R4F) × 100.

based on chemical analysis of HPHCs, *in vitro* genotoxicity, and cytotoxicity assessments. To evaluate the robustness of the data for the products under different conditions, HPHC yields were also measured when using the product under simulated real-life smoking conditions, additional tests were performed under different climatic conditions and with different puffing regimens.

#### 4.1. Aerosol physics and aerosol chemistry; comparison WITH the 3R4F reference cigarette

During this assessment of the THS2.2, following the feedback from taste panels and to ensure sustainability of tobacco sources, the blend FR1 was replaced by blend D2 for the regular product and by blend D1 for the menthol product (Smith et al., 2016). Therefore, it was considered important to present the chemical characterization of all the four products (THS2.2 FR1, THS2.2 FR1 M, THS2.2 D2 and THS2.2 D1 M) in this publication. When comparing the four products, it can be observed that they delivered HPHC yields that were in the same range. The influence of the tobacco blend composition on the HPHC yields is presented in the 3rd publication of this issue (Schaller et al., 2016-b). When comparing menthol and regular THS2.2 products, no substantial influence of menthol on the HPHC yields was detected. Schmeltz and Schlotzhauer have reported that menthol pyrolysis in a closed system produced benzo[a]pyrene leading them to suggest that menthol pyrolysis could act as a potential precursor to benzo[a]pyrene to the smoke of mentholated cigarette products (Schmeltz and Schlotzhauer, 1968). A significant contribution of the menthol to the yield of benzo[a]pyrene was not observed in the THS2.2. The FR1 Menthol product delivered a slightly higher yield of benzo[a]pyrene ( $1.29 \pm 0.10$  ng/stick) than the FR1 Regular product ( $<1.00$  ng/stick), but the yield of menthol in the D1 Menthol product ( $1.08 \pm 0.09$  ng/stick) was on the low side compared to the D2 Regular product ( $1.19 \pm 0.08$  ng/stick). In addition, in the pyrolytic conditions used by Schmeltz and Schlotzhauer, benzo[a]pyrene was detected only on pyrolysis of menthol at 860 °C, but not during pyrolysis at 600 °C. Other studies have reported that when isotopically labeled menthol was added to tobacco of cigarettes, most of the menthol was transferred to the smoke unchanged and the production of labeled benzo[a]pyrene

**Table 12**

Revertant colonies obtained following exposure to the TPM (1 mg per plate) from THS2.2 D2, THS2.2 D1 M, or 3R4F.

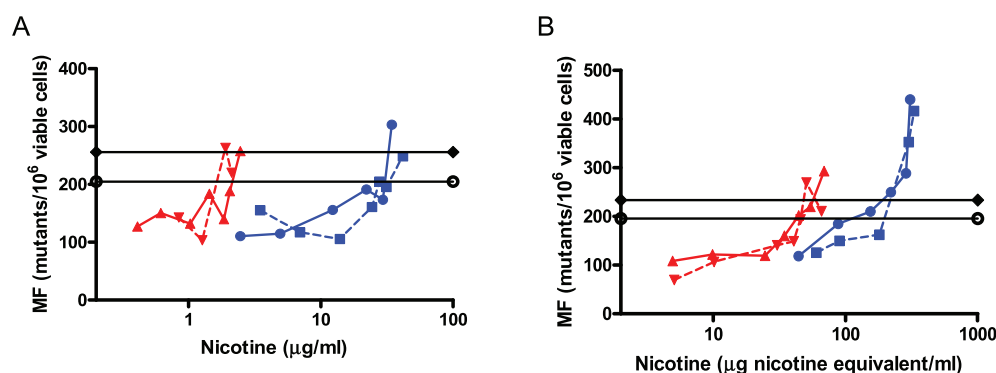
Salmonella typhimurium Strain	THS2.2 D2		3R4F <sup>a</sup>		Solvent Control		Positive <sup>b</sup> Control		THS2.2 D1 M		3R4F <sup>b</sup>		Solvent Control		Positive <sup>b</sup> Control		
	Mean <sup>d</sup>	SD	Mean <sup>d</sup>	SD	Mean <sup>d</sup>	SD	Mean <sup>d</sup>	SD	Mean <sup>d</sup>	SD	Mean <sup>d</sup>	SD	Mean <sup>d</sup>	SD	Mean <sup>d</sup>	SD	
+S9	TA98	22	4	658	89	21	1	109	7	21	2	636	24	25	2	97	17
	TA100	94	21	428	25	87	2	481	22	93	12	440	20	90	6	471	67
	TA102	358	12	409	15	272	22	1005	29	290	15	399	16	265	23	968	25
	TA1535	9	3	17	6	6	1	70	8	15	6	15	6	10	3	113	11
	TA1537	8	5	98	9	6	2	50	5	15	3	94	9	7	2	35	5
-S9	TA98	16	4	17	5	23	6	81	6.2	22	3	10 <sup>c</sup>	6	26	3	93	10
	TA100	61	8	87	13	66	3	195	25	81	11	96	21	62	8	187	17
	TA102	291	15	282	12	267	21	709	5	230	56	264	4	258	26	620	8
	TA1535	12	3	7	3	6	2	37	8	9	4	16	6	12	2	51	6
	TA1537	6	5	3	2	6	3	84	5	15	5	17	5	7	2	83	6

<sup>a</sup> These samples were generated and tested concurrently with the respective THS variant.

<sup>b</sup> Details of dose and substance are provided in the Ames methods section.

<sup>c</sup> Toxicity was detected at this dose.

<sup>d</sup> Each mean and SD value was derived from 3 plates and the values were rounded.



**Fig. 4.** The mutagenic responses induced by aerosol fractions derived from THS2.2 D2 and 3R4F in the 4 h + S9 treatment condition in two independent tests expressed on a per-mg nicotine basis. A. TPM. MFs for the DMSO-treated controls in tests #1 and #2 were  $129.77 \pm 22.57$  and  $78.73 \pm 1.55$  mutants/ $10^6$  viable cells, respectively. THS2.2 D2 #1; THS2.2 D2 #2; 3R4F #1; 3R4F #2; GEF + DMSO MF #1; GEF + DMSO MF #2. B. GVP. MFs for the PBS-treated controls in tests #1 and #2 were  $107.73 \pm 10.40$  and  $69.44 \pm 3.10$  mutants/ $10^6$  viable cells, respectively. THS2.2 D2 #1; THS2.2 D2 #2; 3R4F #1; 3R4F #2; GEF + PBS MF #1; GEF + PBS MF #2.

was not detected (Jenkins et al., 1970; Baker and Bishop, 2004). Since (i) the heater blade temperature in the THS2.2 only reaches a maximum temperature of 350 °C, and (ii) available literature on menthol pyrolysis to yield benzo[a]pyrene is limited, it was concluded that menthol is unlikely to be a significant source of benzo[a]pyrene in the THS2.2 aerosol.

The mainstream aerosols produced by all the analyzed THS2.2 products were similar regarding analyte yields including HPHC yields, but substantially different from the yields in mainstream smoke of the 3R4F reference cigarette. To quantify the exposure reduction, the yields of each HPHC for THS2.2 relative to those in

3R4F were calculated, and are presented on a per Tobacco Stick/cigarette (Fig. 7). The graph presenting the results on a mg-nicotine basis is included in the supplementary material (Figure D).

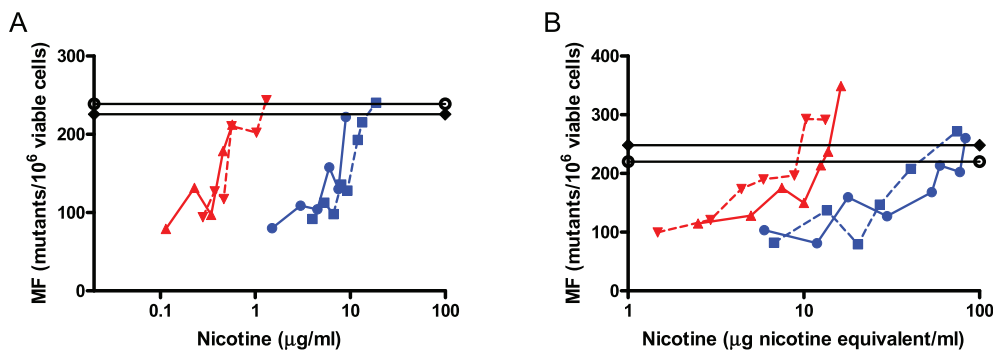
Fig. 7 presents a general view of the ratios between THS2.2 and 3R4F. The 100% line represents the yields of the 3R4F on a per-cigarette basis. When considering the HPHC yields on a Tobacco Stick/cigarette basis, it can be observed that acrylamide, ammonia, butyraldehyde, butyraldehyde, acetamide, and mercury presented ratios between 25 and 50%. Even if this denotes a substantial reduction compared to 3R4F, it was not surprising to see ammonia, acrylamide, acetamide and mercury at this level, since they could be formed or could distill out of tobacco at relatively low temperatures (McDaniel et al., 2001; Stadler et al., 2002; Becalski et al., 2003; Blank et al., 2005; Moldoveanu, 2010; Becalski et al., 2011). The other analytes presented smaller ratios. Therefore, when compared with 3R4F mainstream smoke, all HPHCs are considerably reduced in the THS2.2 aerosol of the four products. This data supports the hypothesis that the controlled heating of tobacco in THS2.2 resulted in a significant reduction in the pyrosynthesis of HPHCs. When compared with 3R4F, the formation of polycyclic aromatic hydrocarbons, aromatic amines, phenols, and aldehydes was reduced by more than 75%, and for the majority of HPHCs, by more than 90% under the HCl machine-smoking conditions. The analyzed HPHCs covered a broad range of chemical compounds, and several of these HPHCs have been described as markers for the pyrolysis of tobacco (Moldoveanu, 2010). Since the reduction of individual HPHCs was consistent across the different HPHC groups,

**Table 13**

The LOGELs (expressed on a nicotine basis) achieved following treatment with TPM (µg/ml) and GVP (µg nicotine equivalent/ml) derived from THS2.2 variants and 3R4F values are shown when GEF threshold was exceeded.

		4 h -S9	4 h +S9	24 h -S9
THS2.2 D2	TPM	18.66	34.48	41.80
	GVP	71.90	83.17	249.87
3R4F	TPM	1.31	2.47	1.91
	GVP	<sup>a</sup>	69.07	50.79
THS2.2 D1 M	TPM	12.55	27.82	21.71
	GVP	83.30	74.53	240.34
3R4F	TPM	0.96	1.11	3.05
	GVP	16.27	10.311	53.74

<sup>a</sup> Concentration-dependent increase in MF observed but below the GEF threshold.



**Fig. 5.** The mutagenic responses induced by aerosol fractions derived from THS2.2 variants and 3R4F in the 4 h–S9 treatment condition in two independent tests expressed on a per-mg nicotine basis. A. TPM from THS2.2 D2 and 3R4F. MFs for the DMSO-treated controls in tests #1 and #2 were  $99.52 \pm 19.21$  and  $112.81 \pm 23.41$  mutants/ $10^6$  viable cells, respectively. ●THS2.2 D2 #1; ■THS2.2 D2 #2; ▲3R4F #1; ▼3R4F #2; ◆GEF + DMSO MF #1; ○GEF + DMSO MF #2. B. GVP from THS2.2 D1 M and 3R4F. MFs for the PBS-treated control(s) in tests #1 and #2 were  $122.33$  and  $94.10 \pm 26.37$  mutants/ $10^6$  viable cells, respectively. ●THS2.2 D1 M #1; ■THS2.2 D1 M #2; ▲3R4F #1; ▼3R4F #2; ◆GEF + PBS MF #1; ○GEF + PBS MF #2.

it may be assumed that other HPHCs, although not measured, were similarly reduced. In addition, it can also be observed that some HPHCs that could distill out of tobacco in 3R4F were also reduced in the THS2.2 aerosol. The transfer of cadmium to the aerosols of the four THS2.2 products could not be quantified (results below LOQ), and the yield of TSNAs was minimal (Tables 5 and 6). Since the nicotine yield was lower in the analyzed THS2.2 products than in 3R4F, the ratios calculated on a per-mg nicotine basis were somewhat higher. However, the trend remained the same, and the reductions expressed on a per-mg nicotine basis were also substantial (Figure D in the supplementary material).

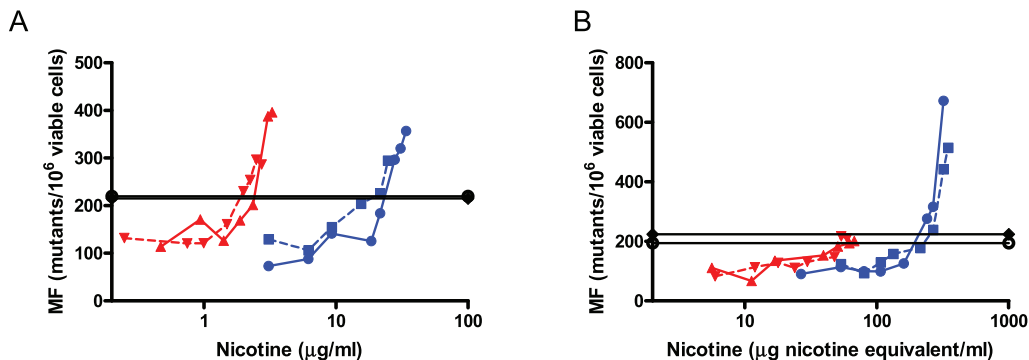
The mainstream smoke of 3R4F and the THS2.2 aerosol generated under the HCI machine-smoking conditions were both shown to be respirable aerosols (Table 9). The MMAD values were similar:  $0.8 \mu\text{m}$  for 3R4F and  $0.7 \mu\text{m}$  for THS2.2. The GSD was somewhat higher for THS2.2 (Section 3.4). Therefore, THS2.2 presents respirable properties that are similar to those of 3R4F, while reducing substantially the levels of the measured HPHCs.

#### 4.2. Chemical composition of the THS2.2 FR1 aerosol collected under different climatic conditions and extreme PUFFING regimens

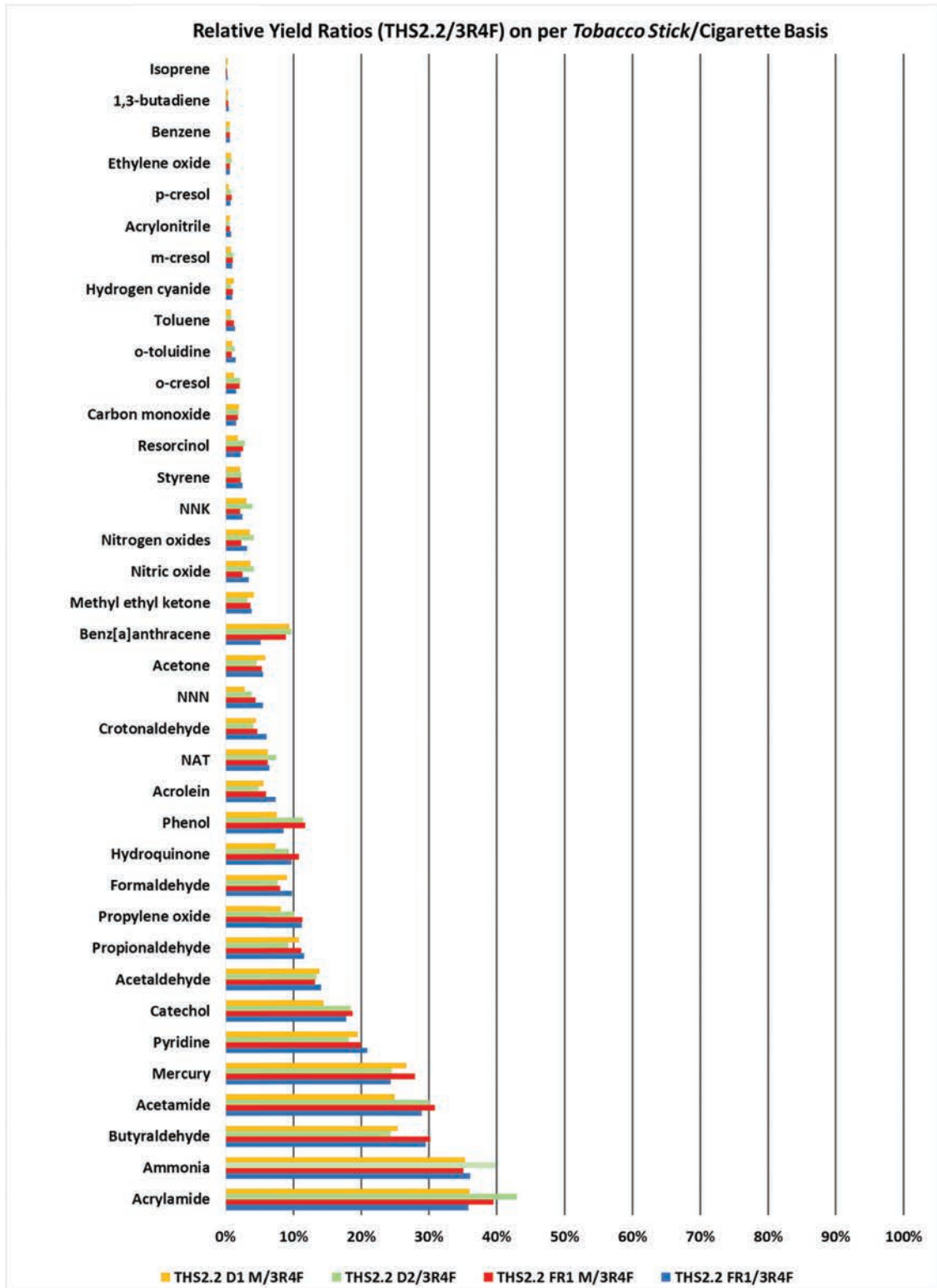
Since the THS2.2 may be used by consumers using puffing regimens under climatic conditions that deviate significantly from those considered in the HCI machine-smoking standard (55 ml puff

every 30 s, 22 °C, 60% RH), the aerosol of the THS2.2 FR1 was collected under different atmospheric and puffing conditions described in sections 2.5 and 2.6.

The climatic conditions may have a significant impact on the deliveries to the mainstream smoke of CC (Dymond and Hirji, 1972; Boder and Senehi, 1984). In previous studies, the filtration efficiency of the tobacco rod and of the filter and the puff count were affected when CC were conditioned and smoked under different climatic conditions. The effect could be observed on both gas phase and particulate phase components. For instance, a temperature increase of 10 °C resulted in an increased delivery of nitric oxide by 18% for an American blend CC, while the phenol delivery decreased correspondingly (Dymond and Hirji, 1972). It was anticipated that the careful control of the heater blade temperature up to a maximum of 350 °C (Smith et al., 2016) and use of a low efficiency filter would make THS2.2 less sensitive to variations in ambient conditions and alterations in the yields of the different analytes. The yield of water in the THS2.2 aerosol was considered the only exception, because of the high humectant content of the tobacco plug which contains glycerin in about 20% of the tobacco plug weight. When the tobacco sticks were conditioned for at least 48 h and used under “desert” conditions, the delivery of water was considerably reduced when compared with the “tropical” conditions (Table 7). The differences in the yield of water under both “tropical” and “desert” conditions explained nearly all the



**Fig. 6.** The mutagenic responses induced by aerosol fractions derived from THS2.2 D1 M and 3R4F in the 4 h + S9 treatment condition in two independent tests expressed on a per-mg nicotine basis. A. TPM. MFs for the DMSO-treated controls in tests #1 and #2 were  $89.01 \pm 6.76$  and  $93.90 \pm 13.64$  mutants/ $10^6$  viable cells, respectively. ●THS2.2 D1 M #1; ■THS2.2 D1 M #2; ▲3R4F #1; ▼3R4F #2; ◆GEF + DMSO MF #1; ○GEF + DMSO MF #2. B. GVP. MFs for the PBS-treated control in tests #1 and #2 were  $97.43$  and  $68.20$  mutants/ $10^6$  viable cells, respectively. ●THS2.2 D1 M #1; ■THS2.2 D1 M #2; ▲3R4F #1; ▼3R4F #2; ◆GEF + PBS MF #1; ○GEF + PBS MF #2.



**Fig. 7.** Mainstream aerosol HPHCs from THS2.2 compared to the mainstream smoke HPHCs from the 3R4F reference cigarette (constituent levels set at 100%) on a per-unit basis under the Health Canada Intense (HCI) machine-smoking regimen. When one value or more was below the LOQ, the results were not presented in the graphs (NAT: N-nitro-soanatabine, NNK: 4-(N-nitrosomethylamino)-1-(3-pyridyl)-1-butanone, NNN: N-nitrosornornicotine).

variability in the TPM yields, since the NFDPM yields obtained from the *tobacco sticks* in the three conditions (“Mediterranean”, “tropical” and “desert”) were similar. The ranges for nicotine, formaldehyde, ethyl methyl ketone, acrylonitrile, 1,3-butadiene, benzene, styrene, toluene, *o*-cresol, *p*-cresol, NNN, ammonia, and acetamide were lower than the Intermediate Precision (IP) (International Council for Harmonisation, 1996; Walfish, 2006) of the respective analytical methods, and the climatic conditions were considered not to have a significant impact on the yields of these HPHCs. The ranges for other HPHCs were low, except for *m*-cresol, phenol, *o*-toluidine, propylene oxide, and nitrobenzene, which had ranges in excess of 35%. For *o*-toluidine, propylene oxide, and nitrobenzene, the highest yield was obtained when conditioning and machine-smoking the *tobacco sticks* at 22 °C and 60% RH. Therefore, collecting the aerosol under “tropical” or “desert” conditions did not increase the yields of these HPHCs. The yield of *m*-cresol was increased from 0.031 to 0.071 µg/stick under the “tropical” condition, while phenol was increased from 1.66 to 2.49 µg/stick under the “desert” condition. However, the values remained low when compared with the yields obtained from 3R4F: *m*-cresol (3.03 µg/stick) and phenol (13.6 µg/stick) (Table 5). Therefore, the variation of the climatic conditions had only a minor influence on the HPHC yields.

The different machine-smoking puffing regimens (Table 2) were selected to cover the puffing behavior reported for users of THS2.2 (Campelos et al., 2016). They induced significant modifications of the air flow and of the quantity of air used to extract aerosol from the tobacco plug in THS2.2. The minimum and maximum yields obtained from the different machine-smoking regimens are presented in Table 8. In general, the yields of polar HPHCs (e.g. phenol and cresol isomers) were more sensitive than apolar HPHCs to the variation of the machine-smoking puffing conditions (Table 8). For each HPHC, the comparison between the maximum yield and the yield obtained with the HCl smoking regimen should enable the identification of the HPHC for which the standard protocol may underestimate the exposure when using more extreme puffing conditions. The ratios of maximum yield to HCl yield are presented in Table 8. These ratios were less than 2 for 42 of 49 analyzed compounds. Again, phenol and cresol isomers were the HPHCs presenting the largest ratios. However, the HCl machine-smoking protocol gave a relevant estimate of the exposure for the majority of the tested HPHCs. Interestingly, the ratio obtained for nitrogen oxides was only 1.1, and the ratio for CO was only 1.2. Since NO<sub>x</sub> and CO can be considered potential markers of combustion (Norman et al., 1983; Reed, 2002; Glarborg et al., 2003; Im et al., 2003; Baker, 2006; Senneca et al., 2007; Cozzani et al., 2016), no evidence of tobacco combustion was found even under extreme machine-smoking puffing conditions. Under extreme machine-smoking puffing conditions, the yields of all toxicologically relevant compounds in the THS2.2 aerosol were lower than those obtained when smoking the 3R4F reference cigarette under HCl machine-smoking conditions (Table 5).

#### 4.3. *In vitro* toxicology

The *in vitro* toxicology results reflect the chemistry data; THS2.2 aerosol fraction-induced effects are distinctly different in terms of potency from those induced by counterpart fractions from 3R4F. The THS2.2 aerosol demonstrates a substantial reduction in toxicological activity compared with 3R4F smoke. In the NRU assay, both the particulate phase and GVP *in vitro* cytotoxicity of THS2.2 Regular (FR1 and D2) and THS2.2 Menthol (FR1 and D1) were reduced by 85%–95% compared with the 3R4F, independent of the basis used to express the activity (per-mg TPM or per-mg nicotine) (Table 10, Table 11 and Tables F and G of the supplementary

material).

The Ames assay did not reveal significant mutagenicity of the TPM fraction for either THS2.2 regular or THS2.2 menthol under the conditions of this test. In contrast, the TPM fraction from 3R4F was mutagenic in tester strains TA98, TA100, and TA1537 in the presence of the S9 metabolizing fraction from Aroclor-treated rat liver. The MLA data show that both the TPM and GVP aerosol fractions derived from THS2.2 D2 and THS2.2 D1 M were mutagenic in this assay. However, the LOGELs demonstrate a lower *in vitro* mutagenic potency of the THS2.2 aerosol fractions compared with 3R4F. While a conclusion underlying the mechanism(s) of this phenomenon cannot be definitively made on the basis of these data, it is reasonable to suggest that the overall reduction in the burden of toxicants present in the THS2.2 aerosols may play a role in the manifestation of reduced cytotoxic and mutagenic potency *in vitro*.

## 5. Conclusion

The low operating temperature of THS2.2 results in significantly lower concentrations of HPHCs in the mainstream aerosol compared with the mainstream smoke of the 3R4F reference cigarette when expressed on either a per-Tobacco Stick/cigarette or a per-mg nicotine basis, while the MMAD of both aerosols remains similar. The reductions in the concentrations of most HPHCs in the THS2.2 aerosol were greater than 90% when compared with 3R4F, and were not affected by machine-smoking of THS2.2 under extreme climatic conditions. No evidence of tobacco combustion was found when using the THS2.2 device with puffing regimens that were significantly more intense than the HCl conditions. The mutagenic and cytotoxic potencies of the mainstream aerosol fractions from THS2.2, when evaluated by the Ames, mouse lymphoma, and NRU assays were reduced by at least 85%–95% compared with the mainstream smoke aerosol of 3R4F.

## Conflict of interest statement

The work reported in this publication involved a candidate Modified Risk Tobacco Product developed by Philip Morris International (PMI) and was solely funded by PMI. All authors are (or were) employees of PMI R&D or worked for PMI R&D under contractual agreements.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.yrtph.2016.10.001>.

## Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.yrtph.2016.10.001>.

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