Inter-Laboratory Reproducibility and Interchangeability of 3R4F and 1R6F Reference Cigarettes in Mainstream Smoke Chemical Analysis and *In Vitro* Toxicity Assays*

by

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SUMMARY

A new reference cigarette, 1R6F, produced by the Kentucky Tobacco Research and Development Center, has been manufactured as a substitute for the 3R4F reference cigarette because of a depletion of 3R4F stock. The purpose of the current study was to investigate the interchangeability of 1R6F and 3R4F by comparing the chemical and biological characteristics of the mainstream smoke and to assess the inter-laboratory reproducibility by comparing the results obtained in the current study with a previous report. We analyzed 45 priority chemicals required by Health Canada for regulatory reporting and assessed the toxicological effects of cigarette smoke using *in vitro* standard toxicological assays recommended by the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) under the International Organization for Standardization (ISO) standard and intense smoking regimens. The results of the chemical analysis and standard toxicological assays showed a good inter-laboratory reproducibility for 1R6F as a reference cigarette, while there were some slight reproducible differences between 1R6F and 3R4F. In addition, we investigated the interchangeability of 1R6F with 3R4F in some additional toxicological assays that detect oxidative stress because oxidative stress is a principle endpoint used in tobacco research with next generation tobacco and nicotine delivery products (NGPs). Both 1R6F and 3R4F elicited comparable responses in the oxidative stress assays. Overall, our results showed inter-laboratory reproducibility in chemical and standard toxicological assessments of 1R6F; thus, suggest-

ing the suitability of 1R6F as a reference cigarette. In addition, the results obtained in the oxidative stress assays provide insight into the interchangeability of 1R6F with 3R4F when used as a comparator for NGPs. [Beitr. Tabakforsch. Int. 29 (2020) 119–135]

KEYWORDS

Mainstream cigarette smoke, inter-laboratory reproducibility, chemical analysis, *in vitro* toxicity

ZUSAMMENFASSUNG

Vom Kentucky Tobacco Research and Development Center wurde eine neue Referenzzigarette mit der Bezeichnung 1R6F als Ersatz für die Referenzzigarette 3R4F hergestellt, weil die Bestände der 3R4F zu Ende gehen. Ziel der vorliegenden Studie war die Untersuchung der Austauschbarkeit von 1R6F und 3R4F durch einen Vergleich der chemischen und biologischen Eigenschaften des Hauptstromrauchs sowie die Bewertung der Interlabor-Reproduzierbarkeit anhand des Vergleichs der Ergebnisse aus dieser Studie mit einem früheren Bericht. Es wurden 45 prioritäre chemische Substanzen entsprechend den regulatorischen Meldeanforderungen des kanadischen Gesundheitsministeriums Health Canada analysiert und die toxikologischen Effekte des Zigarettenrauchs bestimmt. Hierfür wurden die von der CORESTA (Cooperation Centre for Scientific Research Relative to Tobacco) als Standard

empfohlenen toxikologischen *in-vitro*-Testverfahren gemäß den beiden Abrauchverfahren der Internationalen Organisation für Normung (ISO) (normale und intensive Methode) verwendet. Die Ergebnisse der chemischen Analyse und der toxikologischen Standardtests zeigten eine gute Interlabor-Reproduzierbarkeit für die 1R6F als Referenzzigarette. Es gab außerdem einige geringfügige reproduzierbare Unterschiede zwischen der 1R6F und der 3R4F. Zudem wurde die Austauschbarkeit von 1R6F und 3R4F mithilfe einiger zusätzlicher toxikologischer Tests zur Ermittlung von oxidativem Stress untersucht, da der oxidative Stress ein Hauptendpunkt der Tabakforschung im Bereich der Tabak- und Nikotinabgabeprodukte der nächsten Generation (NGPs, Next Generation Products) ist. Sowohl die 1R6F als auch die 3R4F zeigten bei den Tests auf oxidativen Stress vergleichbare Reaktionen. Insgesamt konnte mit den Ergebnissen aus den chemischen und toxikologischen Standardprüfverfahren für 1R6F eine Interlabor-Reproduzierbarkeit gezeigt werden, was auf eine Eignung von 1R6F als Referenzzigarette hinweist. Zudem konnten im Rahmen der Tests auf oxidativen Stress Erkenntnisse hinsichtlich der Austauschbarkeit von 1R6F und 3R4F gewonnen werden, wenn diese als Vergleich für NGPs eingesetzt werden. [Beitr. Tabakforsch. Int. 29 (2020) 119–135]

RESUME

Baptisée 1R6F et mise au point par le Kentucky Tobacco Research and Development Center, une nouvelle cigarette de référence a été fabriquée en remplacement de la 3R4F, dont le stock vient à épuisement. La finalité de la présente étude fut d'analyser l'interchangeabilité de la 1R6F et de la 3R4F en comparant les caractéristiques chimiques et biologiques de la fumée principale et d'évaluer la reproductibilité interlaboratoire en mettant en regard les résultats obtenus par la présente étude et un rapport précédent. Nous passâmes en revue 45 produits chimiques prioritaires requis par Santé Canada en vue des déclarations réglementaires et évaluâmes les effets toxicologiques de la fumée de cigarette à l'aide d'épreuves toxicologiques normalisées *in vitro* recommandées par le Centre de Coopération pour les Recherches scientifiques relatives au Tabac en vertu des paramètres de fumage intenses et normalisés de l'Organisation internationale de Normalisation (ISO). Les résultats de l'analyse chimique et des épreuves toxicologiques normalisées mirent en exergue une bonne reproductibilité interlaboratoire pour la 1R6F utilisée en guise de cigarette de référence tandis que de légères variations reproductibles furent observées entre la 1R6F et la 3R4F. En outre, nous nous penchâmes sur l'interchangeabilité de la 1R6F et de la 3R4F lors de quelques essais toxicologiques complémentaires détectant le stress oxydatif puisque ce dernier est un indicateur de résultat utilisé dans la recherche sur le tabac sur la prochaine génération de produits d'administration du tabac et de nicotine (PNGs). Tant la 1R6F que la 3R4F présentèrent des réactions comparables aux épreuves sur le stress oxydatif. Dans l'ensemble, nos résultats des évaluations toxicologiques normalisées et chimiques de la 1R6F mirent en lumière la reproductibilité interlaboratoire; laissant ainsi à penser que la 1R6F convient en guise de

cigarette de référence. En outre, les résultats obtenus grâce aux épreuves sur le stress oxydatif nous en apprirent davantage sur l'interchangeabilité de la 1R6F et la 3R4F lorsqu'elles servent à comparer des PNGs. [Beitr. Tabakforsch. Int. 29 (2020) 119–135]

1. INTRODUCTION

Various experiments have been performed to investigate the chemical, physiological, and biological properties of cigarette smoke. Some marketed tobacco products have been employed for such tobacco research; however, the properties of cigarette smoke vary depending on the product specification (e.g., blend of tobacco leaf and filter ventilation). Such variations lead to inconsistent and misleading results in tobacco research when independent results obtained using different tobacco products are compared. To avoid such difficulties, reference cigarettes (e.g., 1R5F, 2R4F, or 3R4F) are manufactured by the Center for Tobacco Reference Products at the University of Kentucky as an international standard for cigarettes used for research purposes. Reference cigarettes are useful as a standard research cigarette because they are designed to approximate the typical commercially available cigarettes in the US (1). Consequently, reference cigarettes are widely used in a variety of tobacco research as a basis for comparison with other tobacco products (2–4), and recently reference cigarettes have come to play a key role as comparators for next generation tobacco and nicotine delivery products (NGPs), such as heat-not-burn type products and e-cigarettes, to investigate their reduced-risk potential (5–11). Reference cigarettes are also used as internal laboratory controls in ongoing analytical work and as a common factor during the comparison and interpretation of results from different laboratories in a variety of tobacco research (1).

Over the last decade, researchers have adopted the 3R4F cigarette as a reference cigarette; however, the stock of 3R4F is being depleted. Since 2015, a new-generation reference cigarette, the 1R6F, has been produced as a replacement for 3R4F. JACCARD *et al.* have reported that 1R6F was considered to be a suitable replacement for 3R4F as a reference cigarette, in terms of chemical analysis and standard toxicological assays, although there were some slight differences (12). However, there are still some important features to be investigated in the use of 1R6F as a reference cigarette because reference cigarettes are used not only for internal quality control but also as a benchmark in inter-laboratory studies, as noted above. In this context, the reproducibility of the experimental results in different laboratories needs to be verified to demonstrate that 1R6F can be used as a reference cigarette.

In the present study, we analyzed the chemical and biological characteristics of the mainstream smoke of 3R4F and 1R6F under International Organization for Standardization (ISO) standard and intense smoking regimens (13, 14). Forty-five priority chemicals referred to in the Tobacco Reporting Regulations published by Health Canada were analyzed, and the standard *in vitro* toxicological assays recommended by the Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA), mutagenicity

Table 1. Characteristics of 1R6F and 3R4F reference cigarettes. The data were obtained from literature (12, 19, 20).

Parameter	3R4F	1R6F
<i>Physical data</i>		
Cigarette length (mm)	84	83
Tobacco rod length (mm)	57	56
Filter length (mm)	27	27
Tobacco rod circumference (mm)	24.8	24.6
Cigarette weight (g)	1.1	0.89
Filter ventilation (%)	29	33
Paper permeability (CU)	24	45
Resistance to draw (mm H ₂ O)	128	107
<i>Blend composition</i>		
Flue cured (%)	35	34
Burley (%)	22	24
Maryland (%)	1.4	–
Oriental (%)	12	12
Reconstituted (%)	29.6	20
Expanded flue cured (%)	–	7
Expanded Burley (%)	–	3
<i>Humectants</i>		
Glycerol (%)	2.7	1.7
Propylene glycol (%)	–	1
Isosweet (%)	6.4	6.3
<i>Yield data from supplier (ISO standard smoking regimen)</i>		
Puff count	9.0	7.5
TPM (mg/cig)	11	10
“Tar” (mg/cig)	9.4	8.6
Nicotine (mg/cig)	0.73	0.72
Carbon monoxide (mg/cig)	12.0	10.1
<i>Yield data from supplier (ISO intense smoking regimen)</i>		
Puff count	^a	8.7
TPM (mg/cig)	^a	46.8
“Tar” (mg/cig)	^a	29.1
Nicotine (mg/cig)	^a	1.90
Carbon monoxide (mg/cig)	^a	28.0

TPM: total particulate matter, cig: cigarette,

CU: CORESTA unit (22)

^a: no published data

with the bacterial reverse mutation (Ames) assay, genotoxicity with the micronucleus (MN) assay, and cytotoxicity with the neutral red uptake (NRU) assay (15), were performed. The obtained data were compared with the results in the JACCARD study to investigate the inter-laboratory reproducibility in the chemical and toxicological analysis of 1R6F.

As described above, reference cigarettes are also currently used as comparators for NGPs to investigate their reduced-risk potential. In these comparative studies, oxidative stress assays are often employed because the oxidative stress response is a key toxicological pathway induced by exposure to conventional cigarette smoke (16, 17). Thus, in the present study, we carried out additional toxicological assessments to examine the interchangeability of 1R6F with 3R4F in the oxidative stress assay. We assessed the level of oxidative stress induced by each reference cigarette using the reduced glutathione (GSH) / oxidized glutathione (GSSG) assay and antioxidant response element (ARE)-

luciferase reporter assay (18) with the human bronchial epithelial cell line, BEAS-2B.

Here we show the inter-laboratory reproducibility of 1R6F compared with 3R4F in chemical analysis and standard toxicological assessments and provide an insight into the interchangeability of 1R6F with 3R4F as a comparator for NGPs.

2. MATERIALS AND METHODS

2.1. Test samples

Kentucky reference cigarettes, 3R4F and 1R6F, were purchased from the Kentucky Tobacco Research and Development Center (Lexington, KY, USA). The specifications of these reference cigarettes are shown in Table 1 (12, 19, 20). The cigarettes were stored at 4 °C and conditioned for at least 48 h at 22 ± 1 °C and 60 ± 3% relative humidity before use, according to ISO 3402 (21).

2.2. Chemical analysis

Mainstream cigarette smoke from 3R4F and 1R6F was generated according to the ISO standard (13) and ISO intense smoking regimens (14). The total particulate matter (TPM) was calculated from the difference in the weight of the glass-fiber filter used for smoke collection before and after smoking (23). The nicotine yield was determined by gas chromatography using an Agilent 7890A GC system (Agilent Technologies, Santa Clara, CA, USA) with flame ionization detection from a 2-propanol extract of the TPM filter (24). Carbon monoxide was determined by non-dispersive infrared photometry using a COA205 (CERULEAN, Milton Keynes, UK) (25). The methods for the other chemical analyses are summarized in Table 2. The analysis of each constituent was repeated five times, and the comparison between 1R6F and 3R4F was performed on a per-cigarette basis.

2.3. Smoke generation for *in vitro* assays and sample preparation

Mainstream cigarette smoke was generated using the automatic smoking machine, RM20H (Borgwaldt, Hamburg, Germany), under ISO standard and intense smoking regimens (13, 14). The TPM was trapped on 44 mm-diameter glass-fiber filter pads and extracted with dimethyl sulfoxide (DMSO) (Fujifilm Wako Pure Chemical, Osaka, Japan) to a final concentration of 10 mg/mL for the Ames assay and NRU assay and 40 mg/mL for the MN assay and additional toxicological assays with BEAS-2B. The gas-vapor phase (GVP) passing through the filter was collected by bubbling into ice-cold phosphate buffered saline (PBS) in an impinger and subjected to the NRU assay. The concentration of the GVP was adjusted to be a 6 mg TPM weight equivalent / mL (mg TPM equiv. / mL) solution based on the yield of the TPM concurrently collected on the glass-fiber filter pad. The GVP was subjected to the assay within 1 h after the completion of smoking. Three independent processes of the sample preparation were carried out for each *in vitro* assay.

Table 2. Analysis methods used for specific analytes in mainstream cigarette smoke.

Analytes	Fraction (smoking machine)	Trap	Chromatograph, detection	Notes	Ref.
<i>TSNAs</i> NNK, NNN, NAB, NAT	TPM (RM20H ^a)	A glass-fiber filter	HPLC; Agilent 1290 infinity system ^c , Triple Quad 4500 MS system ^d	The extract with ammonium acetate solution was syringe filtered.	26
<i>Carbonyls</i> Formaldehyde, acetaldehyde, acrolein, crotonaldehyde, acetone, propionaldehyde, <i>n</i> -butylaldehyde, MEK VOCs	WS (SM450RH ^b)	Two impingers with 2, 4-dinitrophenyl-hydrazine solution	HPLC; Agilent 1290 Infinity system ^c , Diode array detection	Derivatized solution was stabilized with Tris base.	27
1,3-Butadiene, benzene, isoprene, acrylonitrile, toluene	GVP (SM450RH ^b)	Two cryogenic impingers with methanol	GC/MS; 5975C GC/MSD ^c , – Electron impact ionization	–	28
<i>Cyanic compound</i> HCN	GVP TPM (SM450RH ^b)	A glass-fiber filter and an impinger with NaOH solution	Continuous flow analyzer; STAT-2000 ^e	The extract with NaOH was syringe filtered.	29
<i>PAH</i> B[a]P	TPM (SM450RH ^b)	A glass-fiber filter	HPLC; HPLC-1100 system ^c , Fluorescence detection	The extract with cyclohexane was syringe filtered and purified using SPE by passing through a silica cartridge and an NH ₂ cartridge, followed by the elution with hexane.	30
<i>Phenols</i> Hydroquinone, resorcinol, catechol, phenol, <i>o</i> -cresol, <i>m</i> -cresol, <i>p</i> -cresol	TPM (SM450RH ^b)	A glass-fiber filter	HPLC; Agilent 1290 Infinity system ^c , Fluorescence detection	The extract with acetic acid was syringe filtered.	31
<i>Ammonia</i> Ammonia	GVPTPM (RM20H ^a)	A glass-fiber filter and two impingers with sulfuric acid	Cation exchange chromatograph; ICS-3000 ^f , Suppressed ion conductivity detection	The extract with ultrapure water was syringe filtered.	32
<i>NO_x</i> NO, NO _x	GVP (RM20H ^a)	–	Online gas phase chemiluminescence analyzer; CLD822Mh ^g	–	33
<i>SVOCs</i> Pyridine, quinoline, styrene	GVP TPM (RM20H ^a)	A glass-fiber filter and two cryogenic impingers with methanol	GC/MS; 5975C GC/MSD ^c , Electron impact ionization	TPM was extracted with the impinger solution.	34
<i>Aromatic amines</i> 1-Aminonaphthalene, 2-aminonaphthalene, 3-aminobiphenyl, 4-aminobiphenyl	TPM (RM20H ^a)	A glass-fiber filter	GC/MS; 5975C GC/MSD ^c , Chemical ionization source (selected ion monitoring)	The extract with hydrochloric acid solution was syringe filtered and loaded to the conditioned SPE-I with ammonia solution and methanol, and then loaded to the SPE-II with toluene. The derivatized solution with heptafluorobutyric acid anhydride solution was loaded to SPE-III.	35
<i>Heavy metals</i> Mercury	GVP (RM20H ^a)	An impinger with acidified potassium permanganate	AAS; FIMS 400 ^h , cold vapor atomic absorption spectrometry	Hydrogen peroxide and ultrapure water were added for microwave digestion. Hydroxylamine hydrochloride was added. Mercury ions in the sample were reduced by stannous chloride.	36
Arsenic, cadmium, chromium, nickel, lead, selenium, beryllium, cobalt	TPM (RM20H ^a)	Glass electrostatic precipitate tube	ICP/MS; Agilent 7500cxc	Collected metals with methanol were mixed with Nitric acid solution, TritonX-100 solution by ultrasonication.	–

TPM: total particulate matter, GVP: gas vapor phase, WS: whole smoke.

TSNA: tobacco specific *N*-nitrosamine, NNN: *N*-nitrosonornicotine, NAT: *N*-nitrosoanatabine, NAB: *N*-nitrosoanabasine, NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, VOC: volatile organic compound, MEK: methyl ethyl ketone, HCN: hydrogen cyanide, PAH: polycyclic aromatic hydrocarbon, B[a]P: benzo[a]pyrene, SPE: solid phase extraction, NO_x: nitrogen oxides, SVOC: semi-volatile organic compound, HPLC: high-performance liquid chromatography, GC: gas chromatography, MS: mass spectrometry, AAS: atomic absorption spectroscopy, ICP: inductively coupled plasma, RM20H: an automatic smoking machine, SM450RH: an automatic linear smoking machine

Instruments purchased from:

^a Borgwaldt, Hamburg, Germany;

^b CERULEAN, Milton Keynes, UK;

^c Agilent Technologies, Santa Clara, CA, USA;

^d SCIEX, Framingham, MA, USA;

^e BL TEC, Osaka, Japan;

^f Thermo Fisher Scientific, Waltham, MA, USA;

^g ECOPHYSICS, Dürnten, Switzerland;

^h PerkinElmer, Waltham, MA, USA.

2.4. Standard toxicological assay

In vitro toxicological assays recommended by CORESTA (Ames assay, MN assay, and NRU assay) were carried out. All experimental data were evaluated as previously described (6). Each experiment was conducted three times independently.

2.4.1 Ames assay

The Ames assay was performed according to the Organization for Economic Cooperation and Development (OECD) guideline 471 (37). *Salmonella typhimurium* strains TA98, TA100, TA1537, TA1535, and TA102 were provided by the National Institute of Health Sciences (Kanagawa, Japan). The TPM samples were tested using the five tester strains with and without metabolic activation by S9 mix (Ieda Trading, Tokyo, Japan) containing rat liver S9 induced by phenobarbital and 5,6-benzoflavone. Eight doses of up to 1000 µg/plate were tested for each TPM. DMSO was used as a solvent control for all conditions. The frozen stock of bacterial culture was inoculated into a flask containing Oxoid Nutrient Broth No. 2 (Thermo Fisher Scientific, Waltham, MA, USA). The bacterial culture was incubated for 9 h at 37 °C on an orbital shaker. A reaction mixture containing 100 µL of bacterial suspension (> 10⁹ cells/mL), 100 µL of TPM, and 500 µL of the S9 mix was incubated in a test tube at 37 °C for 20 min. Following the incubation, 2 mL of molten top agar supplemented with histidine and biotin was added to the reaction mixture. Then, the mixture was poured on to a minimal-glucose agar plate. The S9 mix was replaced with phosphate buffer to determine the mutagenicity without metabolic activation. Plates were incubated at 37 °C for 72 h for TA102, and for 48 h for the other strains, TA98, TA100, TA1535, and TA1537. The number of revertant colonies was counted with an automatic colony counter (System Science, Tokyo, Japan). The mutagenic activity of each TPM obtained in the Ames assay was evaluated based on the slope values of the linear dose-response curves using linear regression analysis.

2.4.2 *In vitro* Micronucleus (MN) assay

The *in vitro* MN assay was performed in principle according to OECD guideline 487 (2014) (38) using the Chinese hamster lung cell line (CHL/IU). CHL/IU was obtained from the National Institutes of Biomedical Innovation, Health and Nutrition (Osaka, Japan). The TPM

samples were assayed using three treatment conditions: short-term exposure (“Short”), with or without S9 metabolic activation by the S9 mix and long-term exposure (“Long”) without metabolic activation. Prior to exposure to the TPM, cells were cultured at 2 × 10³ cells / 200 µL/well in minimum essential medium (MEM) (Thermo Fisher Scientific) supplemented with 10% bovine serum (BS) (Thermo Fisher Scientific) using a 96-well collagen-I coated plate (Thermo Fisher Scientific) at 37 °C in a 5% CO₂ atmosphere for 24 h. Ten doses up to 600 µg/mL were tested for each TPM and DMSO was used as a solvent control. In “Short”, with and without S9, the pre-cultured cells were treated with the medium including the TPM for 3 h. All samples were added to the medium at a final concentration of 2% DMSO. In “Short” with S9, and “Short” without S9, MEM supplemented with 10% BS and MEM supplemented with 1% BS and 5% S9 mix (Ieda Trading) were used, respectively. After removal of the supernatant including the TPM, the cells were further incubated in fresh MEM supplemented with 10% BS for 21 h. In “Long” without S9, the cells were treated with MEM supplemented with 10% BS, including each TPM for 24 h. All samples were added to the medium at a final concentration of 1% DMSO. After exposure, cells were stained with Cell mask orange solution (Thermo Fisher Scientific) for the plasma membrane, Cell stain Hoechst 33342 solution (Dojindo Laboratories, Tokyo, Japan) for the nucleus, and SYTOX Green Nucleic Stain (Thermo Fisher Scientific) for dead cells. Then, cells were fixed with 4% paraformaldehyde for more than 5 min. All plates were scanned for cell and nucleus images using a CellInsight CX5 (Thermo Fisher Scientific). The number of micronucleated cells per 2 × 10³ live cells per well was analyzed and the MN cell frequency (% MN) was calculated.

Logistic regression analysis was performed with data up to the dose at which the relative population doubling (38) was greater than 40%. The slope parameter of the logistic function was defined as the measure for genotoxic activity.

2.4.3 Neutral red uptake (NRU) assay

The NRU assay was performed using the Chinese hamster ovary cell line (CHO-K1) in general accordance with Health Canada Official Method T-502 (39). Ten doses up to 200 µg/mL (TPM) and 200 µg TPM equiv./mL (GVP) were tested for each sample. DMSO and PBS were used as the solvent controls for the TPM and GVP samples, respectively.

The cells were maintained with Ham's F-12 nutrient mixture medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA) in a 5% CO₂ incubator at 37 °C. The cell suspension (5 × 10⁴ cells/mL) was pre-incubated for 24 h in 96-well plates (Sumitomo Bakelite, Tokyo, Japan). The cells were exposed to each smoking fraction for 24 h. After removal of the supernatant including the TPM or GVP, the cells were further incubated in serum-free medium containing 18 µg/mL neutral red dye for 3 h. The cells were then fixed with 1% formalin solution for 2 min. After removal of the fixative, the neutral red dye taken up by the viable cells was extracted by adding 50% ethanol containing 1% acetic acid solution, and the absorbance at 540 nm was measured using a microplate reader (CORONA ELECTRIC, Ibaraki, Japan). The absorbance value at each dose level was normalized to the solvent control.

Non-linear regression analysis for the relationship between the normalized absorbance and concentration was performed based on the least squares method using a logistic function. The half maximal inhibitory concentration (IC₅₀) values were calculated by inverse estimation of the effective concentration at which the normalized absorbance was reduced by 50% as the indicator of toxicity.

2.5. Additional toxicological assays

The methods for the cell viability assay, GSH/GSSH assay, and ARE-luciferase reporter assay have been described in detail previously (5). The experiments were conducted three times independently.

2.5.1 Cell culture and exposure conditions

Immortalized normal human bronchial epithelial BEAS-2B cells, obtained from the American Type Culture Collection (Manassas, VA, USA), were cultured in Dulbecco's MEM (Thermo Fisher Scientific) supplemented with 10% FBS (MP Biomedicals, Santa Ana, CA, USA) in a 5% CO₂ incubator at 37 °C. The cells were seeded at 5 × 10³ cells/well in a 96-well plate (Corning, NY, USA) followed by pre-incubation for 24 h. Then, the cells were exposed to the medium containing each TPM up to 400 µg/mL (1% DMSO) in a 5% CO₂ incubator at 37 °C.

2.5.2 Cell viability assay

Cell viability was determined using a CellTiter-Fluor cell viability assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Briefly, cells were exposed to each concentration of TPM in a 96-well plate (Corning) for 24 h, and the constitutive protease activity within living cells as cell viability was measured with an Infinite 200 PRO microplate reader (Tecan, Männedorf, Switzerland). Tween (3%) was used as a positive control to induce complete cell death. The value of the positive control was deducted from the original values at each dose level, then obtained values were normalized to the concurrent solvent control. The IC₅₀ values were calculated as for the NRU assay.

2.5.3 GSH/GSSG assay

The GSH/GSSG ratio was determined using a GSH/GSSG-Glo assay kit (Promega) according to the manufacturer's instructions. Briefly, after exposure to each TPM for 2 h, cells were lysed with GSH and GSSG lysis buffer to measure total glutathione and GSSG, respectively. Subsequently, enzyme and substrate were added, and then luciferase activity was measured using an Infinite 200 PRO microplate reader. Then, the GSH/GSSG ratio was calculated by interpolation of glutathione concentrations from standard curves. The values at each dose level were normalized to the solvent control. The IC₅₀ values were calculated as for the NRU assay.

2.5.4 ARE-luciferase reporter assay

BEAS-2B cells were transfected with the luciferase gene as the reporter under the transcriptional regulation of ARE (18). The ARE-luciferase reporter assay was carried out using a Luciferase Assay System (Promega) following the manufacturer's instructions. Briefly, cells were lysed after exposure to each TPM for 24 h in a 96-well plate (Corning). Then, the substrate was added, and luciferase activity was measured using an Infinite 200 PRO microplate reader to determine the ARE gene activity. The values at each dose level were normalized to the solvent control. The ARE gene activity for each TPM was evaluated based on the slope values calculated by linear regression analysis over the dose range where cell viability was higher than 50%.

2.6. Statistical analysis

All the statistical evaluations were conducted using JMP version 10.0.2 (SAS Institute Japan, Tokyo, Japan). The comparison of 1R6F and 3R4F was performed based on the critical difference (CD) calculated using 3R4F data variations. The CD value is equivalent to discriminatory power, which is the smallest statistically significant difference that can be detected with an assay based on the variability of data with the significance level of $\alpha = 0.05$ (40).

The range of CD values of 3R4F has been previously described (12);

$$CD(\%) = 3 \times \sqrt{2} \times RSD[\%] \times \sqrt{\frac{1}{n}} \quad [1]$$

We calculated the CD values in the chemical analysis using the 3R4F data sets in the current and JACCARD studies. Each CD of the standard toxicological assays was calculated using the 3R4F historical monitor data set in the current study, while the CD values from the JACCARD study were obtained from literature values. The difference between 1R6F and 3R4F was evaluated as significant when the relative difference of 1R6F compared with 3R4F was out of the CD range. The comparison between 1R6F and 3R4F in the additional toxicological assay was performed based on Student's t-test ($p < 0.05$).

Figure 1. Relative differences in the chemical constituents of mainstream cigarette smoke between 3R4F and 1R6F under the ISO standard smoking regimen. Circles and triangles indicate the relative differences in the chemical yields of 1R6F compared with 3R4F in the current and JACCARD studies (12), respectively. The boxes show the critical difference ranges of 3R4F. “Empty” or “filled” symbols indicate whether the relative difference was within or out of the critical difference range, respectively. LOQ indicates the limit of quantitation.

3. RESULTS

3.1. Chemical analysis

The amount of TPM, nicotine, carbon monoxide, and 45 other chemical constituents in the mainstream cigarette smoke of 1R6F and 3R4F was analyzed under ISO standard and intense smoking regimens (Supplementary Table A1). To compare the mainstream smoke chemistry of 1R6F and 3R4F, the relative differences in the yields of each constituent in 1R6F compared with 3R4F were calculated (circles in Figures 1 and 2), then these differences were compared with the CD range calculated using the data from 3R4F (boxes in Figures 1 and 2). The relative differences for most of the chemicals were out of the CD range under both smoking regimens (filled circles in Figures 1 and 2). Thus, these constituents in the mainstream cigarette smoke of 1R6F were determined to be significantly different from those of 3R4F in the current study.

To investigate the inter-laboratory reproducibility, the results obtained in the current study (circles in Figures 1 and 2) were compared with those from the JACCARD study in a different laboratory (triangles in Figures 1 and 2). Under the ISO standard smoking regimen, the yields of NAT, NAB, NNK, acetone, methyl ethyl ketone (MEK), NO, NO_x, and mercury in 1R6F were significantly lower than those in 3R4F, while the yields of formaldehyde, B[a]P, hydroquinone, catechol, phenol, *p*-cresol, *m*-cresol, ammonia, and quinoline in 1R6F were significantly higher

than those in 3R4F, in both the studies (Figure 1). Although there were significant differences between 1R6F and 3R4F in some constituents observed in only one of the studies, all these constituents, except for acrolein and 3-aminobiphenyl, showed a consistent tendency in the relative differences compared with the JACCARD study. For example, the relative difference of toluene was out of the CD range only in the current study; however, the relative difference was a negative value in both the studies (Figure 1).

Under the ISO intense smoking regimen, the yields of TSNAs, acetaldehyde, acetone, MEK, *n*-butylaldehyde, NO, NO_x, 3-aminobiphenyl, 4-aminobiphenyl, mercury, and cadmium in 1R6F were significantly lower than in 3R4F, while the yield of formaldehyde in 1R6F was significantly higher than in 3R4F in both the current and JACCARD study (Figure 2).

Similar to the ISO standard smoking regimen, some constituents showed significant differences only in one of the studies but there was a consistent trend, except for acrolein and pyridine. The other constituents not mentioned above were similar in both the studies regardless of the smoking regimen.

3.2. Standard toxicological assay

3.2.1 Ames assay

The mutagenicity of each TPM was investigated using TA98, TA100, TA1535, TA1537, and TA102, with or

Figure 2. Relative differences in the chemical constituents of mainstream cigarette smoke between 3R4F and 1R6F under the ISO intense smoking regimen. Circles and triangles indicate the relative differences in the chemical yields of 1R6F compared with 3R4F in the current and JACCARD studies (12), respectively. The boxes show the critical difference ranges of 3R4F. “Empty” or “filled” symbols indicate whether the relative difference was within or out of the critical difference range, respectively. LOQ indicates the limit of quantitation.

without S9 metabolic activation. In the current study, the 1R6F and 3R4F TPM elicited dose-dependent increases in the number of revertants in TA98 with S9, TA100 with and without S9, and TA1537 with S9, regardless of the smoking regimen (Supplementary Table A2). Consequently, the slope values were calculated as the mutagenic activities in these conditions. In the remaining strains and conditions, both the 1R6F and 3R4F TPM showed no reproducible increases in the number of revertants.

To examine the interchangeability of 1R6F and 3R4F regarding the mutagenicity, the relative differences in the slope values between 1R6F and 3R4F in the current study were analyzed (circles in Figure 3A and B for the ISO

standard and intense smoking regimens, respectively). Then, these values were compared with the CD range calculated based on historical variations in 3R4F (boxes in Figure 3A and B). The results indicated that the relative differences were within the range of the CD in TA98 with S9, TA100 with and without S9, and TA1537 with S9, regardless of the smoking regimen (“empty” circles in Figure 3A and B).

The inter-laboratory reproducibility was then examined by comparing the results obtained in the current study (circles in Figure 3A and B) and JACCARD study (triangles in Figure 3A and B). With S9, the relative differences in the mutagenicity were within the range of the CD in TA98, TA100,

Figure 3. Relative differences between 3R4F and 1R6F in the Ames assay. (A) ISO standard smoking regimen; (B) ISO intense smoking regimen. Circles and triangles indicate the relative differences in the mutagenic slope value of 1R6F compared with 3R4F in the current and JACCARD studies (12), respectively. The boxes show the critical difference ranges of 3R4F. “Empty” symbols indicate the relative difference were within the critical difference ranges. Equivocal indicates there was no reproducible significant increase in revertants over three replicates. Non-mutagenic means there was no significant increase in revertants over three replicates.

and TA1537, regardless of the smoking regimen in both the studies (“empty” circles and triangles in Figure 3A and B). Without S9, the mutagenicity of the TPM from both the reference cigarettes was found in TA98 only in the JACCARD study and in TA100 only in the current study, regardless of the smoking regimen (Figure 3A and B). Therefore, the results for the conditions with S9 were reproducible although there were some inter-laboratory differences in the mutagenic response in TA98 and TA100 without S9.

3.2.2 *In vitro* MN assay

The *in vitro* MN assay was conducted using CHL/IU cells under three treatment conditions: “Short”, with or without S9, and “Long” without S9. The 1R6F and 3R4F TPM induced dose-related increases in the MN frequency under all the tested conditions. The slope parameter of the logistic function calculated as genotoxic activity for each condition are shown in Supplementary Table A3.

To compare the genotoxicity between 1R6F and 3R4F in the current study, the relative differences in the slope parameter of the logistic function of 1R6F compared with 3R4F were calculated (circles in Figure 4A and B for the ISO standard and intense smoking regimens, respectively), then compared with the CD range calculated based on historical variations in 3R4F (boxes in Figure 4A and B).

Figure 4. Relative differences between 3R4F and 1R6F in the MN assay. (A) ISO standard smoking regimen; (B) ISO intense smoking regimen. Circles and triangles indicate the relative difference in the genotoxic logit slope values of 1R6F compared with 3R4F in the current and JACCARD studies (12), respectively. The boxes show the critical difference ranges of 3R4F. “Empty” or “filled” symbols indicate whether the relative difference was within or out of the critical difference range, respectively.

The relative differences were within the range of the CD in “Short” with S9, and “Long” without S9 under the ISO standard smoking regimen (“empty” circles in Figure 4A) and in all conditions under the ISO intense smoking regimen (“empty” circles in Figure 4B), while the relative difference was out of the range of the CD in “Short” without S9 under the ISO standard smoking regimen (“filled” circles in Figure 4A). Inter-laboratory reproducibility was then analyzed by a comparison of the results obtained in the current study (circles in Figure 4A and B) and JACCARD study (triangles in Figure 4A and B). The

results from both the studies showed a good agreement. In “Short” without S9 under the ISO standard smoking regimen, the relative genotoxic activity of 1R6F was higher than the upper limit of the CD range (filled circles and triangles in Figure 4A and B). For the other conditions, the relative differences were within the range of the CD (empty circles and triangles in Figure 4A and B). Therefore, inter-laboratory reproducibility in the MN assay was confirmed.

3.2.3 *NRU* assay

Both the TPM and GVP collected from 3R4F and 1R6F elicited dose-dependent decreases in the absorbance, regardless of the smoking regimen. The IC_{50} values for the reference cigarettes are shown in Supplementary Table A4. For a comparison of the cytotoxicity of 1R6F and 3R4F in the current study, the relative differences in the IC_{50} values of 1R6F compared with 3R4F were calculated (circles in Figure 5A and B for the ISO standard and intense smoking regimens, respectively), then compared with the CD range calculated based on historical variations in 3R4F (boxes in Figure 5A and B).

The relative difference was out of the CD range for the TPM under the ISO intense smoking regimen (filled circles in Figure 5B), while the relative differences in the remaining conditions were within the range of the CDs (empty circles in Figure 5A and B).

Figure 5. Relative differences between 3R4F and 1R6F in the NRU assay. (A) ISO standard smoking regimen; (B) ISO intense smoking regimen. Circles and triangles indicate the relative differences in the IC_{50} values of 1R6F compared with 3R4F in the current and JACCARD studies (12), respectively. The boxes show the critical difference ranges of 3R4F. “Empty” or “filled” symbols indicate whether the relative difference was within or out of the critical difference range, respectively.

We further analyzed the inter-laboratory reproducibility by comparing the results obtained in the current study (circles in Figure 5A and B) and JACCARD study (triangles in Figure 5A and B).

For the TPM under the ISO intense smoking regimen, the trend in the relative difference in the IC_{50} values was different from the JACCARD study within $\pm 10\%$ (Figure 5B).

Under the remaining conditions, the results in the current and JACCARD studies were consistent.

Table 3. IC₅₀ values of 1R6F and 3R4F TPM in the cell viability assay. The IC₅₀ values were calculated by inverse estimation of the effective concentration at which the normalized fluorescence was reduced by 50%. S.E. stands for standard error. *p* values were calculated based on Student's *t*-test (*n* = 3).

Smoking regimen	1R6F IC ₅₀ (μg/mL)		3R4F IC ₅₀ (μg/mL)		Relative difference (1R6F/3R4F)		<i>p</i> value (<i>t</i> -test)
	Mean	S.E.	Mean	S.E.	Mean	S.E.	
Standard	75.0	2.44	73.3	2.19	1.02	0.0281	0.62
Intense	79.5	3.98	82.2	4.06	0.975	0.0836	0.67

3.3. Additional toxicological assays

3.3.1 Cell viability

We carried out some additional *in vitro* toxicological assays to investigate oxidative stress with BEAS-2B, a cell line derived from normal human bronchial epithelium. Cell viability was determined following exposure to the TPM collected from 3R4F and 1R6F using both ISO standard and intense smoking regimens. All the TPM exposures induced dose-dependent decreases in cell viability (Figure 6). No significant difference was observed in the IC₅₀ values between 3R4F and 1R6F under either smoking regimen (Table 3).

Figure 6. Dose-response curves in the cell viability assay. Cell viability was measured after 24 h exposure to TPM. The values for the solvent control were set at 100 (%). “Empty” and “filled” symbols represent the means of the viability for 1R6F and 3R4F, respectively. The error bars indicate standard error (*n* = 3). The solid and dotted lines indicate the dose-response curves for the ISO standard and intense smoking regimens, respectively.

3.3.2 Oxidative stress responses

The GSH/GSSG ratio and ARE gene activity were used as indicators of oxidative stress, and changes in these parameters following exposure to the TPM of 3R4F or 1R6F were evaluated under the ISO standard and intense smoking regimens. All the TPM exposures induced reduction of the GSH/GSSG ratio in a dose-dependent manner (Figure 7). A significant difference in the IC₅₀ values between 1R6F and 3R4F was not observed, regardless of the smoking regimen (Table 4).

Exposure to the TPM resulted in significant increases in ARE gene activity regardless of the condition (Figure 8). The decline in luciferase activity observed at a high dose level was possibly because of cytotoxicity. Although 1R6F tended to show higher activity than 3R4F in the ARE-luciferase reporter assay, no significant difference was observed between the slope activities of 3R4F and 1R6F under both smoking regimens, as calculated using Student's *t*-test (Table 5).

Figure 7. Dose-response curves in the GSH/GSSG assay. The intracellular GSH/GSSG ratio was determined after exposure for 2 h to the TPM. “Empty” and “filled” symbols represent the means of the GSH/GSSG ratios of 1R6F and 3R4F, respectively. The error bars indicate standard error (*n* = 3). The solid and dotted lines indicate the dose-response curve under the ISO standard and intense smoking regimens, respectively.

4. DISCUSSION

Reference cigarettes have been widely used for research purposes related to tobacco as the standard monitor cigarette. Recently, 1R6F reference cigarettes have been produced as a replacement for 3R4F because of the depletion of 3R4F stock. Thus, verification that 1R6F can perform the same functions as 3R4F in various tobacco research is required. A comparison study of the chemical and biological characteristics of 1R6F and 3R4F has been reported by JACCARD *et al.* (12) to investigate the interchangeability. The JACCARD study reported that 1R6F was a suitable replacement for 3R4F, while some slight differences were observed in the mainstream smoke between 1R6F and 3R4F in the chemical analysis and the *in vitro*

Table 4. IC₅₀ values of 1R6F and 3R4F TPM in the GSH/GSSG assay. IC₅₀ values in the GSH/GSSG assay were calculated by inverse estimation of the effective concentration at which the normalized luminescence was reduced by 50%. S.E. stands for standard error. *p* values were calculated based on Student's *t*-test (*n* = 3).

Smoking regimen	1R6F IC ₅₀ (μg/mL)		3R4F IC ₅₀ (μg/mL)		Relative difference (1R6F/3R4F)		<i>p</i> value (<i>t</i> -test)
	Mean	S.E.	Mean	S.E.	Mean	S.E.	
Standard	35.0	7.74	40.0	9.36	0.877	0.0128	0.70
Intense	66.5	8.25	73.4	18.9	0.970	0.139	0.76

MN assay for genotoxicity. In this context, we examined the inter-laboratory reproducibility of these results with the results in the current study.

Some significant differences in the results of the chemical analyses between 1R6F and 3R4F were observed in the current study (Figure 1A and B), as expected, because the 1R6F reference cigarette is produced with a different blend design from 3R4F. These results were mostly consistent with the JACCARD study (see also Supplementary Figure A1); therefore, the use of 1R6F was determined to have inter-laboratory reproducibility in terms of the analytical chemistry.

Figure 8. Dose-response curves in the ARE-luciferase reporter assay. Luciferase activity was measured after exposure to the TPM for 24 h to determine ARE gene activity. “Empty” and “filled” symbols represent the means of ARE-luciferase gene activity for 1R6F and 3R4F, respectively. The error bars indicate standard error (*n* = 3). The solid and dotted lines indicate the dose-response curve under the ISO standard and intense smoking regimens, respectively.

The results of the Ames assay under the conditions with S9 in the current study were also consistent with the JACCARD study (Figure 3A and B), in which they reported that no

significant differences were observed between 1R6F and 3R4F. However, there were some differences between the current and JACCARD studies in TA98 and TA100 without S9. In the current study, the mutagenicity in TA98 was equivocal and only TA100 showed a clear mutagenic response to TPM without S9. In contrast, the JACCARD study reported that TA98 without S9 showed a reproducible increase in the revertants in a dose-dependent manner for both reference cigarettes, while TA100 without S9 did not (Supplementary Table A3). Cigarette smoke has been shown to elicit mutagenicity in TA98 with S9, TA100 with S9, and TA1537 with S9, while an inter-laboratory inconsistency has been often observed in the case of TA98 and TA100 without S9 (41, 42), even when using the same TPM collected in a smoking procedure (43). Consequently, although differences were observed between the current and JACCARD study in the response to the TPM of the strains without S9, these differences were not derived from the smoke generation and smoking process.

In the current study, the MN induction by 1R6F was significantly higher compared with 3R4F in “Short” without S9 under the ISO standard smoking regimen (Figure 4A). The JACCARD study reported that although the MN frequency of 1R6F was also higher than 3R4F in “Short” without S9 under the ISO standard smoking regimen, this difference may be a chance finding. However, this difference was reproducibly observed in the current study. Moreover, CHO cells were used in accordance with Health Canada guidelines (44) in the JACCARD study, whereas, we used CHL/IU cells in accordance with OECD TG487 (38), so this tendency may be reproducible despite the differences in cell type and protocol. Thus, we concluded that the MN increase in 1R6F compared with 3R4F was meaningful. This conclusion is reasonable considering the relationship with the results of the chemical analysis. JACCARD *et al.* have suggested that the increases in acetamide and acrylamide in 1R6F possibly contributed to the increased MN induction (12). Although we did not quantify these two compounds, we found that the yields of phenolic compounds such as phenol, which induce MN (45), were higher in 1R6F than in 3R4F, especially under the ISO

Table 5. Slope values of 1R6F and 3R4F TPM in the ARE-luciferase reporter assay. The slope values in the ARE-luciferase reporter assay were calculated by linear regression analysis over the dose range where cell viability was higher than 50%. S.E. stands for standard error. *p* values were calculated based on Student's *t*-test (*n* = 3).

Smoking regimen	1R6F slope value		3R4F slope value		Relative difference (1R6F/3R4F)		<i>p</i> value (<i>t</i> -test)
	Mean	S.E.	Mean	S.E.	Mean	S.E.	
Standard	0.394	0.0441	0.305	0.0456	1.32	0.133	0.23
Intense	0.377	0.0682	0.266	0.0376	1.46	0.292	0.23

standard smoking regimen. Increases in phenolic compounds were also observed in the JACCARD study; therefore, not only acetamide and acrylamide but also phenolic compounds could be responsible for the increase in MN induction.

A significant difference in the IC₅₀ values was observed between the 1R6F and 3R4F TPM under the ISO standard smoking regimen in the NRU assay only in the current study (Figure 5B).

However, the relative difference between the 1R6F and 3R4F TPM under the ISO standard smoking regimen was within $\pm 10\%$, and a significant difference was not observed in the ISO intense smoking regimen. In addition, the difference between the lower limit of the CD (-9.4%) and relative difference compared with 1R6F (-9.9%) was less than 0.5% . Accordingly, the difference in the NRU assay was considered to be not consequential. Thus, inter-laboratory reproducibility in the results of the NRU assay was almost achieved. The results for the GVP in the NRU assay were comparable between the current and JACCARD study regardless of the smoking regimen, although the relative difference in the yields of acrolein obtained in the current study was different from the JACCARD study under both smoking regimens. Acrolein, which is mainly present in the gas phase of cigarette smoke, contributes to cytotoxicity in mammalian cells (46). However, the inter-laboratory difference observed in the relative yield of acrolein in 1R6F compared with 3R4F (within $\pm 15\%$) was not enough to affect the biological endpoints.

We also investigated the interchangeability of 1R6F with 3R4F in oxidative stress assays because such assays are often employed for the comparative assessment of NGPs. No significant differences were observed between 1R6F and 3R4F in dose-related responses in the GSH/GSSG assay and ARE-luciferase reporter assay.

These results were in accordance with a previous report that demonstrated that the yields of free radicals, which are known to cause the oxidative stress, were comparable between 1R6F and 3R4F (47). However, the interchangeability of 1R6F should be studied further in various other assays and investigations, including *in vitro* three-dimensional culture assays and *in vivo* assays used for the comparative assessment of NGPs (7–11) because differences in the smoke chemistry, which did not contribute to the differences observed in the *in vitro* assays in this study, could be critical for these assays.

In addition, the inter-laboratory reproducibility of the oxidative stress assays was not assessed in this study and requires further investigation.

5. CONCLUSION

Overall, our results suggested that the chemical analysis and standard toxicological assays of 1R6F, as compared with 3R4F, could be sufficiently reproducible in inter-laboratory studies for the use of 1R6F as a reference cigarette. Both reference cigarettes could be used interchangeable, although some slight but reproducible differences were observed in the chemical analysis and genotoxicity assay. In addition, 1R6F could replace 3R4F and most likely should be useful for general tobacco research, including the study of NGPs.

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Supplementary Information

Table A1. Chemical constituents in the mainstream smoke of 1R6F and 3R4F.

Analyte	Unit	ISO standard smoking regimen						ISO intense smoking regimen					
		1R6F		3R4F		Ratio (1R6F/3R4F)		1R6F		3R4F		Ratio (1R6F/3R4F)	
		mean	S.E.	mean	S.E.			mean	S.E.	mean	S.E.		
TPM	mg/cig	10.5	0.148	9.9	0.389	106	(–)	42.4	1.19	43.3	0.304	97.9	(–)
Nicotine	mg/cig	0.720	0.0116	0.700	0.0152	103	(111)	1.92	0.0326	1.97	0.0492	97.5	(95.5)
Carbon monoxide	mg/cig	10.0	0.143	10.6	0.371	94.3	(102)	27.5	0.38	30.1	0.622	91.4	(85.9)
<i>TSNAs</i>													
NNN	ng/cig	100	3.58	116	4.02	86.2	(76.3)	221	5.37	279	4.92	79.2	(68.2)
NAT	ng/cig	107	0.894	118	2.68	90.7	(80.9)	253	5.37	291	3.13	86.9	(77.5)
NAB	ng/cig	12.9	0.224	15.0	0.224	86.0	(82.7)	29.6	0.626	35.6	0.358	83.1	(76.7)
NNK	ng/cig	77.8	2.33	103	3.13	75.5	(74.8)	177	3.58	243	4.92	72.8	(65.1)
<i>Carbonyls</i>													
Formaldehyde	µg/cig	37.6	1.21	27.3	1.07	138	(136)	134	4.02	106	3.58	126.4	(119)
Acetaldehyde	µg/cig	548	9.39	594	11.18	92.3	(89.1)	1570	17.89	1697	14.31	92.5	(87.1)
Acetone	µg/cig	179	2.68	202	3.13	88.6	(86.7)	503	3.13	574	5.81	87.6	(84.8)
Acrolein	µg/cig	55.9	1.21	50.2	1.12	111	(97.4)	177	1.34	166	0.447	106.6	(96.1)
Propionaldehyde	µg/cig	40.3	0.626	42.9	0.805	93.9	(90.9)	115	0.894	124	1.34	92.7	(88.1)
Crotonaldehyde	µg/cig	12.2	0.358	11.1	0.358	110	(101)	54.8	0.402	54.9	0.537	99.8	(92.4)
MEK	µg/cig	41.8	0.581	48.3	0.894	86.5	(85.3)	119	0.447	143	1.79	83.2	(82.4)
n-Butylaldehyde	µg/cig	25.0	0.492	27.5	0.76	90.9	(91.2)	68.1	0.939	77.1	1.3	88.3	(86.3)
<i>VOCs</i>													
1,3-Butadiene	µg/cig	40.9	0.358	41.5	1.12	98.6	(104)	96.1	1.21	95.2	1.7	100.9	(102)
Isoprene	µg/cig	314	3.58	341	8.05	92.1	(99.3)	766	15.21	816	16.1	93.9	(94.1)
Acrylonitrile	µg/cig	8.19	0.116	9.39	0.264	87.2	(100)	26.1	0.224	28.9	0.179	90.3	(93.7)
Benzene	µg/cig	42.2	0.358	46.1	1.16	91.5	(100)	91.2	0.671	99.4	1.03	91.8	(92.7)
Toluene	µg/cig	64.7	0.76	78.6	2.1	82.3	(91.8)	160	1.34	191	1.34	83.8	(86.3)
<i>Cyanic compounds</i>													
HCN	µg/cig	98.4	1.52	99.2	2.37	99.2	(107)	404	10.29	470	8.94	86.0	(903)
<i>PAH</i>													
B[a]P	ng/cig	7.51	0.121	6.49	0.17	116	(110)	16.9	0.268	16.9	0.716	100.0	(91.4)
<i>Phenols</i>													
Hydroquinone	µg/cig	35	0.402	30.4	0.313	115	(113)	87.1	0.984	84.1	0.447	103.6	(108)
Resorcinol	µg/cig	0.718	0.0237	0.494	0.0152	145	–	2.15	0.0492	1.6	0.0492	134.4	–
Catechol	µg/cig	40	0.313	36.7	0.313	109	(111)	89.3	0.984	88.1	0.537	101.4	(106)
Phenol	µg/cig	8.79	0.215	6.69	0.085	131	(131)	12.4	0.224	11	0.179	112.7	(105)
p-Cresol	µg/cig	5.09	0.107	4.31	0.0537	118	(115)	8.22	0.157	7.81	0.13	105.2	(100)
m-Cresol	µg/cig	2.01	0.0537	1.78	0.0224	113	(115)	3.08	0.085	2.99	0.0894	103.0	(94.6)
o-Cresol	µg/cig	2.36	0.0537	2.14	0.0268	110	(111)	3.47	0.112	3.72	0.157	93.3	(85.7)
<i>Other amines</i>													
Ammonia	µg/cig	8.72	0.13	8.04	0.0581	108	(130)	30.6	0.76	30.4	0.76	100.7	(106)
<i>NO_x</i>													
Nitric oxide	µg/cig	148	5.63	194	7.06	76.2	(67.3)	358.86	15.07	529	20.89	67.9	(69.9)
Nitric oxides	µg/cig	163	6.46	217	7.92	75.1	(67.8)	399.08	16.94	589	23.11	67.7	(70.4)
<i>SVOCs</i>													
Pyridine	µg/cig	7.33	0.112	7.41	0.148	98.9	(91.6)	36.8	0.581	42.1	0.268	87.4	(103)
Quinoline	µg/cig	0.273	0.00178	0.228	0.00358	120	(152)	0.546	0.0139	0.53	0.00492	103.0	(105)
Styrene	µg/cig	5.68	0.0671	6.79	0.0626	83.7	(76)	23	0.224	27.3	0.134	84.2	(94)

Table A1. (continued)

Analyte	Unit	ISO standard smoking regimen						ISO intense smoking regimen					
		1R6F		3R4F		Ratio (1R6F/3R4F)	1R6F		3R4F		Ratio (1R6F/3R4F)		
		mean	S.E.	mean	S.E.		mean	S.E.	mean	S.E.			
<i>Aromatic amines</i>													
1-Aminonaftalene	ng/cig	13	0.358	12.9	0.134	101	(97.2)	24.5	0.358	26.9	0.179	91.1	(91.8)
2-Aminonaftalene	ng/cig	7.69	0.367	7.82	0.0581	98.3	(122)	15.5	0.581	16.7	0.134	92.8	(89.5)
3-Aminobiphenyl	ng/cig	2.04	0.085	2.2	0.0179	92.7	(103)	4.71	0.183	5.21	0.0224	90.4	(82.5)
4-Aminobiphenyl	ng/cig	1.41	0.0626	1.54	0.0134	91.6	(96.1)	3.35	0.125	3.71	0.0313	90.3	(80.1)
<i>Heavy metals</i>													
Mercury	ng/cig	2.33	0.0179	2.58	0.0313	90.3	(93.8)	5.26	0.0268	6.01	0.0537	87.5	(95.1)
Lead	ng/cig	11.7	0.268	10.6	0.179	110	(-)	31.1	0.581	33.8	0.626	92.0	(-)
Cadmium	ng/cig	36.6	0.671	34.3	0.179	107	(107)	95.3	0.984	114	1.79	83.6	(81.7)
Chromium	ng/cig	<LOD	-	<LOD	-	-	(-)	<LOD	-	<LOD	-	-	(-)
Nickel	ng/cig	<LOQ	-	<LOQ	-	-	(-)	<LOQ	-	<LOQ	-	-	(-)
Beryllium	ng/cig	<LOD	-	<LOD	-	-	(-)	<LOD	-	<LOD	-	-	(-)
Cobalt	ng/cig	<LOQ	-	<LOQ	-	-	(-)	<LOQ	-	<LOQ	-	-	(-)
Selenium	ng/cig	<LOQ	-	<LOQ	-	-	(-)	<LOQ	-	<LOQ	-	-	(-)
Arsenic	ng/cig	2.41	0.0313	2.56	0.0268	94.1	(-)	6.72	0.152	7.92	0.179	84.8	(-)

TPM: total particulate matter, cig: cigarette, TSNA: tobacco specific *N*-nitrosamine, NNN: *N*-nitrosonornicotine, NAT: *N*-nitrosoanatabine, NAB: *N*-nitrosoanabasine, NNK: 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; VOC: volatile organic compound, MEK: methyl ethyl ketone, HCN: hydrogen cyanide, PAH: polycyclic aromatic hydrocarbon, B[a]P: benzo[a]pyrene, NO_x: nitrogen oxides; SVOC: semi volatile organic compound;

S.E.: standard error

LOD: limit of detection, LOQ: limit of quantitation;

Data in (*italics*) indicate results published by JACCARD *et al.* (12).

Table A2. Mutagenic slope values of 1R6F and 3R4F TPM in the Ames assay.

Smoking regimen	Strain and S9 metabolic activation	1R6F slope value (Revetants/μg)		3R4F slope value (Revetants/μg)		Ratio (1R6F/3R4F)	
		Mean	S.E.	Mean	S.E.	Mean	S.E.
Standard	TA98 with S9	2.31	0.0748	2.40	0.145	0.966	(0.841)
Standard	TA98 without S9	equivocal	-	equivocal	-	-	(1.51)
Standard	TA100 with S9	0.849	0.0430	0.886	0.0509	0.959	(0.902)
Standard	TA100 without S9	0.354	0.0213	0.298	0.0365	1.23	(-)
Standard	TA1537 with S9	0.300	0.0301	0.305	0.0455	1.00	(1.05)
Standard	TA1537 without S9	equivocal	-	equivocal	-	-	(-)
Standard	TA1535 with S9	non-mutagenic	-	non-mutagenic	-	-	(-)
Standard	TA1535 without S9	non-mutagenic	-	non-mutagenic	-	-	(-)
Standard	TA102 with S9	non-mutagenic	-	non-mutagenic	-	-	(-)
Standard	TA102 without S9	non-mutagenic	-	non-mutagenic	-	-	(-)
Intense	TA98 with S9	1.10	0.0342	1.02	0.0368	1.072	(0.971)
Intense	TA98 without S9	non-mutagenic	-	equivocal	-	-	(1.18)
Intense	TA100 with S9	0.475	0.0213	0.426	0.0119	1.12	(1.07)
Intense	TA100 without S9	0.233	0.0178	0.190	0.0115	1.24	(-)
Intense	TA1537 with S9	0.162	0.0258	0.187	9.50E-3	0.864	(0.939)
Intense	TA1537 without S9	equivocal	-	equivocal	-	-	(-)
Intense	TA1535 with S9	non-mutagenic	-	non-mutagenic	-	-	(-)
Intense	TA1535 without S9	non-mutagenic	-	non-mutagenic	-	-	(-)
Intense	TA102 with S9	non-mutagenic	-	non-mutagenic	-	-	(-)
Intense	TA102 without S9	non-mutagenic	-	non-mutagenic	-	-	(-)

Data in (*italics*) indicate results published by JACCARD *et al.* (12);

Equivocal: there was no reproducible significant increase in revertants over three replicates;

non-mutagenic: there was no significant increase in revertants over three replicates;

-: calculation or statistical analysis was not done because the sample was determined to be equivocal or non-mutagenic.

S.E.: standard error

Table A3. Genotoxic slope parameters of the logit function of 1R6F and 3R4F TPM in the MN assay.

Smoking regimen	Term and S9 metabolic activation	1R6F slope parameter (mg/mL)		3R4F slope parameter (mg/mL)		Ratio (1R6F/3R4F)	
		Mean	S.E.	Mean	S.E.	Mean	S.E.
Standard	"Short" without S9	10.2	1.26	8.11	1.22	1.27	(1.18)
Standard	"Short" with S9	6.36	1.37	5.67	1.09	1.11	(1.01)
Standard	"Long" without S9	17.8	3.22	15.2	2.22	1.17	(1.05)
Intense	"Short" without S9	7.36	1.10	6.37	1.14	1.17	(1.12)
Intense	"Short" with S9	4.29	0.850	3.87	0.487	1.09	(1.05)
Intense	"Long" without S9	13.8	2.11	11.6	2.02	1.21	(0.894)

Data in (*italics*) indicate results published by JACCARD *et al.* (12).
S.E.: standard error

Table A4. IC₅₀ values of the TPM and GVP of 1R6F and 3R4F in the NRU assay.

Smoking regimen	Fraction	1R6F IC ₅₀ (µg/mL)		3R4F IC ₅₀ (µg/mL)		Ratio (1R6F/3R4F)	
		Mean	S.E.	Mean	S.E.	Mean	S.E.
Standard	TPM	68.6	1.32	70.9	2.67	0.969	(0.979)
Standard	GVP	101	6.89	95.9	7.11	1.05	(1.18)
Intense	TPM	94.2	4.84	105	1.97	0.901	(1.05)
Intense	GVP	104	6.36	107	4.61	0.971	(1.15)

Data in (*italics*) indicate results published by JACCARD *et al.* (12).
S.E.: standard error

Figure A1: Correlation of the absolute values of the relative differences between 1R6F and 3R4F in chemical analysis. (A) ISO standard smoking regimen; (B) ISO intense smoking regimen. X-axis: relative differences in current study (%), Y-axis: relative differences in JACCARD study (%). The coefficients of determination under the ISO standard and intense smoking regimens were $R^2 = 0.73$ and $R^2 = 0.74$, respectively.